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Ph.D. degree in Pathology

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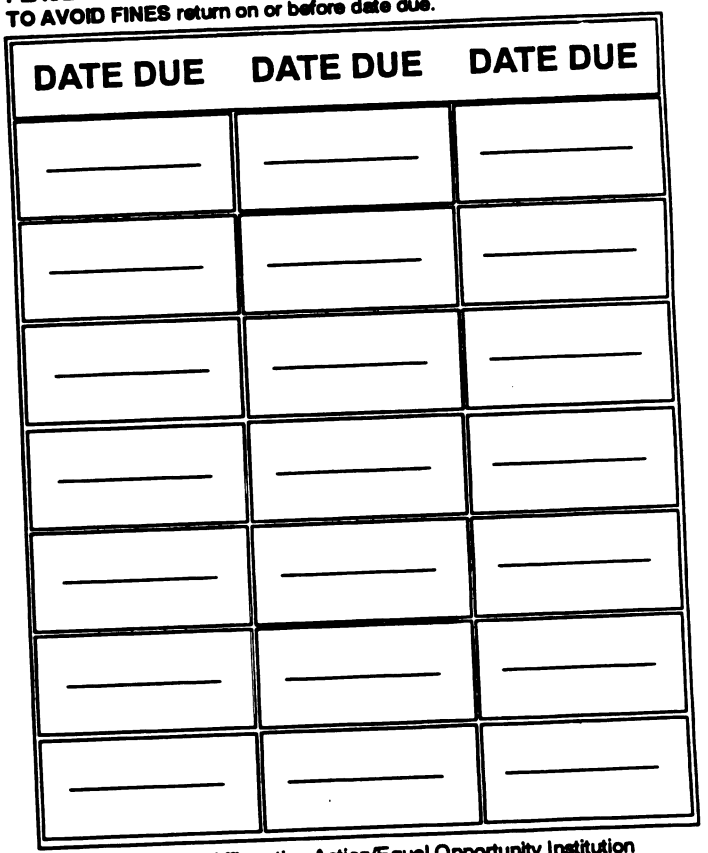

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FUNCTIONAL AND BIOCHEMICAL ABNORMALITIES IN BASSET HOUND HEREDITARY THROMBOPATHY

VOLUME I

By

Mary Frances McConnell

A DISSERTATION

Submitted to

Michigan State University

in partial fulfillment of the requirements

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Department of Pathology

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ABSTRACT

FUNCTIONAL AND BIOCHEMICAL ABNORMALITIES IN

BASSET HOUND HEREDITARY THROMBOPATHY

By

Mary Frances McConnell

Basset hound hereditary thrombopathy is an autosomal recessive defect in dogs in which there is a thrombasthenia-like hemorrhagic diathesis, but normal membrane GPIIb-IIIa content and binding of ^{125}I-labelled fibrinogen. In this study, there was normal shape change, but failure of primary aggregation in response to ADP, PAF and A23187. Affected platelets aggregated in response to thrombin, phorbol ester and dithiothreitol. The rate of thrombin-induced aggregation was very slow and aggregation was more sensitive to inhibition by aspirin than control platelets. In the absence of primary aggregation, ADP induced dense granule secretion which was more rapid than control platelets and sensitive to inhibition by aspirin. Higher concentrations of PAF and thrombin induced secretion which was more sensitive than control platelets to inhibition by aspirin. A23187 and lower concentrations of PAF did not induce secretion by affected platelets. The 20 and 47 kDa proteins were phosphorylated in affected platelets activated by PAF, A23187, thrombin and PMA under non-aggregating conditions. There was a marked decrease in the phosphorylation of a 65 kDa band compared to control platelets. The identity, function and significance of this

protein is not known, but may be of major importance in the pathogenesis of the defect. Resting TxB_2 concentrations were significantly higher in affected than in control platelet rich plasma. Agonists which induced ATP release from affected platelets also induced significantly greater TxB_2 production from affected platelets than controls (ADP, PAF, thrombin). When stimulated by agonists which did not induce aggregation or ATP secretion, affected platelets produced no TxB_2 (0.1 μM PAF) or significantly less TxB_2 (A23187) than control platelets. When affected platelets were stimulated by thrombin, aggregation was associated with TxB_2 production and the extent of aggregation was proportional to the amount of TxB_2 produced. Production of TxB_2 in response to arachidonic acid was similar in both groups of platelets. Neither group produced TxB_2 in response to stimulation by phorbol ester. These results do not fit any described genetic or acquired platelet defect or any situation created <u>in vitro</u> and cannot be explained using current knowledge of platelet function.

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
Ca^{2+}	ionized calcium
DAG	1,2-diacylglycerol
GMP	guanosine monophosphate
GP	glycoprotein
(1)IP	inositol 1-phosphate
(1,4)IP_2	inositol 1,4-bisphosphate
(1,4,5)IP_3	inositol 1,4,5-trisphosphate
PAF	platelet activating factor
PAGE	polyacrylamide gel electrophoresis
PI	phosphatidylinositol
PIP	phosphatidylinositol monophosphate
PIP_2	phosphatidylinositol bisphosphate
PMA	phorbol myristate acetate
SDS	sodium dodecyl sulfate
TxA_2	thromboxane A_2
TxB_2	thromboxane B_2

INTRODUCTION

Current knowledge of platelet function and biochemistry has been gained primarily from investigation of the role of platelets in hemostasis. Much of the motivation for this area of research is provided by the importance of platelets in the pathogenesis of atherosclerosis and thrombosis, which are major causes of death in Western societies. Hereditary disorders of platelet function have contributed greatly to knowledge of platelet structure, function and the relationship between them. Both human and animal hereditary models of platelet dysfunction appear spontaneously, are readily recognized and are able to be characterized in vitro. Platelets are particularly popular tools for cell biology research because they are readily accessible for study, have receptors linked to signal transduction systems and the measurable responses of aggregation and secretion.

The existence of a hemorrhagic diathesis in Basset hounds was first observed by a veterinarian in private practice in the late 1970's. This observation lead to the examination of platelet function in a population of inbred Basset hounds in Michigan. This screening process identified a small subpopulation of these closely related Basset hounds with a severe aggregation dysfunction associated with a bleeding diathesis. These animals were assumed to be homozygous for a hereditary thrombopathy with an apparent autosomal pattern of

inheritance. There was considerable variation in the range of responses to the agonists used within the remaining population. The significance, if any, of this is not known, but it is possible that animals with intermediate responses are heterozygous for the defect.

The initial investigation of Basset hound hereditary thrombopathy included examination of the clinical aspects and genetics of the disorder. These results, plus platelet function studies, identified a platelet dysfunction with many of the functional characteristics of human Glanzmann's thrombasthenia. In this disorder, membrane glycoprotein IIb-IIIa (GPIIb-IIIa) content is reduced or absent. As GPIIb-IIIa has been identified as the fibrinogen receptor on platelets, the functional disorder in Glanzmann's thrombasthenia is the result of the inability of platelets to bind sufficient fibrinogen to support aggregation. However, the membrane GP11b-111a content of platelets from affected Basset hound hereditary thrombopathy dogs was shown to be normal when examined by two-dimensional electrophoresis. Similarly, binding of ^{125}I-labelled fibrinogen was normal, even though the agonist used to initiate binding did not induce platelet aggregation.

While the results of these studies ruled out many known primary defects of platelet function, a definitive conclusion as to the pathogenesis of the defect could not be made from them. The behavior of affected platelets suggested that the complex platelet stimulus-response coupling system was unable to convert the external signal of agonists into the normal sequence and timing of events, ie. shape change, secretion of granule contents and arachidonate metabolites, and

aggregation. This abnormal behavior focused attention on the stimulus-response coupling mechanism as a logical area for further study.

At the beginning of this study, it was hypothesized that there was a defect in the signal transduction pathway in affected platelets, but, given the lack of information available at the time, it was not possible to propose a more specific hypothesis. The information that was available was restricted primarily to the membrane glycoproteins of resting platelets and the responses of affected platelets to a single agonist, adenosine diphosphate. Therefore, a logical starting point was to examine the response of control and affected canine platelets to a wider range of physiologic and non-physiologic agonists. Once this was completed, some aspects of the signal transduction pathway were examined in a attempt to identify a specific area of difference between affected and control platelets.

While a great deal has been learned in the course of this study, the precise pathogenesis of Basset hound hereditary thrombopathy has not been identified. Given the complexity of the problem, such a result is not surprising. However, considerably more is now known about the biochemical nature of the defect. The results of this investigation indicate several paths along which future investigation of this defect should be directed, and the information obtained from such studies has the potential to add to our knowledge of the detailed mechanisms involved in platelet function.

CHAPTER I: LITERATURE REVIEW

Platelets are the smallest circulating cells in mammals and are pivotal for normal hemostasis. The formation of a hemostatic plug represents one the earliest responses to blood vessel injury and any break in the continuity of a vessel wall elicits an instantaneous response from platelets that come into contact with the zone of injury. The process of hemostasis involves rapid adhesion of platelets to the exposed subendothelium followed by platelet-to-platelet adhesion (aggregation) and the formation of a primary hemostatic plug. The conversion of soluble fibrinogen to an insoluble fibrin polymer which enmeshes the platelet thrombus completes the formation of a secondary hemostatic plug.

The formation of a primary hemostatic plug by platelets involves several distinct steps known as the platelet response: adhesion, shape change, aggregation and secretion of granule contents and arachidonate metabolites. These responses can be elicited *in vivo* and *in vitro* by a wide range of stimulating agents and have been studied extensively *in vitro* in suspensions of platelets. Although platelet stimuli and responses are quite varied, the process of platelet activation appears to involve a limited number of pathways or mechanisms. Common to all physiological stimuli is their binding to specific receptors on the platelet surface. The activated receptor transmits the message across

the plasma membrane by GTP-binding regulatory proteins which control the function of specific effector systems such as phospholipase C-induced inositol phospholipid hydrolysis. The effector systems control the generation of second messengers which trigger physiological responses by changes in enzymatic activities and phosphorylation and structural properties of proteins. This process of platelet activation is highly regulated and amplification or inhibition can occur at each step in the process. Although knowledge and understanding of this area have increased considerably over the last ten years, there are many unanswered questions about the mechanisms and control of platelet activation. When compared to human platelets, relatively little is known about the structure and function of canine platelets. However, the limited amount of information published and the results of this study indicate that they are very similar to human platelets. In this review, features which characterize human platelets will be assumed to also characterize canine platelets. Some unique features of canine platelets have been observed, but these are not common and will be described as an exceptional feature where relevant.

MEGAKARYOPOIESIS AND PLATELET PRODUCTION

Megakaryopoiesis

The process by which a pluripotent hematopoietic stem cell becomes committed to megakaryocyte development is not clear. The more differentiated, committed megakaryocyte stem cell is defined by its ability to give rise to colonies of mature megakaryocytes. These

committed stem cells are defined functionally as colony-forming unit-megakaryocyte (CFU-Meg) and are not identifiable morphologically, cytochemically or immunologically.[319] The earliest committed cell recognizable in Wright-stained marrow smears is the megakaryoblast.[225] Megakaryocytes are the largest of the hematopoietic cells in the bone marrow and are uniquely characterized by their size and heterogeneity. The DNA content of megakaryocytes is very variable with most cells having ploidy values between 8 and 64 N.[319] There is evidence that megakaryocytes with higher ploidy values produce larger platelets with greater hemostatic efficiency.[319] Mitotic amplification occurs at the level of the megakaryocyte precursor cells and the megakaryoblast does not undergo cell division.[319] However, the megakaryoblast does undergo endomitosis in which there is mitotic division of the nucleus without division of the cytoplasm to produce cells with a multilobed nucleus and a polyploid DNA content of 4 to 64 N. Promegakaryocytes have more than four distinct nuclei and less basophilic, more abundant cytoplasm than megakaryoblasts. As the cell matures, the nucleus becomes more lobulated, the nuclear chromatin more condensed and the cytoplasm more abundant and granular. Megakaryocytes have a single multi-lobed nucleus and abundant, pale-staining cytoplasm containing small azurophilic granules.[225]

Megakaryocytic Granules and Organelles

The alpha granules represent the largest class of granules in megakaryocytes and platelets and probably arise in the Golgi apparatus.[62] Platelet proteins that have been shown to be synthesized

by megakaryocytes include platelet factor 4, factor VIII:von Willebrand factor, factor V, fibrinogen and thrombospondin. Of these, fibrinogen, thrombospondin and factor VIII:von Willebrand factor have been localized to the alpha granules of megakaryocytes. Platelets contain another class of granules distinct from alpha granules and characterized by a highly osmiophilic core.[62] These dense bodies appear to represent storage sites for the non-metabolic pool of adenine nucleotides, serotonin and calcium.[62] Although dense bodies in megakaryocytes are not readily visualized, megakaryocytes have been shown to store exogenous 5-hydroxytryptamine in organelles that represent the precursors of dense bodies.[62] Electronmicroscopic studies have suggested that dense body maturation occurs very late in megakaryocyte maturation. A third distinct type of granule originates in the Golgi apparatus and contains aryl sulfatase and acid phosphatase.[62] These lysosomal granules have been identified in both megakaryocytes and platelets.

The rough endoplasmic reticulum and free ribosomes are well developed by the megakaryoblast stage.[62] Two enzymes, platelet peroxidase and glucose-6-phosphatase, are present in the endoplasmic reticulum. They are also present in the dense tubular system of platelets which indicates that the dense tubular system is derived from megakaryocyte smooth endoplasmic reticulum.[504] A smooth membrane system called the membrane demarcation system can be identified in the cytoplasm of megakaryoblasts.[62] The cavities of the membrane demarcation system are continuous with the extracellular space, but the origin of this system is not clear. Although it has been proposed that the membrane

demarcation system plays a role in platelet release by delineating platelet boundaries, this proposal has not been universally accepted.[290]

Platelet Formation

The mechanism by which platelets are ultimately formed from mature megakaryocytes and released into the circulation is not clear. The three major theories for platelet formation are: (1) cytoplasmic budding; (2) cytoplasmic dissolution; and (3) pseudopod formation.[290] Although cytoplasmic budding of megakaryocytes has been observed by scanning electronmicroscopy, these blebs do not contain organelles and seem unlikely to be a major source of platelets. Megakaryocytes *in vitro* have been observed to undergo dissolution into small fragments without pseudopodia formation, but there is little evidence that this is significant *in vivo*. The formation of pseudopodia by megakaryocytes has been observed in and outside marrow *in vitro*. The extension of large pseudopodia containing a full complement of cytoplasmic organelles through the endothelium has been described, but there is not yet definite proof of fragmentation of pseudopodia as the major mechanism of platelet formation. In addition, since megakaryocytes can migrate across the vessel wall, the pseudopodia may represent the first step in movement into the sinusoids. A fourth possible mechanism of platelet formation is fragmentation of megakaryocytes which become trapped in the pulmonary capillaries. The extent to which these megakaryocytes contribute to the maintenance of the circulating platelet population remains unclear.

Regulation of Thrombopoiesis

Thrombopoiesis is regulated by a humoral mechanism in which production of platelets from megakaryocytes is responsive to the number or mass of circulating platelets.[194] Acute severe thrombocytopenia causes an increase in size, ploidy, number and rate of maturation of marrow megakaryocytes and accelerated platelet production. The rate and magnitude of the response is proportional to the degree of thrombocytopenia. A thrombopoiesis-stimulating activity (thrombopoietin) has been demonstrated in the plasma of thrombocytopenic animals. Although thrombopoietin alters platelet production through effects on megakaryocytes, its mode of action remains unknown.

Unlike megakaryocytes, the CFU-Meg is relatively insensitive to variations in the circulating platelet concentration, with maintenance of platelet-producing capacity being their major function rather than maintenance of circulating platelet numbers.[319] The CFU-Meg require the presence of stimulators known collectively as megakaryocyte colony-stimulating factors (Meg-CSF) in order to generate colonies of megakaryocytes <u>in vitro</u>.[194] Another growth-promoting activity which potentiates the number of detectable megakaryocyte colonies in the presence of Meg-CSF has been recognized and called megakaryocyte potentiator (Meg-Pot). Other factors that stimulate megakaryopoiesis and thrombopoiesis include interleukin-3, erythropoietin, thrombopoiesis stimulating factor, granulocyte-macrophage colony stimulating factor and megakaryocyte stimulating factor.[194] However,

the humoral regulation of megakaryopoiesis and thrombopoiesis under conditions of normal thrombopoiesis and in vivo is still poorly understood.

PLATELET STRUCTURE AND HERITABLE DISORDERS

Circulating platelets are discoid, anucleate cells which contain a large number of secretory granules. Human platelets are small ellipsoids, 0.5-1.0 μ thick and 1.5-2.5 μ in diameter which circulate in the blood for four to ten days.[207,467] The mean platelet volume for the human and dog is similar (7.6-8.3 fl).[228] Recent studies on the mean survival of ^{111}Inoxine- or ^{51}Cr-labelled canine platelets have indicated that they have a slightly shorter mean survival time of approximately four to seven days.[225] Platelet consumption occurs as the result of normal aging as well as utilization in the hemostatic process. As platelets age, they undergo physical and biochemical changes which identify them for removal from the circulation by the mononuclear phagocytic system in the spleen, liver and bone marrow.

The resting platelet can be divided into four functional zones: (1) the membrane systems; (2) the sol-gel zone; (3) the organelle zone; and (4) the peripheral zone.[508]

1. The Platelet Membrane Systems

The dense tubular system and the open canalicular system form specialized membrane systems within platelets.[504] Close association

between the two systems has been noted, but they are not in direct communication. The open canalicular system is composed of tortuous, tubular invaginations of the plasma membrane which give the platelet a sponge-like structure. Because the open canalicular system is continuous with the platelet surface, almost the entire interior of the cell communicates with the extracellular environment. It is very likely that activated platelets empty their storage granule contents into the open canalicular system during secretion.[507]

The dense tubular system is an internal membrane system that does not communicate with the open canalicular system or extracellular space.[504] It is composed of residual smooth endoplasmic reticulum derived from the rough endoplasmic reticulum of the megakaryocyte. Resting platelets maintain a low cytoplasmic free ionized calcium (Ca^{2+}) concentration and a steep plasma membrane concentration gradient partly by sequestering Ca^{2+} within the dense tubular system.[51] Platelets contain a Ca^{2+}-ATPase that is localized in internal membranes thought to arise from the dense tubular system.[112,325] The dense tubular system is also the major location of cyclooxygenase and thromboxane synthetase activities in platelets.[69,154]

2. The Platelet Sol-gel Zone

The second functional zone is the sol-gel zone which is the matrix of the platelet cytoplasm. It contains the cytoskeleton that supports the discoid shape of resting platelets and provides the contractile system involved in shape change, internal contraction and secretion. The

cytoskeleton of the platelet consists of: (i) actin and myosin filaments; (ii) a membrane skeleton; (iii) microtubules; and (iv) a variety of associated proteins.[150]

(i) Actin and Myosin Filaments

Actin is the major platelet protein and has been estimated to constitute 15 to 20% of the total protein.[150] In addition, many of the other major platelet proteins are those which can bind to actin filaments. These include actin-binding protein, P235, and myosin. Actin monomers are slightly asymmetric, globular molecules (M_r = 42 kDa) which can be induced to polymerize in a noncovalent interaction to form filaments greater than 1 μm long and 5 to 6 nm wide.[367,391] During polymerization, monomers are added rapidly to a nucleus of three or more monomers. Because the molecule is asymmetric, the growing filament has polarity with the two ends being defined as the barbed and pointed ends. Although monomers are added and removed from both ends of a filament, they are added much more rapidly at the barbed end and removed more rapidly at the pointed end. This process has been described as "treadmilling" and will continue unless one or both ends of the actin filament are blocked by a "capping protein". Similarly, polymerization will stop once the concentration of free monomers in a solution drops below a critical level.[501] In non-stimulated platelets, 40 to 50% of actin is in the filamentous form.

When actin filaments interact with myosin filaments, the ionized magnesium-dependent ATPase (Mg^{2+}-ATPase) activity of the myosin is stimulated and a contractile force generated.[259] Myosin consists of two heavy chains of M_r = 200 kDa, two light chains of M_r = 20 kDa and two light chains of M_r = 16 kDa. The light chains are associated with the globular heads of the two heavy chains which also contain the actin-binding sites and actin-activatable ATPase. Platelet myosin polymerizes into short bipolar filaments in solution, although this has not been demonstrated in intact platelets. At physiological Mg^{2+}-ATP concentrations, myosin does not polymerize unless the 20 kDa light chains are phosphorylated.[431] Phosphorylation also converts myosin into a form that can interact with actin and activate its Mg^{2+}-ATPase activity.[2] In the resting platelet, myosin is in the monomeric form. During platelet activation, the myosin light chain kinase is activated and the myosin light chain phosphorylated, presumably allowing myosin polymerization, binding of actin filaments and activation of the Mg^{2+}-ATPase. Conversely, dephosphorylation reverses this process.[143]

(ii) The Membrane Skeleton

The second major component of the platelet cytoskeleton is the membrane skeleton. This is composed of short actin filaments which make up a second pool of actin filaments distinct from the cytoplasmic contractile pool described above.[151] The short actin filaments are cross-linked by actin-binding protein and are

resistant to Ca^{2+}-induced depolymerization. The membrane skeleton forms a lining to the plasma membrane that stabilizes the lipid bilayer, determines the contours of the membrane and is linked to the plasma membrane, primarily through actin-binding protein bound to the glycoprotein Ib-IX (GPIb-IX) complex.[148] Linkage of the membrane cytoskeleton to GPIb-IX determines the organization of the GPIb-IX complex within the membrane and maintains it in a suitable position for binding to von Willebrand factor at the site of vessel injury. The membrane skeleton is also linked to GPIa, GPIIa and a glycoprotein of M_r = 250 kDa on the plasma membrane.[146,148] During platelet aggregation, the Ca^{2+}-dependent protease is activated and hydrolyses actin-binding protein.[147,148] This releases the membrane glycoproteins from the membrane cytoskeleton and may provide a mechanism for regulating the interaction of actin filaments with the plasma membrane.

(iii) The Microtubule Coil

The third component of the cytoskeleton is the microtubule coil which lies at the periphery of the platelet just internal to the membrane cytoskeleton. It is composed of tubulin polymerized into a single long coil which supports the discoid shape of resting platelets.[125,345] During activation of platelets by an aggregating agent, the microtubule coil becomes constricted into a tight ring around the centralizing granules. Opinions differ on whether or not the microtubule coil breaks down and reforms during platelet activation.

(iv) Proteins Associated with the Cytoskeleton

Profilin and gelsolin are proteins that regulate the polymerization of actin in platelets and many other cell types. Profilin forms a 1:1 complex with actin monomers and inhibits actin polymerization. In platelets, monomeric actin is associated entirely with profilin, but dissociates during platelet activation, allowing polymerization of the monomers.[311] When the calcium concentration reaches 1.0 μM or higher, gelsolin binds to actin filaments and causes them to fragment into short filaments.[522,523] The gelsolin remains attached at the barbed end of the filament fragments and prevents the addition of actin monomers to these ends.

P235, vinculin, α-actinin, spectrin and actin-binding protein are the major proteins that may link actin filaments to membranes. P235 has been shown to be similar to talin which is a cytoskeletal protein that binds to vinculin and also binds bundles of actin filaments to the cytoplasmic domain of the fibronectin receptor in fibroblasts and smooth muscle cells.[68,367] Bundles of actin filaments have been shown to be associated with the cytoplasmic domain of the membrane GPIIb-IIIa complex in activated platelets.[385] It has not been proven, but P235 may play a role in linking actin filaments to GPIIb-IIIa. Human platelet P235 has been purified to apparent homogeneity and found to be a dimer with two subunits of M_r = 235 kDa that are rapidly degraded by the Ca^{2+}-dependent protease to a stable fragment of M_r = 200 kDa.[93]

Vinculin has been purified from platelets, but its role in the platelet cytoskeleton is not clear. Based on studies in other cell types, it has been proposed that vinculin may function as a link between actin and P235/talin.[150] α-Actinin is concentrated just beneath the plasma membrane in resting platelets and in the filopodia in activated platelets.[114,273,459] It cross-links or gels actin filaments, stimulates the activity of actomyosin, and may act as a link between actin filaments and the plasma membrane.[272]

Spectrin is major component of the cytoskeleton in red blood cells where it forms a submembranous network that functions in the maintenance of cell shape and structure.[88] Although spectrin has been identified in platelets, it is present in very small amounts and its significance is not understood.[149] Actin-binding protein has been purified from platelets and shown to be associated with the membrane skeleton that is attached to membrane glycoproteins.[146] It is proposed that its major function in platelets is to cross-link actin filaments of the membrane skeleton, similar to the function of spectrin in red blood cells. During platelet activation, it is hydrolysed by the endogenous Ca^{2+}-dependent protease, which may provide a means by which Ca^{2+} could regulate stimulus-induced cytoskeletal changes.[147] Other functions of actin-binding protein are the nucleation of purified actin and the cross-linking of actin filaments into networks.[148]

It appears that the three major proteins involved in organizing actin filaments into their characteristic three-dimensional structure in resting and activated platelets are actin-binding protein, α-actinin and myosin. These interactions are regulated further by tropomyosin and caldesmon.[150] Tropomyosin in platelets may prevent the binding of myosin to actin filaments in localized areas, allowing the development of actomyosin contractile activity only in specific areas of the activated cell. Tropomyosin has also been reported to inhibit the binding of actin-binding protein, spectrin and α-actinin to actin filaments in addition to inhibiting the ability of gelsolin to depolymerize actin filaments.[396] Thus, tropomyosin may have a stabilizing effect on actin filaments in defined areas of the cell. Caldesmon has been detected in platelets and found to bind to actin filaments in the absence of calcium.[117] However, in the presence of calcium it is released from actin and binds to calmodulin, which suggests that it may have a role in regulating the availability of actin filaments for binding to other proteins such as myosin.

Non-stimulated platelets contain an extensive network of filaments containing 40 to 50% of the total platelet actin. The remaining actin is in the monomeric form, capped by profilin which prevents polymerization. The actin filaments are contained in two pools, the short filaments of the membrane skeleton and the cytoplasmic network. The cytoplasmic long actin filaments make up a three-dimensional network that is regulated by its interaction with proteins such as actin-binding protein, α-actinin and tropomyosin. Under resting

conditions, the actin filaments play a structural role by maintaining the discoid shape of the cell and the random distribution of the organelles throughout the cytoplasm. Myosin in the resting platelet is uniformly distributed throughout the cytoplasm.

The membrane skeleton in the resting platelet also contributes to the maintenance of platelet shape as well as playing an important role in stabilizing the plasma membrane and increasing its resistance to shear forces.[151] The membrane skeleton may also regulate the distribution, and possibly the properties, of glycoproteins within the plasma membrane. Preliminary evidence suggests that the membrane skeleton may regulate the distribution of GPIb-IX in the plasma membrane and the ability of the GPIb component of the complex to bind to von Willebrand factor. The attachment of GPIb-IX to the membrane skeleton may help the adherent platelet to resist the shear forces to which it is subjected.

When platelets are activated, there is a rapid increase in actin polymerization.[150] The capping proteins of the barbed end are released and polymerization continues until 70 to 80% of the actin is polymerized. The exact means by which these activation-induced changes are controlled are not known. The function of the cytoplasmic actin filaments is determined by their association with proteins such as α-actinin, tropomyosin and actin-binding protein. They may be organized into loose networks of filaments at the periphery, rings of filaments which constrict around centralizing granules or bundles within the developing filopodia.

Myosin becomes concentrated around the centralized granules of activated platelets. As the cytoplasmic Ca^{2+} concentration increases during platelet activation, the 20 kDa myosin light chain is phosphorylated by the Ca^{2+}-dependent myosin light chain kinase and the myosin becomes almost completely associated with the cytoplasmic actin filaments.[143] Thus, myosin appears to be associated mainly with the centralizing actin filaments rather than the bundles in the filopodia. This association may be responsible for generating the contractile activity necessary for the secretion of granule contents.[143,183] There is also evidence that the association of myosin with actin filaments may be involved in the initial shape change response of platelets to most agonists.[107] Clot retraction has been proposed to depend on the attachment of actin filaments to the internal domain of GPIIb-IIIa and the attachment of fibrin clots to the external domain of GPIIb-IIIa.[89] Clot retraction occurs as the actin-myosin interaction in the cell tends to pull the filaments in the filopodia back into the cell.

The membrane skeleton remains associated with the plasma membrane during activation and may be involved in platelet shape change. After aggregation has occurred, the membrane skeleton is disrupted by activation of the Ca^{2+}-dependent protease which hydrolyses actin-binding protein. This releases the membrane skeleton from its membrane attachment sites and may be a prerequisite for the association of cytoplasmic actin filaments in the filopodia with the GPIIb-IIIa complex during aggregation.[385] With the release of GPIb-IX from its attachment to the membrane skeleton, GPIb loses its ability to bind to von Willebrand factor.

Unlike GPIb-IX and some other membrane glycoproteins, GPIIb-IIIa does not associate with the membrane skeleton, but with the cytoplasmic actin filaments of the cytoskeleton. The association of GPIIb-IIIa with actin filaments only occurs when aggregation takes place; it does not occur if fibrinogen binds to the extracellular domain of GPIIb-IIIa but aggregation is prevented.[385,503] It is likely that the ability of platelets to spread depends on disruption of the membrane cytoskeleton which in turn is dependent on the ability to aggregate and activate the Ca^{2+}-dependent protease.[150,502] Therefore, there are two major cytoskeletal changes which are dependent on the occurrence of aggregation: (1) the activation of the Ca^{2+}-dependent protease which detaches the membrane skeleton from the plasma membrane; and (2) the association of GPIIb-IIIa with cytoplasmic actin filaments.

3. The Platelet Organelle Zone

The third functional zone in the platelet is the organelle zone. This contains granules, electron dense bodies, peroxisomes, lysosomes, glycogen granules and mitochondria randomly dispersed in the cytoplasm.

(i) Platelet Energy Metabolism

Although platelets are anucleate and have minimal ability to synthesize proteins, they do use large amounts of energy in the execution of agonist-induced responses and have a well developed glycolytic system for ATP production.[204] They contain large stores of glycogen that are utilized on energy demand as well as

mitochondria. The availability of ATP is essential for platelet responses and responses are greatly inhibited if glycolysis or oxidative metabolism is blocked, or if platelets are deprived of glucose. Glucose metabolism appears to be the major source for ATP, but oxidation of fatty acids and amino acids also probably play an important role.

(ii) Secretable Storage Granules

Platelets contain three distinguishable types of secretable storage granules: dense granules, alpha granules and lysosomal granules.[201] The dense granules or dense bodies are dense in three respects: they are electron dense, they are the most osmiophilic platelet granules and they have the highest specific weight of any platelet organelle. They are 250 to 300 nm in diameter and store nucleotides, metal ions and serotonin. While all species studied contain high concentrations of adenosine diphosphate (ADP), adenosine triphosphate (ATP) and serotonin, the divalent cation content (Ca^{2+} and/or Mg^{2+}) differs between species.[328] Human platelets have almost exclusively Ca^{2+}, whereas most other species, including the dog, have both Ca^{2+} and Mg^{2+}. Dense granules also contain smaller amounts of inorganic orthophosphate P_i, inorganic pyrophosphate PP_i, guanosine diphosphate (GDP), guanosine triphosphate (GTP) and catelcholamines. The concentrations of adenine nucleotides and divalent cations are extremely high and the granules would be osmotically unstable if these components were free in solution.

Instead, the adenine nucleotides and the divalent cations exist in large, insoluble complexes 25 to 30 kDa in size in which the purine rings of the nucleotides are stacked on top of each other and probably linked together by ring interactions and bridges of divalent cations.

Except for serotonin, the dense granule contents are almost unexchangeable with their cytoplasmic counterparts. The nucleotides exchange very slowly with the cytoplasmic pool and do not participate in energy metabolism.[205] Serotonin is freely exchangeable with extracellular serotonin.[204] The transport across the plasma and granule membranes is driven by a pH gradient created by an ATP-requiring proton pump in the granule membrane. The uncharged serotonin crosses the granule membrane and is protonated, creating a downhill gradient across the granule membrane.

The dense granule contents are secreted during platelet activation in response to appropriate stimuli. Although the precise mechanism by which this takes place is not known, the most widely held theory is that secretory granules centralize and their contents are released into the open canalicular system by exocytosis.[507] Alternatively, they may move to the periphery of the cell where they undergo transition to participate in the formation of membrane-associated multivesicular structures which undergo dissolution and release their granule contents to the environment.[388]

The most abundant organelle seen in the platelet cytoplasm is the alpha granule which may outnumber the dense granules or lysosomes by as much as ten to one.[208] In transmission electronmicrographs, they appear as round or oval vesicles 300 to 500 nm across with a central electron-dense nucleoid surrounded by a less dense peripheral zone.

The alpha granules contain three groups of secretable proteins: (1) platelet-specific proteins (platelet factor 4, platelet derived growth factor, ß-thromboglobulin); (2) coagulation factors (fibrinogen, factor V, factor VIII-von Willebrand factor); and (3) other proteins (thrombospondin, fibronectin, albumin, α-$_2$-plasmin inhibitor, histidine-rich glycoprotein, high molecular weight kininogen, α_1-anti-trypsin, α_2-macroglobulin).[467] The coagulation factors and other non-platelet-specific proteins do not differ from their plasma counterparts, although proteolytic cleavage may occur during or just after secretion.

Platelet factor 4 is a low molecular weight protein that is secreted from platelets in a complex with a high molecular weight proteoglycan carrier.[208] When exposed to heparin, the carrier is dissociated and the platelet factor 4 exerts its heparin-neutralizing activity. Platelet-derived growth factor is a potent mitogen that is able to stimulate growth of fibroblasts, smooth muscle cell and glial cells.[415,416] The proliferative responses characteristic of wound repair are thought to be attributed at least in part to platelet-derived growth factor. The platelet

plug formed at the site of vessel injury in a wound secretes platelet-derived growth factor which may actively stimulate neighboring cells to proliferate. ß-Thromboglobulin is probably the cleavage product of platelet basic protein. It has a low affinity for heparin and is a potent chemotactic agent for fibroblasts. Platelet fibrinogen constitutes approximately 8% of total platelet protein. It does not differ from plasma fibrinogen with respect to size, clottability, affinity for the activated platelet fibrinogen receptor or the capacity to support platelet aggregation.[266] Thrombospondin is a high molecular weight multifunctional protein which is able to interact specifically with macromolecules important in hemostasis such as fibrinogen, collagen and heparin.[283,487] It promotes the aggregation of both non-stimulated and stimulated platelets, but by different platelet surface-receptor mechanisms.

The proposed mechanisms for secretion of alpha granule contents are the same as for dense granules, although it has also been suggested that the alpha granules may fuse together before exocytosis of a multi-alpha-granule complex.[156] The release of alpha granule proteins by activated platelets is thought to play a role in normal hemostasis by providing a localized area of increased concentration of these proteins, but their physiological function has not yet been clearly defined.

Lysosomes have a similar appearance to small alpha granules, but can be differentiated by histochemical techniques.[204,467] They

contain acid hydrolyses which are enzymes with optimal activity in the pH range 3.5 to 5.5. These include acid proteases, acid glycosidases, acid phosphatases and aryl sulfatases. Platelet lysosomes also contain phospholipase A_1 and a heparin-degrading enzyme. In contrast to the dense and alpha granules, release of lysosomal granule contents is partial and slow, even in response to a maximal stimulus. Different proportions of each group of acid hydrolases are released. The mechanism of exocytosis of platelet lysosomal contents and their function is not known. Although platelets can engulf bacteria, they do not have the ability to phagocytose or endocytose extracellular material to destroy it.[532] Based on this, the acid hydrolase-containing vesicles of platelets are not true lysosomes which can fuse with phagosomes to destroy the phagocytosed material. However, the released lysosomal enzymes may play a role in the clearing of thrombi, neutralization of heparin and the inflammatory response.

The term storage pool deficiency encompasses patients with a heterogenous group of deficiencies in the platelet contents of dense and/or alpha granules.[402,509] This group of disorders is characterized by abnormalities in platelet aggregation induced by ADP, epinephrine and collagen. A mild to moderate bleeding diathesis associated with deficiency of the dense granule contents has been described in human platelets as well as several animal species.[28,402] The second wave of aggregation in response to ADP and epinephrine is absent and the response to collagen absent or markedly impaired. The platelets have a marked decrease in dense granules and the total platelet and granule

dense granule contents are decreased.[200,201] Dense granule deficiencies are associated with defects in skin pigmentation in other inherited disorders such as Chediak-Higashi syndrome, Hermansky-Pudlak syndrome and the Wiskott-Aldrich syndrome which suggests that the two types of granules may be interrelated in terms of genetic control. The alpha granule content may be decreased in some cases of dense granule deficiency, or alpha granule deficiency may exist as an isolated defect. The gray platelet syndrome is a rare inherited disorder in which the platelets have an isolated absence of alpha granule contents. The platelet aggregation responses to collagen, thrombin and perhaps ADP are impaired, but the exact mechanism of the platelet dysfunction is unknown. However, it does suggest that the secreted alpha granule proteins play an important role in normal hemostasis.

4. The Peripheral Zone

The first three functional zones of the platelet, the membrane system, the sol-gel zone and the platelet organelles, have now been discussed. The fourth functional zone in the platelet is the peripheral zone which contains: (i) the submembranous zone; (2) the unit membrane; and (iii) the glycocalyx.

(i) The Submembranous Zone

The submembranous zone lies immediately beneath the unit membrane and is the functional area where signals from the cell exterior are translated into the chemical messages and physical

events necessary for platelet activation. It is linked to the unit membrane and the glycocalyx by the transmembrane glycoproteins.

(ii) The Unit Membrane

The middle layer of the peripheral zone is a typical unit membrane which functions to establish a permeability barrier and provide a matrix in which membrane proteins are embedded.[103] The ability of membrane phospholipids to assemble into a fluid bilayer depends on the presence of a hydrophilic head group region and a hydrophobic tail region. The hydrophilic head groups tend to orientate towards the aqueous environment in the cytoplasm or the cell exterior, while the hydrophobic tails orientate toward the non-aqueous inside of the bilayer. At physiological temperatures, most membrane lipids are fluid. This is an important requirement for normal membrane function. Most lipid bilayers are asymmetric with the negatively-charged phosphatidylserine and phosphatidylinositol more often located on the inner leaflet and phosphatidylcholine more often located in the outer leaflet of the membrane.

The platelet lipid bilayer also extends through the multiple channels of the open canalicular system. Asymmetry of the lipid bilayer means that phosphatidylserine and phosphatidylinositol are accessible to cytoplasmic phospholipases during platelet activation. Most of the membrane glycoproteins are integral and

cannot be separated from the lipid bilayer without the use of detergents. However, because lipid bilayers are fluid, membrane glycoproteins are able to move laterally within the membrane. The glycoproteins are also asymmetric with the carbohydrate moiety found exclusively at the external surface of the bilayer. The carbohydrate moieties help the protein to interact with the aqueous environment, as well as function as lectins and play an important role in cell recognition.

(iii) The Glycocalyx

It is the exterior component of the membrane glycoproteins that makes up the third and outermost layer of the peripheral zone, the glycocalyx. The chemical constituents of the glycocalyx provide the receptors for stimuli triggering platelet activation and the substrates necessary for adhesion and aggregation to occur.

PLATELET MEMBRANE GLYCOPROTEINS AND HERITABLE DISORDERS

The membrane glycoproteins are of major importance in platelet function.[366,463] These functions include mediation of adhesion and aggregation, formation of receptors for extracellular ligands, recognition of external phenomena and binding of complement. Glycoproteins also have a role in membrane transport, anti-protease activity, stabilization of the platelet surface and the generation of antigenic specificity and cell surface charge. The membrane

glycoproteins known to mediate platelet adhesion and aggregation are GPIb, GPIIb-IIIa complex, GPIa, and GPV.

Glycoprotein Ib

Glycoprotein Ib plays a major role in primary hemostasis by functioning as the surface receptor that mediates the attachment of platelets to exposed subendothelium via the attachment of von Willebrand factor. This attachment occurs primarily at the high shear rates that are found in small blood vessels. The exact mechanism by which von Willebrand factor binds to platelets is not clear, but it seems probable that the binding of von Willebrand factor to subendothelial collagen induces a conformational change that enables it to bind to GPIb. Binding of von Willebrand factor in itself is sufficient to induce platelet activation and secretion which is of major importance in recruiting other platelets to the site of vessel injury.

Glycoprotein Ib is a two-chain integral membrane sialoglycoprotein ($M_r \approx 170$ kDa) that acts as a receptor for von Willebrand factor on platelets.[33,84,177] Both the α- and ß-subunits are glycosylated, but much of the bound carbohydrate is located on the larger α-subunit. The ß-subunit spans the membrane and is phosphorylated on its cytoplasmic segment.[519] Glycoprotein Ib is tightly complexed within the platelet membrane to a glycoprotein of M_r = 17-22 kDa which has been identified as GPIX.[33] It also interacts with a cytoskeletal protein, actin-binding protein, which links it to the membrane skeleton.[148] The Ca^{2+}-

dependent platelet protease cleaves the α-chain of GPIb to release a high molecular weight (M_r = 140 kDa) glycopeptide called glycocalicin.[509] Thrombin also binds to the α-chain of GPIb.[478] However, the significance of this in thrombin-induced platelet activation is not clear as evidence suggests that other receptors for thrombin may also be involved.[323]

Bernard-Soulier syndrome is a rare, autosomal recessive bleeding disorder in which platelets lack, or have a severe deficiency of, the GPIb-IX complex and GPV.[84,153,338] The hemostatic deficiency is the result of the inability of the platelets to adhere to exposed subendothelium. The response of Bernard-Soulier platelets is delayed and less sensitive to lower doses of thrombin, suggesting that the binding of thrombin to GPIb contributes to, but is not essential for the response of platelets to thrombin. Glycoprotein V is also deficient in Bernard-Soulier platelets, but its relationship to the GPIb-X complex is not known. Bernard-Soulier platelets aggregate normally <u>in vitro</u> in response to agonists such as ADP and fibrinogen binding to the GPIIb-IIIa complex is normal. Bernard-Soulier platelets have been shown to bind ^{125}I-von Willebrand factor in a manner that is specific, saturable and reversible when activated by thrombin, ADP or ADP + epinephrine.[420] This indicates that platelets have at least one other binding site for von Willebrand factor other than GPIb, one of which has been identified as GPIIb-IIIa.

Glycoprotein IIb-IIIa

Glycoprotein IIb-IIIa complexes are major components of the platelet plasma membrane.[366,84] They are distributed as Ca^{2+}-dependent heterodimer complexes and function as the main receptor for fibrinogen and other proteins important in platelet aggregation. Glycoprotein IIb (M_r = 145 kDa) is composed two disulfide-linked α- and ß-subunits. Glycoprotein IIIa is a single polypeptide chain (M_r = 95 kDa) which is cross-linked by intrachain disulfide bonds. Reduction of the disulfide bonds increases the apparent molecular weight to 114 kDa. Sequencing studies have shown that GPIIb and GPIIIa are the products of separate genes and the α- and ß-subunits are synthesized as a common precursor that is then cleaved to give the mature two-chain molecule linked by disulfide bridges.[61,119] Both glycoproteins have the majority of their structure exposed on the exterior surface of the platelet and relatively little buried in the membrane.[71] Glycoprotein IIb is associated with the membrane via the ß-subunit and contains a number of Ca^{2+}-binding domains similar to those on calmodulin. Glycoprotein IIIa has a membrane-crossing domain near the C-terminal and a short cytoplasmic segment.

In non-stimulated platelets at 37°C, the GPIIb-IIIa complex can be dissociated reversibly by short (<10 minutes) incubations with ethylenediaminetetraacetate (EDTA), while longer incubations caused irreversible dissociation.[138] In contrast, the heterodimer is not dissociated with EDTA at room temperature or 4°C. The complexed form is necessary for the expression of fibrinogen receptors, the

dissociated proteins do not bind fibrinogen. The presence of the GPIIb-IIIa complex on the resting platelet has been established by the use of monoclonal antibodies against GPIIb-IIIa complex which bind equally well to resting and stimulated platelets.[31,320] The GPIIb-IIIa complex is the predominant form of these glycoproteins in intact platelets and more complex is exposed on the surface during platelet activation. This may be the effect of increased exposure of the surface-connected open canalicular system during activation or may represent GPIIb-IIIa from the alpha granule membranes that has fused with the plasma membrane after secretion has occurred.[158,518]

The GPIIb-IIIa complex has been demonstrated to function as the platelet fibrinogen receptor.[375,381] Platelets from patients with the genetic bleeding disorder Glanzmann's thrombasthenia lack or are deficient in GPIIb-IIIa and do not bind fibrinogen or aggregate.[90] Several monoclonal antibodies directed against GPIIb and/or GPIIIa block fibrinogen binding and platelet aggregation.[91] ^{125}I-labelled fibrinogen has also been shown to bind to GPIIb-IIIa in crossed-immunoelectrophoresis and by photoaffinity labelling.[30,158]

Platelets possess two high and six low affinity binding sites for Ca^{2+} on their surface which are involved in GPIIb-IIIa complex formation and the binding of adhesive proteins.[53] Extracellular Ca^{2+} exists in rapid equilibrium with Ca^{2+} bound to the surface and it is thought that the requirement of divalent cations for aggregation and Ca^{2+} for GPIIb-IIIa complex formation may reflect a requirement for membrane-bound Ca^{2+}. Even in the presence of divalent cations, the

GPIIb-IIIa complex of resting platelets does not bind fibrinogen. Exposure of the fibrinogen receptor on the surface of activated platelets may depend on changes in the conformation of GPIIb-IIIa and/or changes in the microenvironment around the complexes. Changes in the conformation of GPIIb-IIIa have been shown to take place during platelet activation by development of monoclonal antibodies that bind to the GPIIb-IIIa complex of activated, but not resting platelets.[92,436]

However, the means by which fibrinogen receptor expression is induced in platelets remains unclear. Two routes involving intracellular messengers have been demonstrated, one dependent on phosphoinositide hydrolysis and the other activated by high concentrations of thrombin and independent of phosphoinositide hydrolysis.[438] While binding of fibrinogen to GPIIa-IIIa is essential for aggregation to occur, it alone is not sufficient to induce aggregation. In one study, aggregation and the release reaction were completely abolished by the combination of two monoclonal antibodies, even although fibrinogen binding was normal.[355] This demonstrates that fibrinogen binding to the platelet surface alone is not sufficient to support platelet aggregation and that an additional post-fibrinogen-binding event is required.

The GPIIb-IIIa complex is a member of a family of proteins which function as receptors for adhesive proteins including fibrinogen, fibronectin and von Willebrand factor.[157,399] Fibrinogen, fibronectin and von Willebrand factor all contain the peptide sequence Arg-Gly-Asp which is important in adhesive interactions. Platelet GPIIb-IIIa

functions as a receptor for the Arg-Gly-Asp sequence, although binding of fibrinogen and fibronectin may require recognition of additional information for optimal binding. The binding of fibrinogen, fibronectin or von Willebrand factor to GPIIb-IIIa requires agonist activation whereas the binding of the Arg-Gly-Asp tripeptide does not. Although fibrinogen, fibronectin and von Willebrand factor each bind to GPIIb-IIIa, fibrinogen is much more abundant in plasma and binds preferentially when platelets are activated in the presence of a mixture of adhesive proteins. Therefore, cross-linking of platelets by fibrinogen remains the most likely model of platelet aggregation. It is possible that secreted fibronectin, von Willebrand factor and thrombospondin help to consolidate thrombus formation.

Glanzmann's thrombasthenia is an autosomal recessive bleeding disorder in which there is a deficiency or altered function of the platelet GPIIb-IIIa complex.[84,153,326] It is characterized by the absence of aggregation in response to all physiologic agonists. Functional heterogeneity occurs in Glanzmann's thrombasthenia and the disorder is divided into two basic subgroups. Type I platelets completely lack or contain only traces of GPIIb-IIIa and do not support clot retraction. Stimulated platelets fail to bind detectable amounts of fibrinogen, von Willebrand factor or fibronectin. However, platelet adhesion to the subendothelium and binding of von Willebrand factor to GPIb is normal. The hemorrhagic tendency arises from failure of thrombus formation <u>in vivo</u> as the result of the inability of platelets to aggregate to each other after adhesion to the subendothelium has occurred. Type II platelets contain greatly reduced amounts of GPIIb-

IIIa and support some degree of clot retraction. The clinical and platelet abnormalities are the same as for Type I Glanzmann's thrombasthenia. More recently, cases have been reported where the GPIIb-IIIa content of the platelets is normal, but expression of the functional fibrinogen receptor is abnormal and they fail to bind fibrinogen or aggregate in response to agonists.[365]

Glycoprotein Ia

Membrane GPIa is a large single chain glycosylated glycoprotein (M_r = 153 kDa non-reduced, 167 kDa reduced) that spans the platelet membrane.[84] The function of GPIa is not clear, but it may function as a receptor for collagen. A genetic deficiency of GPIa has been reported and found to be associated with defective aggregation in response to collagen.[246,356] Adherence of these platelets to subendothelium in a flowing blood stream is defective, partly due to an inability to spread after the initial adhesion via von Willebrand factor had occurred. Alternatively, GPIa may represent the binding site for a cofactor of collagen-mediated adhesion such as fibronectin. Recently, it has been reported that what has been referred to as GPIa is in fact two proteins with different properties.[246] Glycoprotein Ia was reported to be poorly expressed in resting platelets relative to stimulated platelets. Two-dimensional electrophoresis separated the GPIa into two different proteins of the same molecular mass. One of these glycoproteins was only exposed on the platelet surface after activation and was also present in the supernatant with other granule contents. It was found to be strongly associated with the

cytoskeleton. Both proteins described as GPIa are involved in collagen-induced activation, but it is the activation-dependent GPIa which has been described in genetic deficiencies.[246,326]

Glycoproteins IV, IX, V, Ic-IIa and GMP-140

Other identified, but less well-characterized, glycoproteins include GPIV, GPIX, GPV and GPIc-IIa. Glycoprotein IV is a single chain polypeptide (M_r = 88 kDa) which may act as the thrombospondin receptor on platelets.[10] Glycoprotein IX is a small (M_r = 17-22 kDa) surface glycoprotein present in the platelet plasma membrane in a 1:1 non-covalent complex with GPIb.[418] The function of GPIX is unknown and its effect, if any, on the function of GPIb is also unknown. Glycoprotein V is a sialoglycoprotein (M_r = 82 kDa) that is the prime candidate for the proteolytic substrate of thrombin on platelets.[509] It is readily cleaved by proteases, including thrombin, to release a 69 kDa water-soluble piece (GPV_{f1}).[528] Although it is cleaved by thrombin, it does not appear to be the thrombin receptor and a definitive role for GPV has not yet been found. It is also deficient in the Bernard-Soulier syndrome. The GPIc-IIa complex consists of two proteins of M_r = 160 and 138 kDa (non-reduced) which are present as a heterodimer on normal and Glanzmann's thrombasthenia platelets.[387] It has been found to mediate the adhesion of platelets to fibronectin-coated surfaces and therefore represents a functional fibronectin receptor. As fibronectin is a chief component of the subendothelial matrix, GPIc-IIa may play an important role in the adhesion of platelets to the subendothelium at the site of blood vessel injury.

An integral membrane glycoprotein, GMP-140, that is located in secretory storage granules has been described in platelets and vascular endothelial cells.[235,321] In platelets, GMP-140 is located in the α-granule membrane where it is a heavily glycosylated protein with a large cytoplasmic domain. It redistributes to the plasma membrane during degranulation and is only detectable on the plasma membrane after the secretory response. The function of GMP-140 is not known, but its structure is compatible with a receptor function. Other glycoproteins in the plasma membrane act as receptors for external ligands, but they are often quantitatively minor glycoproteins which have only been characterized by their interaction with the ligand.

THE BASIC PLATELET RESPONSE

The formation of a platelet plug in response to blood vessel injury involves several steps known as the basic platelet response: the **adhesion** of platelets to sites of vascular injury, **shape change**, **aggregation** of other platelets on the adherent layer and **secretion** of granule contents and liberation of arachidonate which aid in the recruitment of additional platelets.[288,435,467] The reversible responses of adhesion, shape change and primary aggregation are probably involved in the repair of very small defects and maintenance of the vascular system. The release reaction and secondary aggregation are irreversible responses involved in the hemostatic function of platelets and the initiation of thrombosis.

Adhesion

Resting platelets tend to adhere to almost any foreign surface to which they are exposed.[372] In the absence of collagen fibrils or other platelet stimulants, these platelets may not be activated and may remain adhered to the surface or detach, although some degree of activation frequently occurs. In contrast, adherence of platelets to exposed subendothelium within the vascular system at high flow and shear rates is accompanied by further activation of the platelets and initiation of the hemostatic process.[18] Knowledge of the process of adhesion has been greatly aided by study of two congenital bleeding disorders, Bernard-Soulier syndrome and von Willebrand's disease. In both of these, there is a marked decrease in the ability of platelets to adhere to subendothelium.[153,402] Since Bernard-Soulier platelets lack GPIb, it is assumed that GPIb plays an important role in platelet adhesion.[419] A similar defect in adhesion is induced by monoclonal antibodies to GPIb. Platelets in von Willebrand disease contain normal GPIb, but do not adhere normally unless exogenous von Willebrand factor is added. The adhesion defect can be reproduced by incubation with a monoclonal antibody to von Willebrand factor.[213] These results indicate that both von Willebrand factor and GPIb are necessary for normal platelet adhesion.

The role of von Willebrand factor in platelet adhesion is made more complex by its ability to bind to both GPIb and GPIIb-IIIa. As described above, it seems probable that binding of von Willebrand factor to subendothelial fibrillar collagen, or possibly other

subendothelial macromolecules, induces a conformational change that enables it to bind to GPIb. This induces platelet activation and secretion which is of major importance in recruiting other platelets to the site of vessel injury. The identity of the subendothelial molecule(s) recognized by platelets has not been positively identified, although fibrillar collagen is the most probable candidate. Fibrillar collagens in the blood vessel wall are the most thrombogenic of the vessel wall macromolecules. The main collagenous species of the vessel wall are type I and type III, both of which are strong inducers of platelet aggregation.[386] Non-collagenous microfibrils have also been shown to interact with platelets to induce aggregation that is dependent on the presence of GPIb and von Willebrand factor.[128,286]

Glycoprotein Ia has been identified as a probable collagen receptor in platelets and its congenital absence is associated with the failure of collagen-induced aggregation and adhesion.[246,356] Von Willebrand factor has also been reported to mediate collagen-platelet interaction.[386] It was proposed that von Willebrand factor binds to collagen which then interacts with its platelet receptor, probably GPIa. Activation of platelets and exposure of GPIIb-IIIa with binding of von Willebrand factor to GPIIb-IIIa then follows.

The initial phase of adhesion is the contact phase. This is followed by the spreading phase in which there is greater membrane contact between the platelet and the surface. The process of adhesion causes activation in much the same way as a strong agonist does. A three-step model for platelet adhesion has been proposed.[386] The first step is

the binding of von Willebrand factor to the subendothelium. The second step, platelet contact, occurs when von Willebrand factor binds to GPIb. The third step, platelet spreading and thrombus formation, is dependent upon von Willebrand factor binding to GPIIb-IIIa after collagen has bound to GPIa. As this step is not inhibited by fibrinogen, it is possible that released platelet von Willebrand factor has a higher affinity for GPIIb-IIIa than plasma von Willebrand factor.

Shape Change

The first active response of platelets to stimuli is shape change.[207] They undergo a rapid change in shape from smooth, discoid cells to become relatively spherical with long pseudopods as well as blunter protrusions.[506] This response is very rapid, requires metabolic energy and is independent of external Ca^{2+} and fibrinogen. Shape change is associated with the polymerization of cytoplasmic actin and, possibly, breakdown of the microtubule band. The internal organelles, including the granules, are moved towards the center of the cell as the circumferential band of microtubules and filamentous matrix of the sol-gel zone constrict. In fully activated cells, the organelles are clumped together in the center of the cell, surrounded by a ring of microtubules and filaments. Shape change can also be induced by exposure to low temperatures or antimitotic agents such as vincristine. In these circumstances, the shape change is induced by breakdown of the microtubule ring and there is no centralization of the organelles.

Aggregation

The next response of platelets to stimuli is aggregation. Aggregation requires fibrinogen, exposure of fibrinogen receptors on the platelet surface, extracellular Ca^{2+} and/or Mg^{2+} and platelet to platelet collision. Fibrinogen binds to GPIIb-IIIa only after the cell has been activated by an agonist. It appears that platelet activation is associated with a change in the GPIIb-IIIa complex, probably conformational in nature, that results in the exposure of fibrinogen binding sites that had not been functional in the resting state.[91,436] The binding of fibrinogen to its receptor is specific, saturable and not affected by temperatures over the range 10-37°C but is inhibited by chelation of Ca^{2+} and/or Mg^{2+}, metabolic inhibitors, prostaglandin E_1 or an acidic pH of <6.5.[381] The means by which fibrinogen supports platelet-to-platelet aggregation is still not conclusively proven. The most widely accepted theory is that fibrinogen, by virtue of its dimeric structure, is able to bridge between the fibrinogen receptors on two adjacent platelets.[90] In support of this theory is the inhibition of aggregation at very high concentrations of fibrinogen.[381] In another study, fibrinogen-labelled colloidal gold was used in conjunction with platelet whole mount preparations to directly visualize the platelet receptor.[294] On stimulated platelets with maximal binding of fibrinogen, there was redistribution of the fibrinogen receptors toward the central region of the cell after binding. This could provide a mechanism whereby platelets could be drawn closer together during aggregation.

It has also been proposed that the ADP receptor plays an important role in the binding of fibrinogen in response to a range of agonists.[93a] The putative ADP receptor that mediates shape change and aggregation is a 100 kDa membrane protein located close to GPIIb-IIIa and which interferes with fibrinogen binding. Binding of ADP to the 100 kDa receptor produces a conformational change such that it no longer interferes with the binding of fibrinogen to GPIIb-IIIa. This conformational change can be mimicked by partial proteolysis of the 100 kDa protein by chymotrypsin or higher concentrations of thrombin.[93a]

Although fibrinogen binding is essential for aggregation to occur, binding of fibrinogen in itself is not sufficient to cause aggregation. For example, platelets chilled to 4°C bind fibrinogen, but do not aggregate until rewarmed.[380] Platelets fixed after stimulation with ADP bind fibrinogen, but do not aggregate. These findings suggest that membrane fluidity may also contribute to the ability of platelets to aggregate.

A role for unidentified post-fibrinogen events in aggregation is indicated by the inhibition of aggregation and secretion by two monoclonal antibodies to GPIIb-IIIa (AP3 and Tab) which do not inhibit fibrinogen binding.[355] In addition, platelet stimulation by an agonist is not always necessary to expose fibrinogen receptors and allow fibrinogen binding. As described above, incubation of platelets at 4°C induces fibrinogen binding. Incubation of platelets with chymotrypsin or dithiothreitol induces fibrinogen receptor exposure and aggregation if stirred in the presence of fibrinogen.[357,530] With chymotrypsin,

this is likely mediated by proteolysis of the GPIIb-IIIa complex itself or the environment around it. Dithiothreitol is a reducing agent which probably induces fibrinogen receptor exposure by reduction of membrane disulfide bonds. Induction of fibrinogen receptors by cold, chymotrypsin or dithiothreitol is not inhibited by metabolic inhibitors, anticytoskeletal agents or increasing concentrations of cyclic AMP and does not require the continued presence of the agent.

In vitro, two basic types of aggregation responses can be identified. The first is primary aggregation without the release reaction and the second is secondary, irreversible aggregation accompanied by release. Adhesion, shape change and binding of fibrinogen are all potentially reversible processes. Reversible primary aggregation is induced by low concentrations of agonists and the aggregation and disaggregation of platelets is associated with fibrinogen binding to and dissociating from its receptor. However, if the stimulus is sufficiently strong, the primary reversible aggregation response is followed by the platelet release response and secondary irreversible aggregation.[449,467] For most agonists, secondary aggregation is a consequence of the release reaction.

The Release Response

The platelet release response includes secretion of granule contents and the liberation of arachidonic acid from membrane phospholipids.[449] Secretion of alpha and dense granule contents may be induced by all known physiologic platelet agonists. Lysosomal secretion occurs only

in response to strong agonists such as high concentrations of thrombin or collagen. The mechanisms of granule release have been discussed earlier and include fusion of the granule membrane with the membrane of the open canalicular system and formation of multivesicular structures at the plasma membrane which dissolve to release the granule contents to the environment. [388,507]

Physiologically, ADP is the most important substance secreted from the platelet granules because of the positive feedback and amplification role it plays in platelet activation by agonists of weak or intermediate strength.[207] The platelet release reaction includes the formation of arachidonate metabolites, primarily the endoperoxides and thromboxane A_2 (TxA_2). Like ADP, the thromboxane metabolites play a major role in the amplification of the platelet response by agonists of weak or intermediate strength. Therefore a major contribution of the release response is to amplify the response of the platelet to the initial stimulus by positive feed-back loops. However, positive feed-back loops are not involved in the primary and reversible platelet responses of adhesion, shape change and primary aggregation.

Both ADP and the arachidonate metabolites bind to specific receptors on the platelet surface to cause further activation of platelets. The effect of each of these two loops can be studied by the use of drugs which selectively eliminate the contribution of either loop to platelet responses. The ADP-scavenging enzymes (apyrase, creatine phosphokinase) or ADP-receptor blockers [ATP, adenosine monophosphate (AMP)] can be used to block the effects of ADP. The production of

endoperoxides and TxA_2 can be blocked by incubation of platelets with acetylsalicylic acid (aspirin) or other related non-steroidal anti-inflammatory drugs which block the cyclooxygenase component of prostaglandin endoperoxide synthase.[334,417]

Platelet Agonists

Agonists are classified as "weak", "intermediate" or "strong" depending on their ability to induce the release response and irreversible aggregation.[80,449,531] Weak agonists such as ADP or epinephrine, cannot induce irreversible aggregation in the absence of released ADP or arachidonate metabolites. Secretion of granule contents in response to these weak agonists occurs only in association with irreversible aggregation and is abolished by treatment with agents which inhibit production of endoperoxides and TxA_2. In contrast, strong agonists (high concentrations of thrombin and collagen) can induce irreversible aggregation in the absence of the release response and secretion is not dependent on aggregation. Agonists with intermediate potency [platelet activating factor (PAF), vasopressin, prostaglandin and endoperoxide analogues] can induce secretion independently of prior aggregation and cyclooxygenase activity, but aggregation and secretion are inhibited if ADP is removed and cyclooxygenase inhibited. Low concentrations of strong and intermediate agonists act as weak agonists.

SIGNAL TRANSDUCTION MECHANISMS

Physiologic platelet agonists bind to specific receptors on the platelet membrane and do not cross the membrane. Therefore, they must transmit their signal across the plasma membrane to the cell interior where it can be translated into instructions for the physical changes of shape change, aggregation and release. In the platelet, as in many other cell types, the binding of an agonist to its receptor results in the production of second messenger molecules which initiate a large number of intracellular events. There are two major second messenger systems, both of which function in platelets. The first is the hydrolysis of membrane phosphoinositides to generate two main second messengers, 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate [(1,4,5)IP_3]. Arachidonic acid is also released as a consequence of phosphoinositide hydrolysis and is oxygenated to form additional mediators. The second system is the cyclic AMP system in which receptor-induced stimulation or inhibition of adenylate cyclase controls the intracellular level of cyclic AMP.

Phosphoinositide Hydrolysis

Phosphoinositides are the three anionic glycerophospholipids that contain inositol in their head groups.[6,34,211,305,331,433] These acidic phospholipids tend to be located on the inner leaflet of membranes and constitute only 2 to 12% of the total lipid in cell membranes. A minority of the phosphoinositides contain additional phosphate groups as monoesters. Phosphatidylinositol 4-monophosphate (PIP) has a

phosphate group in the 4 position of the inositol ring and phosphatidylinositol 4,5-bisphosphate (PIP_2) has phosphate groups in the 4 and 5 positions of the inositol ring. Simplified formulas of the phosphoinositides are illustrated in Figure 1.

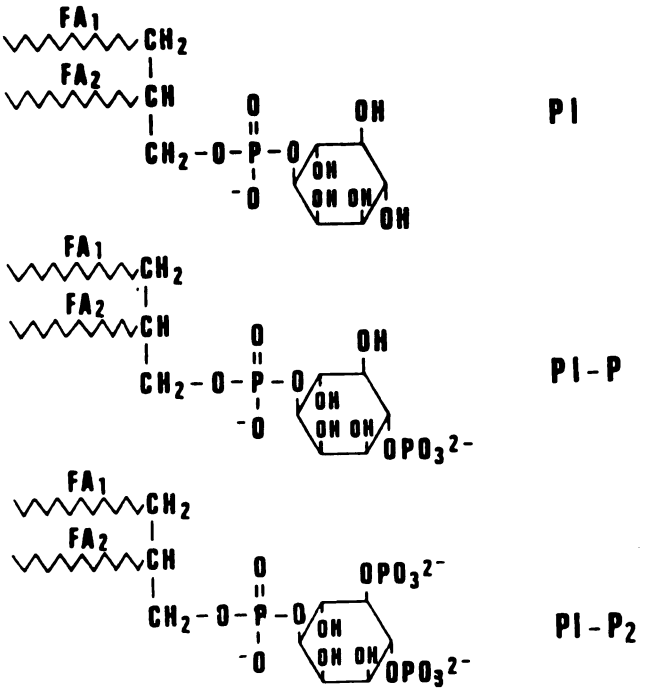


Figure 1. Simplified formulas of the phosphoinositides.[309]

These polyphosphoinositides are in dynamic equilibrium with each other and their parent molecule, phosphatidylinositol (PI). They are constantly being formed from PI by specific kinases and degraded back to PI by specific phosphomonoesterases and are in equilibrium with each other in an endless "futile cycle".[309] Although they are not directly involved in the agonist-induced stimulation of phosphoinositide metabolism, these kinases and phosphomonoesterases are nevertheless very important. Firstly, they are responsible for the rapid turnover

of the monoesterified phosphate groups on the phosphoinositides, and secondly, they provide the membrane with the appropriate phosphoinositide distribution which is required for full cell responsiveness.

The location of these specific enzymes correlates with the location of the phosphoinositides. The synthesis of PI from free inositol plus cytidine diphosphate-diacylglycerol via the activity of PI synthase takes place predominantly in intracellular membranes such as the endoplasmic reticulum or platelet dense tubular system.[191,211,433] In contrast, the polyphosphoinositides are considered to be situated on the cytoplasmic side of the plasma membrane and the Mg^{2+}-dependent PI and PIP kinases are located in or associated with the plasma membrane. The phosphomonoesterases that degrade the polyphosphoinositides are Mg^{2+}-dependent cytoplasmic enzymes. Transport proteins for PI have been found in the cytosol of several cell types, including platelets, and may provide the intracellular transport for PI formed in the membranes of the endoplasmic reticulum or the dense tubular system.[192]

In most mammalian cells, the predominant phosphoinositide is PI, followed by lesser quantities of PIP and PIP_2. For example, in the human platelet, PI, PIP and PIP_2 represent approximately 5, 1 and 0.3%, respectively, of the total membrane phospholipid.[211] Fatty acid analysis of the inositol-containing phospholipids has revealed that they have a rather unique fatty acid composition.[301] The sn-1-position of the glycerol backbone is predominantly occupied by stearic acid and the sn-2-position occupied by arachidonic acid. The predominance of

arachidonic acid in a position where it can be cleaved by phospholipase A_2 is of major significance because of the importance of arachidonate metabolites in platelet responses.

The 1-stearoyl 2-arachidonoyl species represents 71 mol percent of the PI in human platelets compared to 47 mol percent of phosphatidylethanolamine and 10 mol percent of phosphatidylcholine.[301] A similar predominance of the 1-stearoyl 2-arachidonoyl species was found in purified PI, PIP and PIP_2 from human platelet membranes.[210] Tracer studies using glycerol-^{14}C and orthophosphate-^{32}P indicated that newly synthesized species of PI could not account for the predominance of 1-stearoyl 2-arachidonoyl species.[209] It is likely that approximately half the 1-stearoyl 2-arachidonoyl species are generated by way of deacylation-reacylation pathways. The acyl-CoA:lyso(1 acyl) PI transferase in human platelet microsomes exhibits a selectivity towards arachidonyl CoA.[240] Therefore, the fatty acid in the sn-2 position on the glycerol backbone would be removed by a phospholipase A_2 and the position preferentially filled by arachidonic acid.

The phosphoinositides serve as intramembranous storage forms of messenger molecules that can be "released" or generated in response to an agonist binding to its specific receptor.[211,305] The phosphoinositides break down rapidly in response to the occupancy of specific receptors by various hormones, neurotransmitters, growth factors and other stimulants. Receptor occupancy initiates phosphoinositide hydrolysis by a phosphoinositide-specific phosphodiesterase, phospholipase C, which cleaves the phosphodiester

bond between the sn-3 position carbon of the glycerol backbone and the inositol head group. This generates DAG from each of the phosphoinositides plus the corresponding inositol phosphate. Thus, cleavage by phospholipase C of PI, PIP and PIP_2 generates inositol 1-phosphate [(1)IP], inositol 1,4-bisphosphate [$(1,4)IP_2$] and $(1,4,5)IP_3$ respectively.[309,305] The DAG can be phosphorylated in the sn-3 position to yield phosphatidic acid. This can be converted to cytidine diphosphate-diacylglycerol which is recycled to PI via the addition of inositol. A simplified version of the metabolic pathways of the inositol-containing lipids is illustrated in Figure 2.

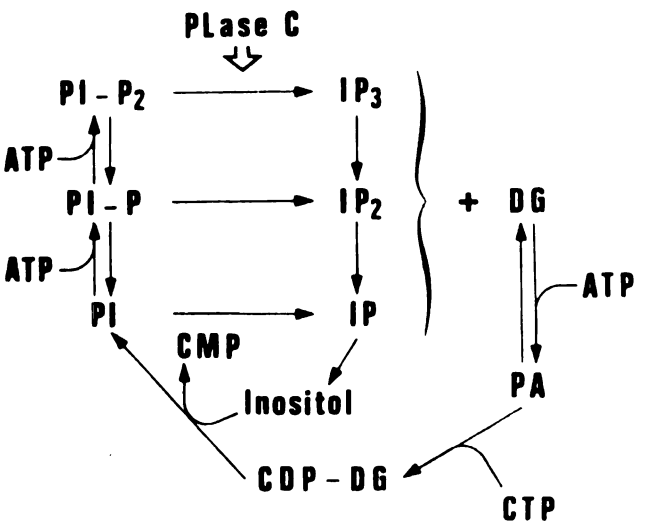


Figure 2. The metabolic pathways of the inositol-containing lipids.[309]

GTP-binding Regulatory Proteins

Phospholipase C in platelets specifically hydrolyses inositol-containing phospholipids and is present in membranes and

cytoplasm.[23,24,295,308,315,411,442] The stimulation of its activity by guanine nucleotides suggests that phospholipase C is regulated by a GTP-binding regulatory protein (G or N protein).[16,17,25] The G proteins are a growing family of closely related regulatory proteins, although at this time only three of them have both a defined structure and a known function.[60,163,292,352] They are responsible for the transmission of information from many membrane-bound receptors to their intracellular effectors. In general, G proteins regulate the activity of specific effectors such as enzymes or ion channels in the plasma membrane. The G protein system operates for a wide range of cell functions including vision, olfaction, control of cell proliferation and cellular regulation by a host of hormones, neurotransmitters and stimulants.

The G proteins have several common properties including an oligomeric structure, GTP-binding activities and GTPase activities.[163,292] All have a common heterotrimeric structure with the three protein subunits being designated α, ß and γ, in order of decreasing mass. The different G proteins have quite a lot of structural similarity, although their amino acid sequences differ and specific antibodies can differentiate between them. The α-subunits appear to be different for each type of G protein that has so far been identified.[60,163] They have apparent molecular weights of 39 to 51 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and contain the sites for guanine nucleotide binding and hydrolysis and possibly the sites for interaction with receptors and effectors. The ß-subunits are very similar with apparent molecular weights of 35 or 36 kDa. The γ-

subunits usually have molecular weights of 8 to 10 kDa, but the relationship between the γ-subunits of the different G proteins is not yet clear, partly because they are difficult to separate from the ß-subunits under non-denaturing conditions. The ß-γ complex may function partly to anchor the intact G protein to the plasma membrane.[297]

The inactive state of an individual G protein is the oligomer with GDP bound to the α-subunit (GDP•αßγ). Under these conditions, the rate of dissociation of GDP from the complex is extremely slow. When it is solubilized from membranes, GDP•αßγ behaves as a monomer of approximately 100 kDa.[466] When an agonist binds to its receptor, interaction of the G protein with the agonist-receptor complex promotes dissociation of GDP from the α-subunit and permits the binding of GTP, which is the more prevalent cellular guanine nucleotide. The binding of GTP alters the α-subunit in some way, possibly conformational, such that it dissociates from the complex. It also causes a decrease in affinity of the agonist for its receptor. It is the dissociated α•GTP subunit that activates the next stage in the communication pathway in which the function of an effector, such as phospholipase C, is modulated. The mechanism by which this modulation occurs is not known. The effect is terminated by the slow hydrolysis of the GTP to GDP by the intrinsic GTPase activity of the α-subunit. This leads to reassociation of α•GDP with the ßγ-dimer and cessation of the signal. The role played by the ßγ-dimer may be considerably more important than earlier studies suggested and it is possible that, at least in some cases, the interaction between the G protein and the effector is mediated by the ßγ-dimer.[243]

The three G proteins that have been isolated, and in which structure and function have been defined, are G_s and G_i of the adenylate cyclase system and transducin.[292] G_s is involved in the transduction of information leading to the elevation of cyclic AMP levels whereas G_i is involved in the inhibition of adenylate cyclase by hormones. Although their α-subunits differ, amino acid sequence analysis shows that the ß- and γ-subunits are identical. In platelets, the GTP•α complex of G_i is a weak inhibitor of adenylate cyclase and the ß•γ dimer a strong inhibitor.[243] Another possible function for G_i may be to stimulate Na^+/H^+ exchange which leads to cytoplasmic alkalinization and activation of phospholipase A_2.[474] In vertebrate retinal rod cells, activation by light of rhodopsin stimulates cyclic GMP phosphodiesterase activity through a G protein called transducin. This photolysis of rhodopsin by light leads to a decrease in the concentration of cyclic GMP and subsequent hyperpolarization.

Investigation of G proteins has been aided greatly by the ability of GTP and GDP analogues and certain bacterial toxins to alter G protein function.[60] Because normal cells are impermeable to these compounds, analogues of GTP and GDP are used in isolated membranes or in cells which have been permeablized. Normal platelets are also impermeable to cholera and pertussis toxin.[217,489] Commonly used methods of permeabilizing cells are exposure to low concentrations of detergents such as saponin or repetitive high-voltage electrical discharge. In the absence of agonists, non-hydrolyzable analogues of GTP, such as Gpp(NH)p or GTPγS, persist at the GTP binding site and cause prolonged activation of G proteins. Analogues of GDP, such as GDPßS, inhibit

activation. Cholera or pertussis toxin transfer the ADP-ribosyl moiety of NAD to the α-subunits of some intact oligomeric G proteins. Cholera toxin ADP-ribosylates the α-subunit of G_s which inhibits its intrinsic GTPase activity and causes persistent activation of adenylate cyclase. Pertussis toxin ADP-ribosylates the α-subunits of G_i and transducin which uncouples them from their receptors and blocks the effect of agonist-binding.

Both G_s and G_i are present in platelets. The G_s that is coupled to activation of platelet adenylate cyclase contains an α-subunit of 45 kDa which is ADP-ribosylated by cholera toxin. The G_i subunit that is coupled to inhibition of adenylate cyclase has an α-subunit of 41 kDa (α_{41}) which is ADP-ribosylated by pertussis toxin.[59,173]

GTP-binding Regulatory Protein Control of Phospholipase C

There is considerable evidence that the ability of hormones or agonists to activate phospholipase C is mediated by a G protein.[16,25,87,159,335] This G protein is usually identified as G_p, which refers to its effect on the phosphodiesterase phospholipase C. G_p has not been unequivocally identified, but a role for G_p is believed to exist in a cell when the following criteria can be demonstrated in permeablized cells and/or isolated membranes: (1) guanine nucleotides stimulate PIP_2 hydrolysis and/or the production of (1,4,5)IP_3 or DAG; (2) guanine nucleotides mimic the functional effects of agonists known to stimulate hydrolysis of the phosphoinositides; (3) exogenous GTP enhances the ability of agonists to cause phosphoinositide hydrolysis;

(4) pertussis toxin or GDP analogues inhibit agonist-induced phosphoinositide hydrolysis or processes which depend on the products of phosphoinositide hydrolysis; and (5) inhibition of the agonist-induced effects of pertussis toxin correlates with ADP-ribosylation of G proteins.[60]

The growing list of tissues in which G_p appears to exist suggests that regulation of phospholipase C by a G protein is a widespread phenomenon. However, some of the properties of G_p differ from tissue to tissue. The tissues studied so far are divided into two groups; those in which pertussis toxin inhibits phosphoinositide hydrolysis and those in which it does not. This is quite different to the effects of G_i and G_s on adenylate cyclase which are consistent from cell to cell.

Evidence for G protein involvement in the control of phospholipase C in platelets has come from studies on permeabilized platelets, isolated platelet membranes and solubilized phospholipase C.[17,56,187,188,216,255,370] In the earlier studies, electro-permeabilized platelets in which the extracellular and intracellular Ca^{2+} concentrations are equal were used.[187,188] Secretion of serotonin occurred when the free Ca^{2+} concentration was raised from 1.0 μM to 10 μM. Non-hydrolyzable GTP analogues dramatically reduced the Ca^{2+} concentration required to induce secretion to as low as 0.1 μM. This suggests that guanine nucleotides can lower the Ca^{2+} concentration threshold to the range present in the intact cell at the time of activation.[234] It was also demonstrated that GTP increased thrombin-induced [^{3}H]DAG formation and that GTPγS could stimulate as much

[^{3}H]DAG formation as thrombin. Similar studies in isolated platelet membranes concluded that a G protein mediated thrombin-induced phosphoinositide hydrolysis.[17,216,370] In sonicated platelets, most of the phospholipase C activity is found in the supernatant.[442] Using supernatant as a source of soluble phospholipase C and lipid vesicles with radiolabelled phosphoinositides as substrate, GTP analogues stimulated phosphoinositide hydrolysis.[115]

In saponin-permeablized platelets, non-hydrolyzable GTP analogues reproduced the effects of thrombin by causing DAG formation, Ca^{2+} release from the dense tubular system and serotonin secretion.[56] In the same study, the GDP analogue GDPßS and pertussis toxin inhibited the ability of thrombin to induce these events. However, increasing the thrombin concentration overcame the effects of GDPßS on secretion without restoring DAG formation. It was concluded that a G protein sensitive to inhibition by pertussis toxin couples thrombin receptors to the phosphoinositide hydrolysis pathway in platelets. However, a pathway for thrombin-induced secretion also exists that is independent of phosphoinositide hydrolysis. The identity of this additional pathway(s) is(are) not known.

In addition to thrombin, three other platelet agonists appear to have receptors that cause G-protein-controlled phosphoinositide hydrolysis. These are PAF, vasopressin and thromboxane analogues such as U46619 and U44069.[214,215] Each of these agonists has been shown to bind to specific receptors on the platelet surface, stimulate GTPase activity and to cause phosphoinositide hydrolysis. It was observed that

pertussis toxin did not affect platelet responses to all agonists to the same degree. The GTPase activity in thrombin-stimulated platelets was partially inhibited by preincubation with pertussis toxin.[215] In contrast, the increase in GTPase activity measured in platelets activated by PAF, vasopressin and U46609 was not affected, which suggested that the G_p involved with these agonists is pertussis toxin-resistant. In another study, the effect of pertussis toxin on phosphoinositide hydrolysis and [^{32}P]ADP-ribosylation of α_{41} in saponin-permeabilized platelets were examined.[59] Thrombin, which elicited responses mediated by both G_i and G_p, decreased radiolabelling by more than 90%. Epinephrine elicited responses mediated by G_i, not G_p, whereas vasopressin and PAF elicited responses mediated by G_p, not G_i. Both decreased radiolabelling by 50%. The thromboxane mimetic U46619 had no effect on ADP-ribosylation, even although it stimulates GTPase activity. It was concluded that: (1) in platelets a single pertussis toxin-sensitive, α_{41}-containing G protein may be involved in the regulation of both adenylate cyclase and phospholipase C; or (2) platelet α_{41} is composed of equal amounts of the [^{32}P]ADP-ribosylated α-subunits of G_i and G_p.[59,60]

Platelet Phospholipase C Activity

The contribution of each of the cytoplasmic and membrane-bound pools of phospholipase C in platelets to hydrolysis of the phosphoinositides in response to agonists is not known at this stage. Although membrane and cytoplasmic phospholipase C have been partially purified, there is not yet agreement over the intracellular distribution, number of forms

and molecular mass of platelet phospholipase C.[23,24,295] It has been suggested, but not proven, that most of the enzyme activity for the hydrolysis of PI (and PIP and PIP_2) is located in the cytosol and is Ca^{2+}-dependent, requiring millimolar concentrations of Ca^{2+} to obtain maximal hydrolysis of PI.[23,24] At lower, micromolar, Ca^{2+} concentrations, the enzyme preferentially hydrolyses the polyphosphoinositides PIP_2 and PIP. The rest of the phospholipase C activity is associated with the membrane and shows a preferential hydrolysis of PIP_2 with maximal activity at 4.0 μM Ca^{2+}.[24] Although the cytoplasmic Ca^{2+} concentration and phospholipid composition may influence the relative rate of hydrolysis, a single phospholipase C can account for the hydrolysis of all three phosphoinositides seen in agonist-induced activation.[513]

Within five seconds of platelet stimulation by thrombin, PAF, ADP, arachidonic acid or thromboxane mimetics, a rapid decrease in PIP_2 is measured.[40,41,63,316,413,440,492] The extent of the decrease varies between different agonists and species, but it is always transient. Levels of PIP_2 return to normal or increase above control baseline values approximately 10 seconds after the addition of agonist. The decrease in PIP_2 could be the result of phospholipase C-induced hydrolysis, reduced phosphorylation of PIP by PIP kinase, or increased dephosphorylation of PIP_2 by phosphomonoesterase. The simultaneous increase in production of inositol phosphates, in particular (1,4,5)IP_3, provides evidence that phospholipase C-mediated hydrolysis is the major cause of the measured decrease in PIP_2.[5,104,414,446]

It is generally assumed that, after the binding of an agonist to its receptor on the platelet membrane, phospholipase C initially hydrolyses the polyphosphoinositides, PIP and PIP_2, and later degrades PI.[304,513] The preferential hydrolysis of the polyphosphoinositides at low Ca^{2+} concentrations is consistent with the role of (1,4,5)IP_3 as a second messenger that triggers a rise in cytoplasmic Ca^{2+}. Therefore, agonist-induced stimulation leads directly to PIP_2 hydrolysis and the generation of small amounts of DAG and (1,4,5)IP_3. The (1,4,5)IP_3 raises cytoplasmic Ca^{2+} which initiates the breakdown of PI by phospholipase C. The bulk of PI breakdown and generation of DAG and (1)IP that occurs in thrombin-stimulated platelets has been shown to occur via direct phospholipase C hydrolysis of PI rather than via conversion of PI to PIP_2.[491,514] As the amount of PI hydrolysed is ten times greater than that of PIP_2, the production of (1,4,5)IP_3 and DAG are virtually separate events with much more DAG being produced than (1,4,5)IP_3.[304]

The Generation and Function of the Inositol Phosphates

The formation and metabolism of (1,4,5)IP_3 in platelets is extremely rapid. The IP_3 isomer initially formed is (1,4,5)IP_3 which is rapidly converted to either inositol 1,4-bis phosphate (1,4-IP_2) by a specific 5-phosphomonoesterase or to inositol 1,3,4,5-tetrakisphosphate [(1,3,4,5)IP_4] by a specific 3-kinase.[96,98,109] Degradation of the (1,3,4,5)IP_4 by the 5-phosphomonoesterase results in the formation of (1,3,4)IP_3. The further metabolism of the inositol phosphates is very complex.[305,449] The enzymes involved differ in Mg^{2+} requirements and

sensitivity to lithium and there are alternative degradation paths for some of the inositol polyphosphates. Ultimately, the inositol polyphosphates are degraded to isomers of inositol phosphate which are further dephosphorylated to inositol that is available for recycling.

In addition to inositol polyphosphates, thrombin-stimulated platelets also generate cyclic inositol polyphosphates.[42,224] In another study, both inositol 1:2-cyclic 4-bisphosphate [(1:2-cyclic,4)IP_2] and inositol 1:2-cyclic 4,5-trisphosphate [(1:2-cyclic,4,5)IP_3] were generated from vesicles containing phosphoinositide and phosphatidylethanolamine by phospholipase C.[515] Both the non-cyclic (1,4,5)IP_3 and the (1:2-cyclic,4,5)IP_3, but not the (1:2-cyclic,4)IP_2, were able to release $^{45}Ca^{2+}$ from permeabilized platelets in a concentration-dependent manner. This suggests that (1:2-cyclic,4,5)IP_3 as well as (1,4,5)IP_3, may function as a second messenger in platelets. The (1:2-cyclic,4,5)IP_3 is not a substrate for the 3-kinase and is only slowly dephosphorylated by the 5-phosphomonoesterase.[398,515] If formed *in vivo*, it might cause persistent activation of Ca^{2+} mobilization. However, the role, if any, of the cyclic polyphosphates in platelet activation is not clear at this time. The cyclic products are also metabolized back to inositol and recycled.

The (1,4,5)IP_3 that is generated by the phospholipase C-mediated hydrolysis of PIP_2 is a water-soluble molecule that is released into the cytoplasm. It has been shown that (1,4,5)IP_3 mobilizes sequestered Ca^{2+} from the endoplasmic reticulum and membrane vesicles from many cell types, including platelets.[3,12,44,515] Inositol 1:2-cyclic,4,5-

trisphosphate and (1,3,4)IP_3 also mobilize Ca^{2+}, but (1,3,4)IP_3 has only one tenth the potency of (1,4,5)IP_3 and (1:2-cyclic,4,5)IP_3.[67,221,515] The mechanism by which (1,4,5)IP_3 mobilizes Ca^{2+} from intracellular stores not been elucidated, but it is postulated that it activates a Ca^{2+} channel in the endoplasmic reticulum by a ligand-binding reaction.[55,369] Specific binding sites for (1,4,5)IP_3 have been demonstrated by specific binding of radiolabelled (1,4,5)IP_3 to membranes.[166,464] In platelets, a monoclonal antibody to (1,4,5)IP_3 was found to block the release of Ca^{2+} from membrane vesicles by (1,4,5)IP_3.[370] Recently, (1,4,5)IP_3 receptors have been characterized in neutrophils, hepatic plasma membranes and rat brain.[167,471]

It has also been demonstrated that (1,4,5)IP_3 can release Ca^{2+} from platelet plasma membrane vesicles, which suggests that (1,4,5)IP_3 may also be able to translocate Ca^{2+} across the plasma membrane.[406] Inositol 1,2,4,5-tetrakisphosphate may also function to allow an influx of extracellular Ca^{2+}.[222,223] However, its function in platelets is not known. When (1,4,5)IP_3 is added to saponin-permeablized cells, TxA_2 production, phospholipase C activation, protein phosphorylation, secretion and aggregation occur.[13,499] However, all of these events, except Ca^{2+} mobilization, are blocked by cyclooxygenase inhibitors. This indicates that platelet activation by (1,4,5)IP_3 is initiated by Ca^{2+} mobilization which activates phospholipase A_2. The thromboxane and endoperoxides that are subsequently generated induce activation by binding to specific receptors on the platelet membrane. The Ca^{2+} mobilization induced by (1,4,5)IP_3 is, in itself, not sufficient to induce secretion or aggregation of permeabilized platelets.

The Generation and Function of 1,2-Diacylglycerol

The (1,4,5)IP_3-mediated release of sequestered Ca^{2+} from the dense tubular system allows phospholipase C to hydrolyze PI and generate the bulk of DAG measured upon cell activation. Within five seconds of exposure to thrombin, platelets generate DAG.[410] Production is transient, peaking at approximately 15 seconds and then decreasing. Concomitant with DAG production is a decrease in PI. 1,2-Diacylglycerol is a neutral lipid which remains within the membrane and is rapidly phosphorylated to phosphatidic acid by 1,2-diacylglycerol kinase.[43,44,276] The major function of DAG is to activate protein kinase C which is a Ca^{2+}- and phospholipid-dependent protein kinase ubiquitous in tissues and organs.[360,361]

1,2-Diacylglycerol is enriched in stearic and arachidonic acids and may function as a source of arachidonic acid during platelet activation. The DAG second messenger signal is transient and rapidly attenuated via two different pathways.[43] The majority of DAG is phosphorylated by 1,2-diacylglycerol kinase to phosphatidic acid which is enriched in arachidonic acid at the sn-2 position. The arachidonic acid can be cleaved from this position by the action of a particulate phospholipase A_2 which appears to act selectively upon phosphatidic acid.[39] The remaining DAG is degraded by the sequential action of diacylglycerol and monoacylglycerol lipases.[27,42] Arachidonic acid is cleaved from the 2-monoacylglycerol by the action of monoacylglycerol lipase. The arachidonic acid released from DAG is then available for eicosanoid formation and amplification of platelet activation.

Phosphatidic Acid and Lysophosphatidic Acid

Both phosphatidic acid and lysophosphatidic acid are able to translocate Ca^{2+} across membranes *in vitro*. However, whether or not either has any physiological significance as a second messenger is not known. Phosphatidic acid increases the permeability of multilamellar liposomes for Ca^{2+}, but has been shown to be unable to affect translocation of Ca^{2+} across pure phosphatidylcholine membranes.[199,434] The addition of oleoylacetylglycerol to permeabilized platelets causes the mobilization of Ca^{2+} from the platelet dense tubular system.[57] This effect was dependent on the conversion of oleoylacetylglycerol to its phosphatidic acid and/or lysophosphatidic acid, but independent of arachidonic acid metabolism or protein kinase C activation. It was suggested that DAG metabolites might augment changes in Ca^{2+} homeostasis caused by (1,4,5)IP_3 in platelet activation. Phosphatidic acid added exogenously to platelets does not exert any effect, probably because it cannot physically reach a site at which it might have an effect.[32] The role of phosphatidic acid in physiologic platelet activation remains unknown.

Lysophosphatidic acid formed by deacylation of phosphatidic acid is formed in small amounts in stimulated platelets and may be released to the external medium.[39,278] Lysophosphatidic acid added to intact platelets induces shape change, secretion and aggregation.[88,154] It is also able to mobilize Ca^{2+} from Ca^{2+}-loaded membrane vesicles and has been reported to function as an ionophore *in vitro*.[154,456] It is suggested that lysophosphatidic acid-induced platelet activation

involves an initial mobilization of intracellular Ca^{2+} with subsequent activation of phospholipase A_2 and formation of arachidonate metabolites which activate phospholipase C and induce the platelet response.[497] The activation of phospholipase C by lysophosphatidic acid is blocked by inhibitors of cyclooxygenase.

The Generation and Function of Cyclic AMP

The role of the cyclic AMP second messenger system in platelets is not as well-defined as that of the inositol lipid system. The control of adenylate cyclase is mediated by binding of agonists to adrenergic receptors on the platelet membrane. Human platelets possess both $ß_2$-adrenergic receptors (60 sites/platelet) which stimulate adenylate cyclase and α_2-adrenergic receptors (150-450 sites/platelet) which inhibit adenylate cyclase.[1,175,228,247] The density of each of these receptors on platelets varies between species and determines the effect of epinephrine on adenylate cyclase and platelet function.[248] The α_2-adrenergic receptor from human platelets has been purified and characterized as a single glycoprotein of M_r = 64 kDa.[354,404] The gene that codes for the human platelet α_2-adrenergic receptor has also been cloned and sequenced.[257] Less is known about the platelet $ß_2$-adrenergic receptor than the α_2-adrenergic receptor. It is weakly coupled to the adenylate cyclase system and has an inhibitory action of questionable significance.[516]

In contrast to many cell systems, elevation of cyclic AMP in platelets is inhibitory to platelet activation.[274,333] Cyclic AMP is

produced from ATP by adenylate cyclase and is degraded by cyclic AMP phosphodiesterase. Therefore, agents which either stimulate adenylate cyclase or inhibit cyclic AMP phosphodiesterase will increase the intracellular levels of cyclic AMP and inhibit platelet function. Activators of adenylate cyclase include prostacyclin, prostaglandin D_2, prostaglandin E_1, isoprenaline and adenosine.[467] Prostacyclin is produced from arachidonic acid in endothelial cells, binds to specific receptors on the platelet membrane and increases intracellular cyclic AMP by activation of adenylate cyclase.[333,486] It is the most potent endogenous inhibitor of platelet responses. Agents that have been demonstrated to inhibit cyclic AMP phosphodiesterase include papaverine, dipyridamole and the methylxanthines.

The mechanism by which cyclic AMP inhibits platelet function is not clear, but a major effect of cyclic AMP seems to be the suppression of the generation of second messengers.[423] It has been shown to be able to inhibit the increase in intracytoplasmic Ca^{2+} concentration due to both Ca^{2+} influx and release from internal stores in response to physiological agonists.[45,409] It also enhances the sequestration of Ca^{2+} into internal storage sites.[45,242] In contrast, it does not inhibit the rise in Ca^{2+} evoked by calcium ionophores.[423] It also inhibits agonist-induced hydrolysis of inositol lipids.[277] There is some evidence that the inhibition of second messenger generation occurs by inhibition of phospholipase C, but the mechanism is not known.[254,410]

Cyclic AMP is known to modulate the activity of a specific cyclic AMP-dependent protein kinase. This kinase phosphorylates, and

therefore regulates, a number of enzymes and proteins. Increased intracellular cyclic AMP is associated with enhanced phosphorylation of several proteins, the two most prominent of which have molecular weights of approximately 22 and 24 kDa.[135] The Ca^{2+}-ATPase activity and Ca^{2+} transport across platelet internal membranes are stimulated by cyclic AMP-dependent kinase, probably via phosphorylation of a 22 kDa protein similar to phospholamban of cardiac muscle.[4] This protein in platelets has been called thrombolamban and has been found to be covalently bound to the Ca^{2+}-ATPase.[135,136]

Activation of α_2-adrenergic receptors in the presence of GTP or by stable guanine nucleotides leads to three different events: (1) inhibition of adenylate cyclase; (2) conversion of the α_2-adrenergic receptor binding site from high to low affinity; and (3) stimulation of GTPase activity.[7,79,196,227,330] Only a brief interaction between ligand and receptor is necessary to induce a persistent inhibition of adenylate cyclase in intact platelets.[299] The inhibition of adenylate cyclase is GTP-dependent, mediated through G_i and inhibited by pertussis toxin.[214,230] Addition of stable guanine nucleotides to platelet membranes converts the high affinity state of the receptor for agonists to the low affinity state.[196,329] The high affinity state of the receptor seems to represent the α_2-adrenergic receptor coupled to the G_i protein. Upon activation, the G_i dissociates into its subunits and is uncoupled from the receptor, converting it to the low-affinity state.[329,461] It has been suggested that the high affinity form of the receptor cannot exist in the resting cell because of the high levels of GTP. Instead, binding of agonist to the receptor induces the high

affinity state in which G_i is coupled to the receptor and able to modulate adenylate cyclase activity.[330,461] This high affinity state cannot be induced by α_2-adrenergic antagonists. However, recent work in which the endogenous G_i co-purified with the α_2-adrenergic receptor has indicated that resting platelets may contain high affinity state α_2-adrenergic receptors.[79,490]

Epinephrine added to human platelet rich plasma has two separate effects: (1) it induces aggregation; and (2) it inhibits adenylate cyclase. Epinephrine also potentiates aggregation by other agonists. Although there is evidence that aggregation and inhibition of adenylate cyclase are both mediated by a single α_2-adrenergic receptor, they are separate and independent events. Epinephrine induces primary aggregation and an increase, not a decrease, in cyclic AMP levels.[182] The potentiation of aggregation and secretion by epinephrine is not mimicked by inhibitors of adenylate cyclase.[486] It has been demonstrated that human platelets have an α_2-adrenergic receptor reserve, with only 10% of receptors needing to be occupied to elicit a 50% inhibition of adenylate cyclase.[287] Platelets from patients with a familial defect in which there is a pronounced reduction in the number of platelet α_2-adrenergic receptors have impaired epinephrine-induced aggregation and secretion responses, but normal inhibition of adenylate cyclase.[403] It was concluded that fewer platelet receptors are required for epinephrine-induced inhibition of adenylate cyclase than for platelet aggregation. A difference in the means by which α_2-adrenergic receptors induce aggregation and inhibition of adenylate cyclase is also suggested by the stimulus-specific desensitization of

platelets exposed to epinephrine. Exposure of platelets to epinephrine for short periods of 5-30 minutes desensitizes the ability of α_2-adrenergic receptors to both initiate and potentiate aggregation, but does not affect their ability to inhibit adenylate cyclase.[340]

The Generation and Function of Cyclic GMP

In parallel to the cyclic AMP system, cyclic GMP is produced and degraded by guanosine cyclase and cyclic GMP phosphodiesterase respectively. Stimulation of platelets by thrombin induces an elevation in intracellular cyclic GMP, but it is not apparent if it is serving a stimulatory or inhibitory role.[184] However, evidence suggests that cyclic GMP is a negative rather than positive stimulus in platelets. The effects of exogenous cyclic GMP on platelet function have been examined using a stable analogue. Aggregation, secretion, (1,4,5)IP_3 production and the rise in intracytoplasmic Ca^{2+} were inhibited in thrombin-stimulated platelets.[347] When guanylate cyclase was stimulated by incubation with sin 1 to increase cyclic GMP levels, aggregation and serotonin release in response to thrombin were inhibited slightly and Ca^{2+} influx from the external medium was inhibited markedly.[458] It was concluded that effects on phospholipase C activation were secondary to inhibition of the Ca^{2+} influx.

THE ROLE OF CALCIUM IN CELLULAR PROCESSES

Calcium plays a central role in both resting and stimulated platelets. Extracellular Ca^{2+} exists in rapid equilibrium with Ca^{2+} bound to the platelet surface and at least part of the requirement of extracellular Ca^{2+} for platelet functions such as fibrinogen binding to GPIIb-IIIa may reflect a requirement for surface-bound Ca^{2+}.[53] Platelets possess two exchangeable Ca^{2+} pools.[50] One is a slowly exchangeable Ca^{2+} pool representing 54% of exchangeable Ca^{2+} that is presumably intracellular because it is not affected by incubation with ethyleneglycoltetraacetic acid (EGTA). The other represents 46% of exchangeable Ca^{2+} and is a rapidly exchangeable pool which is likely to be surface-bound because it is removed by EGTA.

Platelets and Surface-bound Ca^{2+}

When platelets are exposed to agonists, the amount of surface-bound Ca^{2+} increases due an increase in the number of binding sites. The rate of exchange into the intracellular pool also increases. It has been determined that GPIIb and GPIIIa represent the major Ca^{2+}-binding glycoproteins on non-stimulated platelets while neither of these glycoproteins or GPIb represents the new Ca^{2+}-binding sites induced on stimulated platelets.[53] Removal of the Ca^{2+} from the platelet surface inhibits platelet function, even in the presence of 1.0 mM Mg^{2+}. This suggests that binding of extracellular Ca^{2+} to GPIIb and GPIIIa, and possibly other membrane proteins, on stimulated platelets is essential for maximal platelet responses to ADP and epinephrine. Therefore the

requirement for extracellular Ca^{2+} may represent a requirement for surface-bound Ca^{2+}.

Intracellular Ca^{2+} Pools

The exchangeable intracellular Ca^{2+} pool consists of a more rapidly exchanging pool ($t_{\frac{1}{2}}$ = 17 minutes) that corresponds to the cytoplasmic pool of Ca^{2+} and a slowly exchanging pool ($t_{\frac{1}{2}}$ = 300 minutes) that corresponds to the dense tubular system and mitochondria.[54] The cytoplasmic Ca^{2+} pool is not affected by changes in the extracellular Ca^{2+} concentration and is maintained at 0.1 μM as measured by the fluorescent indicator quin2.[407] The more slowly exchanging pool varies with changes in the extracellular Ca^{2+} concentration.

In most cell types, the maintenance of the cytoplasmic Ca^{2+} concentration below that of the external medium is accomplished by: (i) sequestration of intracellular Ca^{2+} within internal storage sites such as the endoplasmic reticulum and mitochondria; (ii) active promotion of Ca^{2+} efflux; and (iii) limiting Ca^{2+} influx. In platelets, the best characterized of these is the sequestration of Ca^{2+} into an intracellular storage site which appears to be primarily the dense tubular system, a structure derived from the megakaryocyte endoplasmic reticulum.[51] The mechanisms that mediate Ca^{2+} influx and efflux across the plasma membrane are not as well-characterized, but observations suggest that influx occurs through specific channels in the plasma membrane and Ca^{2+} efflux occurs via an undefined energy-dependent process.[51,52]

(i) Sequestration of Ca^{2+} into Internal Storage Sites

Membrane Ca^{2+}-ATPases are responsible for the energy-dependent transport of Ca^{2+} across membrane barriers against concentration gradients. The sequestration of Ca^{2+} into the dense tubular system is stimulated by Ca^{2+} and ATP and requires the presence of Mg^{2+}. A Ca^{2+}-ATPase responsible for the sequestration of Ca^{2+} into the dense tubular system has been identified in platelet dense tubular system membranes.[168,325] It has been purified and characterized and found to be a polypeptide of M_r = 100 kDa that is immunochemically related to the Ca^{2+} pump from muscle sarcoplasmic reticulum.[112] A monoclonal antibody to a 100 kDa protein has been shown to greatly inhibit Ca^{2+} uptake into platelet intracellular vesicles and the dense tubular system of platelets permeabilized with saponin.[169,170] The antibody did not affect the (1,4,5)IP_3-induced release of pre-stored Ca^{2+} in either permeabilized platelets or membrane vesicles. It was concluded from this study that the antibody bound only to the translocating channel protein associated with the Ca^{2+}-ATPase and not to calcium channels responsive to (1,4,5)IP_3.

The energy-dependent Ca^{2+} transport into the dense tubular system consumes one molecule of ATP for each two molecules of Ca^{2+} transported and proceeds through one or more phosphorylated intermediates.[113] It has been proposed that this phosphorylation of a series of intermediates is associated with a transmembrane decrease in affinity for Ca^{2+} such that the free ion can

eventually be liberated into the intravesicular space.[168] During Ca^{2+} uptake, two major polypeptides of 90 kDa and 95 kDa are slowly, but progressively, phosphorylated. As discussed earlier, the uptake of Ca^{2+} into the dense tubular system is known to be regulated, at least in part, by the cyclic AMP levels in the cell. Activation of the cyclic AMP-dependent protein kinase phosphorylates a 22 kDa protein (thrombolamban) that stimulates the activity of the Ca^{2+}-ATPase and Ca^{2+} uptake. In the absence of cyclic AMP, calmodulin may also stimulates Ca^{2+} uptake.[427] Therefore, similar to cardiac muscle cells, the platelet Ca^{2+}-ATPase activity may be under the dual control of cyclic AMP and calmodulin.

(ii) Active Promotion of Ca^{2+} Efflux Across the Plasma Membrane

The second means by which the cytoplasmic Ca^{2+} concentration is maintained at a lower concentration than in the surrounding medium is by active Ca^{2+} efflux from the cell. In most cell types, Ca^{2+} efflux is mediated by either a Ca^{2+}-ATPase or by a Na^{+}-Ca^{2+} exchanger in the plasma membrane. In platelets, the ATP-dependent Ca^{2+}-accumulating activity has been reported to be almost exclusively associated with the internal membranes.[168,325] However, recent reports describe a Ca^{2+}-activated ATPase associated with the plasma membrane.[122,123] A Na^{+}-Ca^{2+} exchanger which mediates $^{45}Ca^{2+}$ uptake in exchange for intravesicular Na^{+} has been described in human platelet plasma membrane vesicles.[405] While changes in the plasma membrane Na^{+} gradient have been shown

to affect Ca^{2+} homeostasis in resting platelets, the results of these studies also indicate that is unlikely that direct exchange of extracellular Na^{+} for intracellular Ca^{2+} is a major contribution to Ca^{2+} efflux.[52] Therefore, some other mechanism(s) must also be involved in Ca^{2+} efflux from platelets, but has not been clearly identified as yet.

(iii) Limitation of Ca^{2+} Influx Across the Plasma Membrane

Limitation of influx across the plasma membrane is the third means by which the intracytoplasmic Ca^{2+} concentration is maintained at 1.0 μM. Very little is known about Ca^{2+} influx into platelets, although presumably it represents passive Ca^{2+} movement down the plasma membrane gradient. The rate of Ca^{2+} exchange has been investigated in Glanzmann's thrombasthenia and EGTA-treated platelets as well normal platelets stimulated by a range of agonists in the presence of monoclonal antibodies to GPIIb-IIIa.[54,394] From these studies it was concluded that: (1) intact GPIIb-IIIa complex may be necessary for maintenance of the relatively slow influx of Ca^{2+} in resting platelets, but not for the very rapid influx that occurs during activation; and (2) Ca^{2+} influx in response to weak agonists occurs predominantly via a channel that is very close to, but separate from, the GPIIb-IIIa complex.

Intracytoplasmic Ca^{2+} Fluxes During Platelet Activation

Fluxes in the cytoplasmic free Ca^{2+} levels in response to agonists are most commonly measured in platelets by the fluorescent Ca^{2+} chelators quin2 and fura-2 and the photoprotein aequorin.[86] Both quin 2 and fura-2 measure the "average" free Ca^{2+} throughout the cytoplasm, compared to aequorin which is presumed to measure more localized areas of increased cytoplasmic free Ca^{2+}, possibly close to the plasma membrane. The measured Ca^{2+} flux in the absence of external Ca^{2+} is a reflection of the mobilization of Ca^{2+} from intracellular stores.

In the absence of external Ca^{2+}, there is a rapid and transient increase in cytoplasmic Ca^{2+} as measured by quin 2 or fura-2 in response to stimulation by thrombin, ADP, PAF, arachidonic acid, U46619, vasopressin and calcium ionophores.[137,174,300,393,407,483] Under these conditions, collagen has been reported to both elicit and not elicit a Ca^{2+} peak from aspirin-treated platelets.[9,350,408] Collagen does mobilize intracellular Ca^{2+} when measured by aequorin.[495] Phorbol ester does not elicit a Ca^{2+} peak when measured with quin2 or fura-2, but does with aequorin.[408,494] Epinephrine does not induce a Ca^{2+} peak in the absence of external Ca^{2+} when measured by quin2, fura-2 or aequorin.[495]

There are three possible sources for the rapid and transient increase in platelet cytoplasmic Ca^{2+} that occurs in response to the binding of an agonist to its external receptor on the platelet plasma membrane: (i) release of Ca^{2+} from the dense tubular system; (ii) release of Ca^{2+}

from internal storage sites in the membranes; and (iii) entrance of Ca^{2+} into the platelet from the external medium.

(i) Release of Ca^{2+} from the Dense Tubular System

The major internal storage site for released Ca^{2+} in platelets is the dense tubular system. The hydrolysis of PIP_2 by phospholipase C generates the water-soluble (1,4,5)IP_3 molecule which has been shown to promote the release of Ca^{2+} from non-mitochondrial stores in a variety of cell types.[36,371] In studies using both vesicles prepared from platelet intracellular membranes and intact saponin-permeabilized platelets, (1,4,5)IP_3 induced rapid release of Ca^{2+}.[12,55] Half-maximal Ca^{2+} release occurred at 1.0 μM (1,4,5)IP_3, which is compatible with the amount of (1,4,5)IP_3 produced during platelet activation.[414] The (1,4,5)IP_3-mediated release is not affected by Na^+ or Ca^{2+}, but is potentiated by increases in pH.[55] As discussed earlier, the mechanism by which (1,4,5)IP_3 mobilizes Ca^{2+} from intracellular stores has not been identified, but it is postulated that it activates a Ca^{2+} channel by a ligand-binding reaction.[55,369]

Rapid termination of the (1,4,5)IP_3 signal is of great importance in the control of Ca^{2+} release from internal stores. Dephosphorylation of (1,4,5)IP_3 and (1:2-cyclic,4,5)IP_3 by a 5-phosphomonoesterase is one means by which the Ca^{2+}-mobilizing signal may be terminated.[98] It has been proposed that the 5-phosphomonoesterase is the 47 kDa protein phosphorylated by

protein kinase C during platelet activation.[97] Purified 5-phosphomonoesterase has been shown to be activated by phosphorylation by protein kinase C. It is proposed that the initial (1,4,5)IP_3-mediated Ca^{2+} release allows phospholipase C to hydrolyze PI to generate the DAG that activates protein kinase C, which in turn terminates the Ca^{2+} signal by activating the 5-phosphomonoesterase.

Alternatively, (1,4,5)IP_3 may be phosphorylated by a specific 3-kinase to (1,3,4,5)IP_4.[98] The 3-kinase has been partially purified and is activated by the Ca^{2+}-calmodulin complex.[110,422] It is proposed that the Ca^{2+} mobilized from the dense tubular system forms a complex with calmodulin and the Ca^{2+}-calmodulin complex then stimulates the conversion of (1,4,5)IP_3 to (1,3,4,5)IP_4. Recent studies suggest that (1,3,4,5)IP_4 might modulate the entry of Ca^{2+} from the extracellular environment.[222] The (1,3,4,5)IP_4 molecule is also dephosphorylated by the 5-phosphomonoesterase to form (1,3,4)IP_3 which is the major form of IP_3 found in human platelets.[109]

(ii) Release of Ca^{2+} from Membrane Storage Sites

The second internal storage site for Ca^{2+} and possible source of released Ca^{2+} are the membranes themselves. The affinity of the PIP_2 molecule for Ca^{2+} may provide a membrane-bound Ca^{2+} store which releases Ca^{2+} to the cytoplasm when PIP_2 is hydrolyzed by phospholipase C during platelet activation.[65] It is not known if

this source makes a significant contribution to the release of Ca^{2+} from internal stores in response to platelet agonists. It is possible that the divalent cation binding sites on PIP_2 are saturated with Mg^{2+} which is present in high concentrations in the platelet cytoplasm. The release of membrane-bound Ca^{2+}, as measured by a decrease in chlortetracycline fluorescence, in platelets stimulated by thrombin or arachidonic acid does correspond closely to the breakdown of PIP_2 and may be sufficient to account for the increase in cytoplasmic Ca^{2+}.[63,132,284]

(iii) Influx of Ca^{2+} from the External Medium

The third source of the elevated cytoplasmic Ca^{2+} is influx from the external medium. The contribution of Ca^{2+} influx from the external medium to the cytoplasmic Ca^{2+} rise in response to an agonist can be determined by measuring Ca^{2+} peaks in the presence and absence of external Ca^{2+}. The maximal Ca^{2+} peak measured by quin2 or fura-2 fluorescence is much greater in the presence of external Ca^{2+} when platelets are stimulated with thrombin, collagen, ADP, PAF, U44619, calcium ionophore or vasopressin.[174,175,300,393,407,424] Not only is the cytoplasmic Ca^{2+} rise much greater in the presence of external Ca^{2+}, it is also prolonged. The ability of Mn^{2+} to quench quin2 fluorescence and the buffering capacity of quin2 were utilized to establish that Ca^{2+} influx occurs during platelet activation.[14,175]

The two major pathways for Ca^{2+} entry into cells are voltage-dependent channels and receptor-operated channels. Studies of the effects of inhibitors of voltage-dependent channels on platelet Ca^{2+} fluxes and examination of platelets for binding sites for these agents suggest that Ca^{2+} entry occurs via a pathway other than potential-sensitive channels.[373]

Platelets are presumed to possess receptor-operated divalent cation channels.[175] Very little is known about the regulation of Ca^{2+} channels in platelets. Stimulated platelets very rapidly produce (1,3,4,5)IP_4 which has been demonstrated to effect Ca^{2+} influx in other cell types. It has been proposed that Ca^{2+} mobilized from internal stores by (1,4,5)IP_3 enhances the formation of Ca^{2+}/calmodulin which activates the 3-kinase. This enzyme phosphorylates (1,4,5)IP_3 to (1,3,4,5)IP_4 which causes the second phase of Ca^{2+} mobilization from the extracellular pool.[422] This would explain why the Ca^{2+} signal is relatively prolonged in the presence of external Ca^{2+}.

Ca^{2+}-Dependent Events in Platelet Activation

The platelet proteins regulated by Ca^{2+} directly or via binding to calmodulin include myosin light chain kinase, phospholipase A_2, Ca^{2+}-dependent neutral proteases, the Ca^{2+}-ATPase and cytoskeletal proteins. The latter two have already been discussed. The intracellular Ca^{2+}-binding regulator protein calmodulin has been characterized in platelets and found to be present in high concentrations.[525]

Calmodulin is a low molecular weight (M_r = 17 kDa) activator protein whose ability to activate is dependent upon the binding of Ca^{2+} at micromolar or higher Ca^{2+} concentrations.[82] The Ca^{2+}/calmodulin complex formed when Ca^{2+} is released in activated platelets interacts with various calmodulin-binding proteins to propagate the Ca^{2+} signal to a number of platelet enzymes and structural proteins.[493] Ten distinct calmodulin-binding proteins have been observed in platelets. These include myosin light chain kinase, calmodulin-dependent phosphatase, phosphorylase kinase, cyclic nucleotide phosphodiesterase and caldesmon, but the remaining proteins have not been identified.

(i) Myosin Light Chain Phosphorylation

Platelet stimulation leads to the phosphorylation of a 20 kDa protein that has been identified as the myosin light chain.[2,106] The binding of Ca^{2+} to calmodulin in the stimulated platelet leads to the association of the Ca^{2+}-calmodulin complex with the myosin light chain kinase which is then activated. Myosin phosphorylation results in increased actin-activated ATPase activity, the association of myosin with the cytoskeleton and the assembly of myosin into filaments. When intracellular cyclic AMP levels are elevated, the cyclic AMP-dependent protein kinase is activated and can phosphorylate the myosin light chain kinase.[99] In the presence of Ca^{2+}-calmodulin complex, phosphorylation by the cyclic AMP-dependent protein kinase has no effect, but in the absence of Ca^{2+}-calmodulin the phosphorylation markedly decreases the activity of the myosin light chain kinase and its affinity for

calmodulin. This may explain the cyclic AMP-induced inhibition of myosin light chain phosphorylation, but not the reversal of phosphorylation that occurs when adenylate cyclase stimulators are added after stimulation of platelets.

(ii) Activation of Phospholipase A_2

The active hydrolysis of membrane phospholipids by Ca^{2+}-dependent phospholipase A_2 releases arachidonic acid which is converted via the cyclooxygenase complex to prostaglandins, including TxA_2 which is a potent potentiator of platelet aggregation.[220] Weak agonists such as ADP, epinephrine and low concentrations of PAF and thrombin all share the requirement that arachidonic acid must be liberated and metabolized to endoperoxides and TxA_2 for irreversible aggregation and dense granule secretion to occur. Stable prostaglandin endoperoxide analogues, like endoperoxides and TxA_2, bind to specific receptors on the platelet surface, stimulate phospholipase C and phosphoinositide hydrolysis and the platelet responses of shape change, aggregation and secretion.[445]

Two of the three major routes for arachidonate liberation from platelet membranes have been discussed earlier: phospholipase C-mediated release of diacylglycerol from phosphoinositides followed by sequential activation of diacyl- and monoacylglycerol lipases, and phosphorylation of diacylglycerol to phosphatidic acid and the activation of a phosphatidic acid-specific, Ca^{2+}-dependent

phospholipase A_2. The third pathway is the direct deacylation of phospholipids, particularly phosphatidylcholine and phosphatidylethanolamine, by a Ca^{2+}-dependent phospholipase A_2. The proportion of arachidonate derived from each of these is unclear, but the majority appears to be derived from the action of phospholipase A_2 on membrane phospholipids. It has been estimated that a maximum of 15% of arachidonic acid liberated is derived from phosphoinositides.[302,317]

Phospholipase A_2 cleaves arachidonic acid from the 2-position of phosphatidylcholine, phosphatidylethanolamine and, to a lesser extent, phosphatidylinositol. The phospholipase A_2 activity measured in platelet homogenates in earlier studies appeared to be insufficient to account for the agonist-induced release of arachidonate.[27] However, it has now been demonstrated that the activity of phospholipase A_2 in human platelet homogenates is much higher than previously thought because its activity is markedly inhibited by unsaturated fatty acids present in the homogenate.[19,20]

Phospholipase A_2 activity has been identified in both the cytoplasmic and membrane fractions of platelet homogenates.[19,262] This is similar to most cell types where membrane-bound forms of phospholipase A_2 are Ca^{2+}-dependent with pH optima in the neutral to basic range and those in the cytoplasmic or lysosomal compartments are Ca^{2+}-independent and are optimally active in the acid range.[430] It is possible that stimulation of platelets

induces redistribution of phospholipase A_2 leading to an increase in the membrane-bound Ca^{2+}-dependent enzyme. Presumably, association with membranes could be caused by an increase in cytoplasmic Ca^{2+}.[262]

Although it is frequently assumed that a major controlling factor in the liberation of arachidonic acid by phospholipase A_2 is the intracellular Ca^{2+} concentration, the control of phospholipase A_2 may be more complex. Phospholipase A_2 is a Ca^{2+}-dependent enzyme which can be activated by elevated Ca^{2+} concentrations, but this does not necessarily mean it is Ca^{2+}-controlled.[220] There is now substantial evidence that G proteins regulate phospholipase A_2 activity.[15,348,349] It is proposed that distinct G proteins modulate the activities of phospholipase C and phospholipase A_2. Since phospholipase A_2 is a Ca^{2+}-dependent enzyme which responds to increased levels of Ca^{2+}, G proteins may modulate its activity by either opening Ca^{2+} channels or by (1,4,5)IP_3-induced release of Ca^{2+} from intracellular stores.[15,220]

(iii) Activation of Ca^{2+}-dependent Protease

Two forms of Ca^{2+}-dependent protease, or calpains, have been identified in platelets: calpain I which requires 5-50 μM Ca^{2+} and calpain II which requires millimolar concentrations of Ca^{2+} for activation.[384,425] More recently, a single Ca^{2+}-dependent protease from human platelets has been purified to near homogeneity.[306] The protease consisted of two polypeptide chains of M_r = 74 kDa

and M_r = 28 kDa and was fully active at 0.06 μM Ca^{2+}. The Ca^{2+}-dependent protease has been shown to be activated when platelets are induced to aggregate by the addition of thrombin, collagen or the calcium ionophore A23187.[145] When aggregation is prevented, the Ca^{2+}-dependent protease is not activated. The major substrates for Ca^{2+}-dependent protease in the platelet that have been identified are cytoskeletal proteins (actin-binding protein, α-actinin, P235/talin and spectrin) and GPIb and GPV.[147,148,150,384] The effect of proteolysis on these proteins was discussed earlier. The Ca^{2+}-dependent protease can also irreversibly activate protein kinase C, particularly the membrane-bound form. The role of proteolytic activation of protein kinase C is not yet known.

Calcium Ionophores

Divalent ionophores such as A23187 or ionomycin facilitate the movement of divalent cations such as Ca^{2+} across membranes. The calcium ionophores are non-physiologic platelet agonists which induce shape change, aggregation and secretion.[129,312] They do not bind to membrane receptors and do not activate phospholipase C to hydrolyse the phosphoinositides.[40,412,413] Instead, they penetrate membranes and directly mobilize Ca^{2+} from internal stores. The responses of platelets, except for aggregation, are independent of external Ca^{2+}. This suggests that they mobilize Ca^{2+} from internal stores rather than from the external medium.[202] The increase in cytoplasmic Ca^{2+} concentration activates phospholipase A_2 which releases arachidonic acid from membrane phospholipids. The TxA_2 and endoperoxides formed

activate phospholipase C and stimulate the formation of the inositol lipid second messengers. In the presence of cyclooxygenase inhibitors, phospholipase C is not activated and these second messengers are not produced.[413] However, under these conditions, in the absence of phospholipase C activation, A23187 can induce phosphorylation of the 20 and 47 kDa proteins.[281] This suggests that the increase in Ca^{2+} alone is sufficient to activate the myosin light chain kinase and protein kinase C. Relatively high concentrations of A23187 also deplete the polyphosphoinositides in aspirin-treated platelets, presumably by stimulation of the phosphomonoesterases.

THE ROLE OF DIACYLGLYCEROL AND PROTEIN KINASE C IN CELLULAR PROCESSES

Protein Kinase C

The major function of DAG is to activate protein kinase C which is a Ca^{2+}- and phospholipid-dependent enzyme that is ubiquitous in tissues and organs.[360,361] Currently, seven subspecies of protein kinase C have been identified in mammalian tissues.[70,362] Protein kinase C is composed of a single polypeptide chain of M_r = 77-80 kDa and the presence of Ca^{2+} and phosphatidylserine is essential for its activation.[250,360] The preferential location of phosphatidylserine in the inner leaflet of the plasma membrane may play a role in the activity of the enzyme. 1,2-Diacylglycerol dramatically increases the affinity of protein kinase C for Ca^{2+} such that it can be activated at the cytoplasmic Ca^{2+} levels present in the resting cell.[237,477] The transient production of DAG in activated platelets is always associated

with activation of protein kinase C.[428] Protein kinase C, particularly the membrane-bound form, can also be activated irreversibly by proteolysis.[251,342,361] The Ca^{2+}-dependent protease splits protein kinase C into two fragments. The 32 kDa hydrophobic domain contains the binding sites for Ca^{2+}, phospholipid and diacylglycerol while the 51 kDa hydrophilic domain contains the catalytic site responsible for phosphorylating serine and threonine residues on target proteins. Although proteolytic conversion of protein kinase C into a Ca^{2+}- and phospholipid-independent irreversibly activated enzyme occurs in platelets activated by phorbol ester, the role of proteolytic activation of protein kinase C by physiologic agonists is not known.

In most tissues, protein kinase C is recovered mainly from the soluble fraction in an inactive form and is apparently translocated to the membrane in a Ca^{2+}-dependent fashion when the cell is stimulated.[261,517] If platelets are lysed in the presence of Ca^{2+} chelators, the protein kinase C is found in the cytosol, whereas if they are lysed in the presence of Ca^{2+} and protease inhibitors, the protein kinase C is bound to the membrane. When platelets are activated, Ca^{2+} and DAG may act together in binding protein kinase C to the membrane.[448,517] The mobilization of Ca^{2+} is associated with an increase in the number of binding sites for phorbol esters on intact platelets which may be a reflection of translocation of protein kinase C from the cytosol to the platelet membrane.[481]

The 47 kDa Platelet Substrate of Protein Kinase C

In platelets, the major substrate for protein kinase C is a 47 kDa, cytoplasmic protein which comprises 0.65% of total platelet protein.[298,485] It has been purified and found to normally exist as a monomer.[485] Two-dimensional electrophoresis of proteins from resting platelets labelled with ^{32}P demonstrated that the 47 kDa protein consists of two or three major forms that incorporate very little ^{32}P. After activation of platelets by thrombin, there are seven to nine major highly phosphorylated forms containing 83% phosphoserine and 17% phosphothreonine. It is not known at this time whether the microheterogeneity of the 47 kDa protein is the result of post-translational modification or because they are isoforms.

Although the function of the 47 kDa protein is not known with any certainty, possibilities include a lipocortin and the 5-phosphomonoesterase[97,485] The lipocortins are a group of proteins that have been reported to inhibit phospholipase A_2 by direct interaction with the enzyme.[142] It has been reported that the 47 kDa substrate of protein kinase C in platelets is a lipocortin whose inhibitory activity is suppressed by phosphorylation by protein kinase C.[485] More recent work has established that agonist-induced phosphorylation of the 47 kDa substrate by protein kinase C does not necessarily activate phospholipase A_2 and cause liberation of arachidonic acid.[102] In addition, lipocortins are phosphorylated by tyrosine kinases on tyrosine residues whereas protein kinase C phosphorylates proteins in serine and threonine residues.[64] These results indicate that the 47

kDa protein is unlikely to be lipocortin. Recent evidence has suggested that the in vitro inhibition of phospholipase A_2 by lipocortin is due to sequestration of the phospholipid substrate by the inhibitor rather than a direct interaction with the phospholipase.[111] This raises questions about the physiological significance of the inhibition of phospholipase A_2 by these proteins. It appears unlikely that lipocortin is the 47 kDa substrate.

The 5-phosphomonoesterase contributes to the termination of the Ca^{2+} signal by hydrolyzing the 5-phosphate from (1,4,5)IP_3, (1:2-cyclic,4,5)IP_3 and (1,3,4,5)IP_4 to form products that do not mobilize Ca^{2+}.[96,98] In one study, purified 5-phosphomonoesterase co-migrated with the 47 kDa protein on SDS-polyacrylamide gels.[97] When the two proteins were digested with proteases, the resulting phosphorylated peptide maps were similar. It was concluded that the 47 kDa protein and 5-phosphomonoesterase are the same protein. Protein kinase C phosphorylates, and thereby increases, the activity of the 5-phosphomonoesterase which would terminate the Ca^{2+}-mobilizing signal. This is compatible with the general role of protein kinase C as a regulator of cellular activation. It is also compatible with the ability of pretreatment with phorbol ester to cause diminished Ca^{2+} mobilization and decreased (1,4,5)IP_3 production in response to agonists.[300,389,414,526] In contrast, Ca^{2+} mobilization in response to calcium ionophore is not affected by pretreatment with phorbol ester.[526]

Recently, the 47 kDa substrate has been cloned from a human promyelocytic leukemia cell line.[488] Searches of current databases did not reveal any striking similarities between the 47 kDa protein and any known sequences. It was not similar to the human lipocortin sequence. In this same publication, reference was made to work in progress in which the 5-phosphomonoesterase could be partially separated from the 47 kDa protein kinase C substrate.

Activation of Protein Kinase C by Diacylglycerols and Phorbol Esters

The effect of DAG can be mimicked by chemically modified sn-1,2-diacylglycerols such as 1-oleoyl-2-acetylglycerol which, when added to intact platelets, intercalate into the membrane bilayer and activate protein kinase C.[238] Like DAG, they are rapidly metabolized to the corresponding phosphatidic acid. 1,2-Diacylglycerol can also be substituted for by tumor-promoting phorbol esters such as phorbol myristate acetate and 12-O-tetradecanoylphorbol-13-acetate. These have a similar structure to DAG and are able to increase the affinity of protein kinase C for Ca^{2+}, resulting in full activation of the enzyme at physiological Ca^{2+} levels.[73,74] In contrast to DAG and chemically modified diacylglycerols, phorbol esters activate protein kinase C permanently.

The addition of phorbol esters or bioactive diacylglycerols to platelet rich plasma induces a slow aggregation response and delayed dense granule secretion without causing platelet shape change.[529] Phorbol esters or diacylglycerols do not induce inositol phospholipid

hydrolysis, arachidonic acid release or formation of arachidonate metabolites and do not evoke a rise in cytoplasmic Ca^{2+} that is detectable by quin2 or fura-2.[78,279,341,408,448] However, they do induce a Ca^{2+} rise that is detectable by aequorin. This suggests that local Ca^{2+} fluxes beneath the membrane may occur, but not an increase in the overall average cytoplasmic Ca^{2+} concentration.[124,494] Bioactive diacylglycerols induce a maximal and reversible phosphorylation of the 47 kDa protein, whereas phorbol esters induce an irreversible phosphorylation.[73,238] The slow secretion by phorbol ester-stimulated platelets may be accounted for by the failure of development of a contractile skeleton.[72] Phorbol ester-stimulated platelets develop a pseudopodal cytoskeleton containing actin filaments bundled with α-actinin and actin-binding protein, but not the contractile component of actin filaments combined with myosin which probably is the means by which secretion occurs.

There are significant differences between the actions of phorbol esters and bioactive diacylglycerols such as 1-oleoyl-2-acetylglycerol. The aggregation and phosphorylation of the 47 kDa protein induced by 1-oleoyl-2-acetylglycerol is inhibited by apyrase to a much greater extent than that of phorbol ester.[11] This indicates that released ADP plays a significant role in the induction of the responses to 1-oleoyl-2-acetylglycerol. 1-Oleoyl-2-acetylglycerol has been shown to release intracellular stores of Ca^{2+} in permeabilized platelets.[57] This response was not inhibited by aspirin or reproduced by phorbol ester, but was considerably slower than the release of Ca^{2+} induced by exogenous (1,4,5)IP_3. It was concluded that the Ca^{2+} release induced

by 1-oleoyl-2-acetylglycerol was actually due to its conversion to phosphatidic acid and/or lysophosphatidic acid. However, this Ca^{2+} mobilization does not appear to be detectable by quin2, possibly because of the high buffering capacity of quin2.[408] The Ca^{2+} rise that is detected by aequorin is not greatly affected by removal of extracellular Ca^{2+}, which supports the theory that 1-oleoyl-2-acetylglycerol can mobilize intracellular Ca^{2+}.[494]

The Role of Protein Kinase C in Platelet Activation

The ability of phorbol esters to mimic the effect of DAG on protein kinase C has been used widely to investigate the role of protein kinase C in platelet activation. One of the limitations of the use of phorbol esters is that they activate protein kinase C irreversibly, which presumably has very different effects to the transient production of endogenous DAG and activation of protein kinase C by physiologic agonists. The results of these studies are somewhat confusing and often conflicting. On one hand, activation of protein kinase C is synergistic with Ca^{2+} mobilization in platelet activation. On the other hand, there are many reports of negative feedback on many aspects of platelet activation by protein kinase C.

If subthreshold concentrations of phorbol ester are added to platelets with subthreshold concentrations of calcium ionophore, rapid platelet aggregation and release occurs.[238,279] Similarly, subthreshold concentrations of phorbol ester and thrombin are synergistic in inducing platelet responses.[521] Phorbol ester or diacylglycerol also

enhances arachidonic acid release in response to calcium ionophore, even in aspirin-treated platelets.[172] Agonists which mobilize Ca^{2+} have a potentiating effect on the activation of protein kinase C.[238,448] These results have been interpreted as indicating that either 47 kDa protein phosphorylation or Ca^{2+} mobilization alone is necessary, but not sufficient by itself, for the induction of platelet activation. Both are synergistic in causing the full cellular response of aggregation and release.

There are numerous reports of inhibitory or desensitizing effects of protein kinase C activation on platelet activation induced by a wide range of agonists. The most commonly reported inhibitory effect of phorbol esters is on the mobilization of Ca^{2+} by thrombin, arachidonic acid, ADP, PAF or vasopressin.[101,264,300,389,524] Where measured, the inhibition of Ca^{2+} mobilization was accompanied by an inhibition of dense granule content secretion.[101,262,389] Similarly, preincubation with phorbol ester or diacylglycerol is reported to inhibit phosphoinositide hydrolysis as measured by production of phosphatidic acid and inositol phosphates in response to thrombin, PAF or collagen.[414,498] Surprisingly, activation of protein kinase C stimulates the accumulation of polyphosphoinositides.[171,498] Whether or not this reflects direct phosphorylation of the polyphosphoinositides by protein kinase C or an effect on the specific kinases and/or phosphomonoesterases is not known. The relationship, if any, between this accumulation and the inhibition of polyphosphoinositide hydrolysis has not been identified.

There have also been reports of potentiation of platelet secretion by shorter preincubation times with phorbol esters.[101,264,447] It appears that the rate and extent of protein kinase C activation induced by phorbol esters or bioactive diacylglycerols before addition of the second agonist determine whether platelet activation is to be inhibited or potentiated. In one study, phorbol esters added to aspirin-treated platelet rich plasma 5 or 15 seconds before epinephrine, ADP, prostaglandin endoperoxide analogue, collagen, PAF or vasopressin potentiated the rate and extent of aggregation and ATP secretion.[447] At lower concentrations of the second agonist, only aggregation was potentiated, not secretion. Similar results, although less pronounced, were obtained with preincubation periods of up to 12 minutes and in washed platelets. The exception was epinephrine which was potentiated at all concentrations and for all incubation periods. The synergism correlated with potentiation of protein kinase C activation as measured by 47 kDa protein phosphorylation. However, both phosphorylation of the myosin light chain and Ca^{2+} fluxes were reduced. This suggests that the potentiation may be associated with activation of protein kinase C, but possibly inhibition of phospholipase C.

In contrast, when non-aspirin-treated platelet rich plasma was used, the effect of phorbol ester pretreatment on secretion depended on the effect on thromboxane formation.[265] When the second agonist was a weak agonist such as ADP, epinephrine or low dose of PAF, preincubation with phorbol ester inhibited thromboxane formation, and therefore dense granule secretion. Collagen-induced thromboxane formation and dense granule secretion were enhanced by short preincubation with phorbol

ester. When cyclooxygenase was blocked by indomethacin, thromboxane production and secretion in response to weak agonists were abolished, but, if the platelets were preincubated with phorbol ester, secretion was enhanced. Apparently, inhibition of endogenous thromboxane synthesis allows potentiation of secretion by synergism between phorbol esters and the weak "thromboxane-dependent" agonists.

Treatment of intact platelets with phorbol esters has been reported to reduce the adenylate cyclase inhibition in membranes from the platelets induced by GTP and inhibitory agonists (thrombin and epinephrine) via a specific alteration in the G_i-mediated signal transduction to adenylate cyclase.[231] The free 41 kDa α-subunit of the inhibitory G protein, G_i, is a substrate for protein kinase C.[244,496] This phosphorylation is inhibited by the addition of free ß-subunit. Thus, only the free subunit, not oligomeric G_i, serves as a substrate for protein kinase C. From these findings, it was proposed that the functional state of the inhibitory coupling component for adenylate cyclase is controlled by phosphorylation of the α-subunit by protein kinase C.

In a more recent study in intact platelets, the predominant effect of phorbol ester on adenylate cyclase activity was found to be inhibitory.[512] The degree of inhibition depended on the activator of adenylate cyclase used. The effects of prostaglandin D_2 and forskolin were inhibited and the effects of prostaglandin E_1 were initially inhibited and later enhanced. The inhibition by epinephrine and high thrombin concentrations of the cyclic AMP accumulation induced by

prostaglandin E_1 was attenuated by phorbol ester, but the effect of ADP was little affected. It was concluded that, in the intact platelet, the major effects of protein kinase C are inhibitory, which suggests that the enzyme has effects other than inactivation of G_i.

The role of protein kinase C in platelet activation is complex and far from clear. The mass of conflicting data is also a reflection of the very variable conditions used in different studies, including intact platelets versus membrane preparations and the time of preincubation with phorbol ester. However, protein kinase C activation does appear to be a positive signal for platelet aggregation and secretion while suppressing signal transduction between the membrane receptor and phospholipase C.

THE ROLE OF Na^+/H^+ EXCHANGE IN CELLULAR PROCESSES

Over the last few years, considerable evidence implicating Na^+/H^+ exchange in platelet activation has accumulated, particularly in response to weak agonists. The regulation of intracellular pH is a necessary function of all cells. Cells have a large inside-negative membrane potential, therefore, passive movement of H^+ across the plasma membrane would create a very acid intracellular environment that is not compatible with normal metabolic function.[432] Each cell must have an efficient mechanism for extruding H^+ against a concentration gradient into the extracellular environment. In many cell types, including platelets, active H^+ secretion is accomplished by Na^+/H^+ countertransport.[293] There are three major functions of the Na^+/H^+

exchanger or antiporter: (1) regulation of intracellular pH and the metabolic processes that are dependent on pH; (2) provision of a mechanism of cell entry for Na^+ which serves an important role in determining the distribution of water between the cell and the extracellular space; and (3) net transportation of solutes between extracellular fluid and a transcellular space, such as intestinal or renal tubular lumina.[432]

Since the stoichiometry of Na^+/H^+ exchange is one-for-one, the exchanger is electrically neutral and insensitive to manipulation of the membrane potential.[164,454] The driving force is generated by the Na^+/K^+ ATPase which builds up an inwardly directed Na^+ gradient. The exchanger is fairly specific with Li^+ being the only cation that can replace Na^+. The Na^+/H^+ exchanger can be inhibited by amiloride, a potassium-sparing diuretic, and its analogues as well as competitively by Li^+. Amiloride and its analogues are used widely in the investigation of the role of Na^+/H^+ exchange in cell processes.

Na^+/H^+ Exchange and Cell Activation

In many non-stimulated non-epithelial cells, the exchanger appears to be nearly quiescent when the cytoplasmic pH is in the physiological range, but it can be activated by variety of stimuli including hormones, growth factors, tumor promoters and hypertonic shrinking.[164] The biological actions of most of the agents that activate the exchanger are known to be mediated by protein kinases. Therefore, it has been suggested that stimulation of the exchanger may be mediated by

phosphorylation of the exchanger or of a regulatory protein. This hypothesis has not been proven. In contrast, parathyroid hormone and cyclic AMP diminish the activity of the exchanger.

The term "activation of Na^+/H^+ exchange" is often used to describe the increase in pH that occurs when cells are stimulated by specific agonists, although the exchanger is not dormant in resting cells.[164,454] When resting platelets are treated with amiloride analogues to inhibit the exchanger, the internal pH (pH_i) decreases by about 0.05 units within eight minutes, indicating that the exchanger normally functions to continuously remove metabolically generated protons.[527] Stimulation of platelets by thrombin has been reported to cause a biphasic change in pH_i with a rapid decrease of 0.01-0.05 from a resting level of 7.09 units followed after 10-15 seconds by a sustained rise of 0.1-0.15 units.[212,527] This effect was prevented by incubation with amiloride or its analogues.

Activators of protein kinase C such as phorbol esters increase pH_i and enhance the effect of other platelet stimuli and the recovery from an acid load.[453,521] Although the mechanism by which protein kinase C alters the exchanger's properties are not yet known, it is likely that either protein kinase C or one of its phosphorylated derivatives affects the regulatory site of the exchanger.

There is also evidence that Ca^{2+} mobilization and Na^+/H^+ exchange are related, although which one affects the other is not clear. Concurrent analysis of changes in pH_i and cytoplasmic Ca^{2+} in platelets stimulated

by high concentrations of thrombin indicate that Ca^{2+} mobilization precedes the cytoplasmic alkalinization.[527] In addition, the calcium ionophore A23187 triggers a proton efflux in aspirin-treated platelets that is inhibited by amiloride. There is also evidence in favor of an effect of the exchanger on Ca^{2+} translocation. Removal of Na^+ from the extracellular medium inhibits Na^+/H^+ exchange and almost completely abolishes Ca^{2+} mobilization from internal storage sites and greatly reduces Ca^{2+} influx, although phosphoinositide hydrolysis is not affected.[451,452] It has also been demonstrated that the $(1,4,5)IP_3$-mediated release of Ca^{2+} from intracellular stores in permeabilized platelets is pH-dependent with increased pH potentiating release, especially at low concentrations of $(1,4,5)IP_3$.[55]

Although it appears that cytoplasmic alkalinization is an important step in platelet activation, an artificial increase in pH_i does not affect Ca^{2+} translocation or initiate platelet aggregation.[451,452] It appears that the role of cytoplasmic alkalinization is in the enhancement of the signal processing induced by platelet agonists. While the greatest increase in pH_i is seen with strong activation of platelets, such as with high concentrations of thrombin, the enhancement of signal processing by alkalinization becomes relevant when platelets are weakly activated.

Weak Platelet Agonists and Na^+/H^+ Exchange

The role of Na^+/H^+ exchange in activation of platelets by the weak agonists is an area in which there has been considerable interest over

the last few years. Weak agonists are those whose secondary aggregatory and secretory effects are dependent upon formation of arachidonate metabolites and inhibited by aspirin and other cyclooxygenase inhibitors. If insufficient arachidonate metabolites are produced, aggregation is reversible and secretion absent or markedly inhibited. The signals produced by these agonists are small and harder to detect; therefore, relatively little is known about their signal transduction. In addition, synergism between different weak agonists is an important mechanism of platelet activation, possibly via multiple effector mechanisms. It is also difficult to separate the initial signal from that produced as a result of positive feedback loops.

Most, if not all, weak agonists decrease the cyclic AMP content of platelets. However, a decrease in cyclic AMP production is not the major signalling mechanism used by these agonists to induce platelet aggregation and secretion. The main means by which this effect of weak agonists is detected in intact cells is their ability to inhibit the cyclic AMP increase induced by prostaglandin E_1 or forskolin. In broken cell preparations, epinephrine, ADP, PAF and low concentrations of thrombin have been shown to inhibit adenylate cyclase in a GTP-dependent and Na^+-dependent manner.[8,100,229,511] The decrease in cyclic AMP can be separated from the signal which mediates aggregation and secretion. Exposure of platelets to a low concentration of epinephrine desensitizes them to the aggregatory effect of subsequent exposure to higher concentrations of epinephrine, but does not affect the ability of epinephrine to inhibit adenylate cyclase.[340] Similarly, removal of

extracellular Na^+ does not alter the ability of epinephrine to inhibit adenylate cyclase, but it does prevent aggregation and secretion in response to epinephrine, ADP and low concentrations of thrombin.[94] These results suggest that either aggregation and secretion are controlled by events distal to cyclic AMP production, or that the two processes are mediated by two separate signalling pathways. Although these paths may be separate, they are still connected functionally in that an elevation in cyclic AMP levels inhibits epinephrine-induced platelet activation.[232]

Monovalent cations, preferably Na^+, as well as GTP, are necessary for the epinephrine-induced inhibition of the increase in cyclic AMP levels induced by prostaglandin E_1 in platelet membranes.[229] This requirement for Na^+ applies to all agents that are able to inhibit adenylate cyclase. In a study in which platelets were activated in Na^+-free extracellular medium, there was complete inhibition of secondary aggregation and secretion in response to weak agonists.[476] Primary aggregation, and therefore agonist binding and fibrinogen binding were not affected.

The effect of changing the Na^+ concentration on the responses of platelets to weak agonists is consistent with a role for Na^+/H^+ exchange in platelet activation. To examine this theory, Na^+/H^+ exchange has been blocked by three different manipulations: (1) preparation of platelets in Na^+-free buffer; (2) increased extracellular H^+ concentration by decreasing the pH of the suspending buffer from 7.35 to 6.95; and (3) addition of the amiloride analogue

ethylisopropylamiloride to directly inhibit the Na^+/H^+ exchanger.[474] These manipulations caused complete inhibition of secondary aggregation and secretion in response to epinephrine, ADP or low concentrations of thrombin. Therefore, the responses eliminated by removal of extraplatelet Na^+ are the same as those eliminated by treating platelets with a cyclooxygenase inhibitor, suggesting a strong link between the presence of extracellular Na^+ and the operation of platelet function mediated by the cyclooxygenase pathway.[95]

Na^+/H^+ Exchange and Arachidonic Acid Mobilization

Subsequent studies provided evidence that inhibition of Na^+/H^+ exchange by any of the above manipulations inhibits mobilization of arachidonic acid in response to these weak agonists.[472] The conversion of exogenous arachidonic acid to TxA_2 was not affected. Epinephrine has been reported to be a unique platelet agonist in that it does not induce shape change, requires extracellular divalent cations and does not directly activate phospholipase C.[444] The activation of phospholipase C by epinephrine is totally dependent upon released ADP and arachidonate metabolites. When Na^+/H^+ exchange, or cyclooxygenase, was blocked, the activation of phospholipase C in response to epinephrine, and ADP, was also completely blocked.[474] These results suggest that these agonists activate phospholipase C secondary to activation of Na^+/H^+ exchange and mobilization of arachidonic acid. It was proposed that blockade of Na^+/H^+ exchange would block epinephrine-induced mobilization of arachidonic acid which would in turn prevent phospholipase C activation. Inhibitors of Na^+/H^+ exchange or

cyclooxygenase have no effect on the activation of phospholipase C by high concentrations of thrombin or the endoperoxide analogue U46619, indicating that these inhibitors do not affect phospholipase C itself.[474]

The technique of gas chromatography/mass spectrometry has been used to assess the mobilization of arachidonic acid in response to epinephrine in the presence of a specific inhibitor of phospholipase C.[474,476] In these experiments, a very small pool of arachidonic acid mobilized in response to epinephrine was detected. This mobilization could be blocked by ethylisopropylamiloride, removal of extraplatelet Na^+ or reduction of extraplatelet pH. From this, it was postulated that a phospholipase A_2 controlled by Na^+/H^+ exchange might exist. This was supported by the finding that lysophosphatidylinositol production in response to epinephrine and ADP was not affected by thromboxane receptor antagonists, but was inhibited by perturbants of Na^+/H^+ exchange.[473,476] Inhibition of Na^+/H^+ exchange decreased lysophosphatidylinositol production in response to high concentrations of thrombin and completely inhibited production in response to low concentrations.

Epinephrine also caused a rapid decrease in membrane-bound Ca^{2+} as measured by chlortetracycline fluorescence, which suggests that epinephrine is able to mobilize a small pool of local Ca^{2+}.[473] The activation of Na^+/H^+ exchange may provide localized Ca^{2+} mobilization plus an alkaline environment, both of which are conducive to activation of phospholipase A_2.[38] Platelet phospholipase A_2 has a pH optimum of

8.0, and, at this pH, enzymatic activity can be detected at lower Ca^{2+} concentrations.[476] The small pool of mobilized arachidonic acid is converted to TxA_2 which activates phospholipase C when it binds to the TxA_2/PGH_2 receptor. Phospholipase C activation leads to mobilization of much larger pools of arachidonic acid which are detectable by measurement of ^3H-arachidonic acid release or by radioimmunoassay for TxB_2. Strong agonists, such as high concentrations of thrombin, activate phospholipase C more directly and do not need prior formation of cyclooxygenase products or activation of Na^+/H^+ exchange.

Na^+/H^+ Exchange, Fibrinogen Binding and the α_2-Adrenergic Receptor

In another study, the ability of epinephrine to activate phospholipase C was found to be blocked completely by inhibitors of the α_2-adrenergic receptor, cyclooxygenase, phospholipase A_2, Na^+/H^+ exchange or fibrinogen binding to GPIIb-IIIa and by removal of fibrinogen.[22] In addition, the cytoplasmic alkalinization which was measured in response to epinephrine was inhibited by ethylisopropylamiloride or by an antibody that prevents fibrinogen interaction with GPIIb-IIIa. It was proposed that epinephrine promotes exposure of GPIIb-IIIa sites for fibrinogen binding which leads to cytoplasmic alkalinization by activation of Na^+/H^+ exchange. This, in conjunction with local shifts in Ca^{2+}, promotes low-level activation of phospholipase A_2. The released arachidonic acid is converted to cyclooxygenase products which, potentiated by epinephrine, activate phospholipase C.

There is considerable evidence that α_2-adrenergic receptors may be very closely linked to the Na^+/H^+ exchange mechanism, if not themselves part of the mechanism. Agents such as amiloride analogues, Na^+ or H^+, which interact with Na^+/H^+ exchangers all affect α_2-adrenergic receptor affinity for agonists.[229,291,329,364] The α_2-adrenergic receptor itself may play a dual role as both receptor and ion-transporter.

Platelet Activating Factor and Na^+/H^+ Exchange

Platelet activating factor shares some of the features of weak platelet agonists. Vigorous aggregation is inhibited by cyclooxygenase inhibitors and thromboxane antagonists and it elicits a GTP-dependent and Na^+-modulated inhibition of adenylate cyclase.[475,511] There is however, considerable variation in opinion concerning the role of arachidonic acid metabolites in PAF-induced aggregation. At low concentrations of PAF it acts as a weak agonist, but at high concentrations it is less dependent on arachidonate metabolites. While a thromboxane antagonist completely inhibits dense granule secretion, ethylisopropylamiloride causes partial inhibition of release.[476] This suggests that PAF may utilize two separate pathways to elicit full platelet activation with inhibition of Na^+/H^+ exchange not causing complete blockade of arachidonic acid release in response to high concentrations of PAF.

Calcium Ionophores and Na^+/H^+ Exchange

Activation of platelets by the calcium ionophore A23187 has been shown to activate Na^+/H^+ exchange in addition to mobilizing intracellular Ca^{2+} stores.[450] The release of protons mediated by the accelerated Na^+/H^+ exchange was markedly inhibited by amiloride or decreasing the Na^+ in the medium. Aggregation of platelet rich plasma by 20 μM A23187 was completely inhibited by inhibition of Na^+/H^+ exchange. Although inhibition of aggregation in response to very high concentrations of A23187 (40 μM) appeared to be only partial, at this concentration cell lysis may occur. The release of arachidonic acid or measurement of TxB_2 production was not measured, therefore it is not known if inhibition of Na^+/H^+ exchange affects the activation of phospholipase A_2 by A23187. As aggregation in response to A23187 is totally dependent upon formation of arachidonate metabolites, failure to generate these may explain the complete inhibition of aggregation. Alternatively, cytoplasmic alkalinization may be essential for aggregation to occur, possibly by pH-dependent effects on the cytoskeleton.[289]

Thrombin and Na^+/H^+ Exchange

Thrombin accelerates Na^+/H^+ exchange and blockade of this exchange inhibits platelet responses to lower, but not high, concentrations of thrombin. However, the mechanism by which thrombin stimulates Na^+/H^+ exchange is not known. At low concentrations, thrombin acts as a weak agonist and can only activate phospholipase C secondary to activation

of Na^+/H^+ exchange and mobilization of arachidonic acid. Higher concentrations of thrombin activate phospholipase C directly and aggregation is not dependent upon Na^+/H^+ exchange or arachidonate metabolites. It is probable that the production of DAG and activation of protein kinase C enhances or mediates acceleration of Na^+/H^+ exchange. The thrombin-induced activation of Na^+/H^+ exchange in hamster fibroblasts has been demonstrated to be at least partly mediated by the activation of protein kinase C.[374] Activation of protein kinase C by addition of phorbol ester to platelets has been shown to evoke Na^+-dependent alkalinization.[453]

The responses of thrombin, and possibly PAF, indicate that there are at least two ways in which platelet agonists can accelerate Na^+/H^+ exchange: (1) a pathway used by weak agonists which lies close to the receptors themselves, is enhanced by fibrinogen occupancy of GPIIb-IIIa and is probably independent of protein kinase C; and (2) secondary to phosphoinositide hydrolysis, DAG production and protein kinase C activation.

THE RELATIONSHIP OF SECOND MESSENGERS TO PLATELET FUNCTION

Although quite a lot is known about the formation of second messengers and their biochemical functions in platelets, relatively little is known about their relationship to the physical events of shape change, aggregation and secretion of granule contents. Many events occur concurrently during platelet activation, making it very difficult to establish cause-and-effect relationships between them.

A wide range of platelet agonists stimulate phosphoinositide hydrolysis by phospholipase C in platelets. These agonists include thrombin, collagen, PAF, vasopressin, arachidonic acid, and endoperoxide and thromboxane analogues.[40,41,239,413,441,443,444] Whether or not ADP stimulates phospholipase C is not clear, but recent work suggests that ADP can generate (1,4,5)IP_3 and phosphatidic acid in aspirin-treated platelets.[108,448] As discussed above, epinephrine does not activate phospholipase C directly, but indirectly activates phospholipase C via cyclooxygenase products.

Shape Change

All agonists that induce direct hydrolysis of phosphoinositides by phospholipase C induce shape change. Epinephrine does not induce shape change that is detectable on an aggregometer tracing. Both phosphoinositide hydrolysis and shape change are independent of extracellular Ca^{2+} and Mg^{2+} and are not blocked by calmodulin antagonists, inhibitors of arachidonic acid release or cyclooxygenase inhibitors.[318,441,444]

Shape change is also associated with the phosphorylation of the 20 kDa myosin light chain.[107] Because myosin phosphorylation is very rapid and precedes the onset of shape change, it may play a role in the initiation of shape change.[116] Myosin light chain phosphorylation is regulated at least in part by Ca^{2+}, which may also play a role in the induction of shape change. However, myosin phosphorylation may occur at near resting levels of cytoplasmic Ca^{2+} under some circumstances,

such as in the absence of external Ca^{2+}.[176] The physiological significance of this is not known, but it does suggest that Ca^{2+} and calmodulin may not be the only means by which myosin light chain kinase is regulated. An increase in cytoplasmic Ca^{2+} alone does not induce shape change by physiologic agonists.[407] Although myosin phosphorylation is reversible, shape change induced under these circumstances is irreversible, indicating that myosin phosphorylation may be required for the initial change in platelet shape, but is not necessary for maintenance of the change.[107]

There is no evidence linking activation of protein kinase C and phosphorylation of the 47 kDa protein to shape change. Phorbol esters or diacylglycerol induce maximal activation of protein kinase C without inducing shape change that is detectable on an aggregation tracing.[529] Treatment of platelets with phorbol esters induces the extension of pseudopods, but does not induce spheration. Phorbol ester-stimulated platelets develop a pseudopodal cytoskeleton containing bundles of actin filaments, but not the contractile component of actin filaments combined with myosin.[72]

Secretion of Granule Contents

Agonists such as high concentration of thrombin, collagen, A23187, PAF, vasopressin and endoperoxide analogues can induce platelet secretion independently of extracellular Ca^{2+} and in the absence of aggregation.[441,448] The dense granule secretion induced by weak agonists such as ADP and epinephrine is dependent upon extracellular

Ca^{2+} and Mg^{2+} and the secondary aggregation induced by arachidonate metabolites. Secretion only occurs in response to ADP in medium that is low in Ca^{2+} and high in Mg^{2+}, such as in citrated platelet rich plasma.[343]

There is a strong relationship between phosphoinositide hydrolysis and secretion of dense granule contents from platelets. The phospholipase C activation associated with shape change may be separated from the phospholipase C activation associated with secretion.[444] The inositol lipid hydrolysis associated with secretion is quantitatively greater than that associated with shape change, with the extent of secretion being generally proportional to the extent of phosphoinositide hydrolysis. Strong agonists such as high concentrations of thrombin induce greater activation of phospholipase C and release of dense granule contents than weaker agonists.[276,443] The phosphoinositide hydrolysis associated with secretion by weak or intermediate agonists may be partly or entirely mediated by activation of phospholipase C by arachidonate metabolites or released ADP.[444] However, there are circumstances under which secretion may occur in the absence of phosphoinositide hydrolysis. In one study using permeabilized platelets in the presence of high (1.0 mM) concentrations of Ca^{2+}, thrombin induced secretion without phosphoinositide hydrolysis or protein phosphorylation.[280] In another study, thrombin was unable to activate phospholipase C, mobilize intracellular Ca^{2+} and cause secretion in platelets inhibited with GDPßS which inhibits G protein function.[56] However, increasing the thrombin concentration could overcome the inhibition of secretion, but not the inhibition of

phospholipase C activation. These findings indicate that there must be a pathway for thrombin-induced secretion that is independent of phosphoinositide hydrolysis.

Secretion is very likely initiated by an increase in the cytoplasmic Ca^{2+} concentration. If platelets are permeabilized by exposure to saponin or an intense electric field, the intra- and extracellular Ca^{2+} concentrations equilibrate. Under these conditions it has been found that release of dense granule contents can be induced by an increase in the Ca^{2+} concentration in the medium, with half-maximal release occurring at 1-10 μM free Ca^{2+}.[55,253] This Ca^{2+} sensitivity in permeabilized platelets is greatly enhanced by the addition of thrombin, GTP analogues, phorbol ester or diacylglycerol.[186,188,256] The dose-response curve for Ca^{2+}-induced secretion is shifted to the left and the half-maximal stimulatory dose decreases to 0.5 μM, which is within the cytoplasmic Ca^{2+} range reached during stimulation by physiologic agonists. Agonists such as ADP or epinephrine which cause little or no activation of phospholipase C and protein kinase C do not enhance the Ca^{2+} sensitivity of secretion.[255] Thus it appears likely that the increase in cytoplasmic Ca^{2+} and activation of protein kinase C act synergistically to mediate secretion.

Myosin light chain phosphorylation is further increased during secretion and may be responsible for inducing secretion.[185,363] Phosphorylation of myosin light chain increases the activity of actin-activated myosin ATPase and the resultant contraction presumably mediates the secretion of granule contents. The calmodulin antagonist

trifluoperazine has been shown to inhibit platelet aggregation and secretion in response to thrombin, trypsin, collagen, epinephrine, A23187 and arachidonic acid.[133] Trifluoperazine also inhibited thrombin-induced phosphorylation of the 20 and 47 kDa proteins. However, trifluoperazine has also been shown to be able to inhibit secretion without significantly inhibiting myosin light chain phosphorylation.[203] It may be that the Ca^{2+}-dependent myosin phosphorylation is essential for secretion, but does not regulate the response.[448]

Phosphorylation of the 20 and 47 kDa proteins are probably both important steps in secretion. Secretion of dense granule contents and phosphorylation of the 47 kDa protein have been closely linked in several studies.[183,185,363,368] Protein kinase C activation alone is not sufficient to induce secretion, but when combined with an increase in cytoplasmic Ca^{2+}, secretion can be triggered.[238,279] The synergistic actions of Ca^{2+} mobilization and phorbol ester-induced activation of protein kinase C are associated with potentiation of secretion and aggregation.[448]

The role of protein kinase C activation in secretion has been investigated by the use of inhibitors of protein kinase C such as sphingosine and staurosporine.[178,500] These studies suggest that protein kinase C is a positive signal for secretion and secondary aggregation, but some caution needs to be used in the interpretation of the results of these studies because of the lack of specificity of available inhibitors of protein kinase C. Preincubation with

sphingosine resulted in the inhibition of platelet secretion and secondary aggregation in response to ADP, γ-thrombin, collagen, arachidonic acid and PAF.[178] This inhibition was overcome by the addition of phorbol ester, which suggests that it was the result of inhibition of protein kinase C. The initial shape change and primary phase of aggregation were not affected. Higher concentrations of agonists induced irreversible aggregation and partial secretion in the presence of sphingosine, suggesting the existence of protein kinase C-independent pathways for platelet aggregation. Staurosporine fully inhibited phosphorylation of the 47 kDa protein, but also inhibited phosphorylation of the 20 kDa myosin light chain.[500] This was associated with inhibition of thrombin-induced secretion and slowing of the aggregation response. Activation of phospholipase C measured by phosphatidic acid and inositol phosphate production was not affected.

Aggregation

Aggregation is dependent upon the presence of either extracellular Ca^{2+} or Mg^{2+} for the binding of fibrinogen to its receptor in addition to the requirement for membrane-bound Ca^{2+} for the integrity of the GPIIb-IIIa complex. The role of cytoplasmic Ca^{2+}, if any, in the exposure of fibrinogen receptors and aggregation is not known. Aggregation can be observed in the absence of phosphoinositide hydrolysis, such as the primary aggregation response of epinephrine and aggregation induced by phorbol ester. Phorbol esters also enhance fibrinogen binding which suggests that activation of protein kinase C is involved in the exposure of fibrinogen receptors and

aggregation.[358,438] The ability of epinephrine to induce fibrinogen binding and primary aggregation indicates that there are also mechanisms that are independent of protein phosphorylation.[437]

A hypothetical model for platelet activation involving two major steps has been proposed by Siess.[449] The first step is the formation of (1,4,5)IP_3, Ca^{2+} mobilization and myosin light chain kinase phosphorylation. These events cause shape change. In the second step, mobilization of Ca^{2+} primes protein kinase C for activation by DAG formed from hydrolysis of PI. Protein kinase C activation is related to platelet aggregation and secretion. Although this scheme fits for some agonists, it still leaves many questions unanswered.

BASSET HOUND HEREDITARY THROMBOPATHY

The study of inherited platelet disorders has contributed greatly to the knowledge of normal platelet function. Inherited abnormalities of the membrane glycoproteins such as Glanzmann's thrombasthenia (deficiency of GPIIb-IIIa) and Bernard-Soulier syndrome (deficiency of GPIb/IX and GPV) have already been discussed. These syndromes have been invaluable in defining the normal structural and functional roles of these glycoproteins. The inherited abnormalities involving platelet granules have also been briefly discussed. These platelet abnormalities are usually part of a genetic defect which affects many tissues and organs. There are numerous other congenital platelet defects described, most of which are sporadic and not yet well understood. They include abnormalities of adhesion, secretion,

thromboxane production, Ca^{2+} mobilization, glycoproteins, specific agonist receptors, enzymes and phosphatidylinositol metabolism.[120,121,180,206,258,269,270,271,400,401,402,403,479]

Basset hound hereditary thrombopathy is an inherited platelet disorder in purebred Basset hounds. To this date, no similar disorder of human or other animal species platelets has been described. The disease was first identified in the 1970's after a veterinarian requested help with the problem of hemorrhagic diatheses in Basset hounds. In the initial investigation, affected dogs were found to have prolonged bleeding times and complete failure of primary aggregation in response to all concentrations of ADP used.[29,236] Testing of 92 related Basset hounds in Michigan suggested an autosomal recessive inheritance pattern.[29]

In a more detailed investigation of 45 of these dogs, Basset hound hereditary thrombopathy was found to have many features in common with Glanzmann's thrombasthenia of man.[29] These included prolonged bleeding times, normal coagulation parameters and von Willebrand factor levels, unaltered platelet numbers, normal morphology by light microscopy and transmission electronmicroscopy and failure of primary aggregation in response to ADP. One major difference between Basset hound thrombopathy and Glanzmann's thrombasthenia is the normal clot retraction seen in Basset hound hereditary thrombopathy. All affected dogs had a moderately severe hemorrhagic diathesis typical of defective platelet function with easy bruising, petechiation, mucosal hemorrhages and excessive bleeding associated with shedding of deciduous teeth and

estrous cycles. Primary aggregation in response to all concentrations of ADP and collagen was completely absent.

It was found that animals with abnormal responses could be divided into two groups. There were those described above with complete failure of primary aggregation in response to ADP. There was also a group of dogs who aggregated in response to collagen, but whose aggregation response to ADP was weaker both in rate and extent than those dogs that were classified as normal. This group were assumed, but not proven, to be heterozygotes. The exact status of dogs with subnormal aggregation responses to ADP remains to be clarified.

Earlier investigation into the nature of the defect in Basset hound hereditary thrombopathy was based on the hypothesis that the defect may be a model for Glanzmann's thrombasthenia in human platelets. This work included investigation of the aggregation and dense granule ATP secretion patterns of affected platelets in response to ADP, examination of the membrane glycoprotein content and investigation of the ability of affected platelets to bind fibrinogen.

In addition to the failure of primary aggregation, affected platelets also exhibit abnormal dense granule ATP secretion in response to ADP.[377] Release of ATP in response to 10 μM ADP occurred approximately six times more rapidly than from control canine platelets, but the quantity of ATP released did not differ significantly. The aggregation and secretion defect in response to ADP was confirmed by transmission electronmicroscopy.

Three different techniques were used to compare the GPIIb-IIIa content of control canine and affected platelets: (1) crossed immunoelectrophoresis; (2) nonreduced-reduced two-dimensional electrophoresis; and (3) O'Farrell two-dimensional electrophoresis.[378] In the crossed immunoelectrophoresis procedure, the canine GPIIb-IIIa cross-reacted with the AP-2 monoclonal antibody and a commercially available anti-human platelet antibody. All three methods detected normal quantities of GPIIb-IIIa and no other glycoprotein differences. A minor difference in a small cluster of low molecular weight lipid and/or sialoglycoprotein constituents was detected on the O'Farrell two-dimensional gels. The significance of this difference is not known.

Although affected platelets were shown to have normal GPIIb-IIIa content, this did not indicate if the complex is functional and able to bind fibrinogen. Recently, there have been reports of cases of Glanzmann's thrombasthenia in which the GPIIb-IIIa content is normal, but the complex is not functional.[365,480] The ability of affected platelet GPIIb-IIIa to bind fibrinogen was examined by measuring their ability to bind both liquid and solid phase fibrinogen. When affected platelets were activated with ADP in the presence of liquid phase ^{125}I-fibrinogen, affected platelets bound labelled fibrinogen to the same extent as control canine and human platelets.[376]

Fibrinogen binding was also examined directly by scanning electronmicroscopy utilizing colloidal gold probes.[126] Using ADP-induced adhesion and spreading, affected platelets were labelled with

either albumin-gold, fibrinogen-gold or fibrinogen-gold with blocking unlabeled fibrinogen. Platelets from humans and normal unrelated dogs were used as controls in a comparative study. The surface labelling of fibrinogen receptors seen in affected dogs was identical to control canine and similar to human platelets. The binding in all groups was blocked by physiologic concentrations of unlabelled fibrinogen. These results clearly demonstrated that affected platelets are able to bind fibrinogen in response to ADP, even although there is failure of primary aggregation.

These results are in conflict with the work of Catalfamo et al.[75] in which it was reported that affected platelets expressed fibrinogen binding sites only when activated by thrombin, not when activated by ADP. The fibrinogen binding test utilized in their experiments was the fibrinogen-coated bead method which depends on bead agglutination after the addition of platelets.[90] This method gave negative results with affected platelets for all agonists that failed to induce aggregation. This method was originally devised to examine thrombasthenic platelets for GPIIb-IIIa and the ability to bind fibrinogen. As thrombasthenic platelets lack GPIIb-IIIa, they cannot bind fibrinogen or aggregate and therefore do not agglutinate the coated beads. If platelets are able to bind to fibrinogen on the beads, but are unable to aggregate is seems probable that the beads would not agglutinate as there is no platelet-to-platelet binding. This is compatible with the negative results for agonists which do not induce aggregation and the positive result for thrombin, which was able to aggregate affected platelets. If this is so, the bead agglutination test is a test of fibrinogen

binding and aggregation rather than a specific test for fibrinogen binding. This could explain the discrepancy between these two studies.

Because platelet adhesion is defective in Glanzmann's thrombasthenia, the adhesion of affected platelets was investigated.[314] Affected platelets have been shown to have normal plasma and platelet fibrinogen and plasma von Willebrand's factor levels.[29,75] The levels of the other adhesive proteins thrombospondin and fibronectin have not been examined. Contact reactivity of affected platelets was studied by whole mount electron microscopy.[314] Affected platelets attached poorly to a formvar substrate and contact-induced shape change was inhibited compared to control platelets. The addition of ADP to affected platelets induced almost a four-fold increase in the number of spread forms and a five-fold increase in adherent affected platelets, but did not cause aggregate formation.

It has also been reported that affected platelets have basal intracellular cyclic AMP levels elevated 30-90% over controls due to impaired regulation of cyclic AMP phosphodiesterase activity.[47,48] When affected platelets were incubated with forskolin, which directly activates adenylate cyclase, the cyclic AMP levels elevated further and did not return to basal levels. This elevation was inhibited by epinephrine and augmented by prostacyclin.[47] There was no significant difference between the cytoplasmic cyclic AMP phosphodiesterase activities of control and affected platelets, although both were higher than human platelets. Affected platelets were also found to be more sensitive to the inhibitory effects of forskolin and MIX, a competitive

inhibitor of cyclic AMP phosphodiesterase.[48] The concentrations of forskolin or MIX required to inhibit γ-thrombin-induced aggregation were significantly less than that required to inhibit control platelet aggregation. From these two studies it was concluded that affected platelets have functionally intact G_i and G_s proteins and cyclic AMP phosphodiesterase with impaired regulation of the cyclic AMP phosphodiesterase.

It is apparent that Basset hound hereditary thrombopathy is not a model for Glanzmann's thrombasthenia and represents a new and unique platelet defect. A logical approach to identifying the defect is to expand the range of physiologic and non-physiologic agonists used to investigate the aggregation defect to see if this provides any direction for further study. While this project was being undertaken, some similar work was published.[75] In this study, affected platelets aggregated in response to thrombin and high concentrations of calcium ionophore A23187, but failed to aggregate in response to ADP, sodium arachidonate and collagen. These results imply that affected platelets are capable of binding fibrinogen and aggregating, if the signal transduction system can carry the appropriate information to the cell. It is proposed that affected platelets have a defect in either the second messenger systems or in the regulation of these systems. While this covers a very broad area, it is hoped that the results of these studies will narrow the field and identify an area that can be investigated in more detail.

CHAPTER II: STATEMENT OF THE PROBLEM

Like most such endeavors, this dissertation is a study that does not begin exactly at the beginning, or end at the end. The very beginning is the clinical research that made it possible to identify the existence of the platelet problem. The end may be the identification of the Basset hound hereditary thrombopathy gene, its product, and the role of this product in the control of platelet responses. The middle of the study is the identification of the precise pathway or enzyme system that is defective. It is this middle area that was the focus of this dissertation study, with the aim being to identify the biochemical nature of the defect in Basset hound hereditary thrombopathy.

The experiments planned to investigate this defect were not without precedent. Because of the high frequency of fatalities associated with arteriothrombosis in humans, it is important to determine the role that platelets play in the pathogenesis of this disease. The classic theory of the role of platelets in thrombus formation has been constructed principally from observations of normal platelet function. Investigation of congenital platelet defects has revealed unexpected relationships between the various events that occur during platelet activation. These "experiments of nature" have contributed greatly because they force scientific reconciliation between data obtained from genetically defective platelets and that obtained by investigation and

manipulation of normal platelets. Hereditary defects in man frequently evolve as the consequence of familial consanguinity. A similar origin is responsible for animal models of hemorrhagic disease.

Although there existed only a narrow data base at the start of this investigation, there was ample evidence of a hemorrhagic diathesis associated with hereditary Basset hound thrombopathy. The heritability, clinical signs, prolonged bleeding times and presence of an aggregation defect with abnormal granule release and normal fibrinogen binding had all been established prior to the onset of these studies. Since there was now substantial and convincing evidence for normal fibrinogen binding by affected platelets, further investigation of this area was unlikely to identify the defect.

In the initial investigation of Basset hound hereditary thrombopathy, adenosine diphosphate (ADP) was the only agonist which had been methodically investigated with respect to aggregation, dense granule release and fibrinogen binding. Comparison of these results with those of normal canine platelets did not reveal an obvious location of the defect in affected platelets. Therefore, it was judged that a broader knowledge of the aggregation and dense granule secretion patterns of control and affected canine platelets could be an important initial step in localizing the area of investigation. Thus, the first study involved a complete review and extension of the aggregation studies that had been done previously.

The choice of agonists for all the studies was based primarily on the ability of the various agonists to activate platelets at different points in the proposed pathway of events that occurs during activation. The non-physiologic agonist phorbol myristate acetate directly activates protein kinase C and therefore bypasses phospholipase C-dependent inositol lipid hydrolysis. Similarly, high concentrations of the physiologic agonist, thrombin, are also proposed to be able to activate platelets independently of inositol lipid metabolism. In contrast, the weak agonists ADP, low concentrations of platelet activating factor (PAF) and thrombin require the release of dense granule ATP and arachidonate metabolites for irreversible aggregation to occur. The non-physiologic agonist calcium ionophore A23187 is dependent upon the formation of arachidonate metabolites for activation of phospholipase C and inositol lipid hydrolysis to occur. Factors such as availability and knowledge of the responses of canine platelets based on reports in the literature and preliminary studies done in our laboratory were also taken into consideration in the choice of agonists.

The areas of investigation for the second and third studies were chosen because they both examine the generation of second messengers by platelets in response to a range of agonists. Phosphorylation of platelet proteins in response to physiologic agonists is dependent upon the generation of the second messengers 1,2-diacylglycerol and 1,4,5-inositol trisphosphate. Although this does not directly measure the production of these second messengers, phosphorylation of the 20 and 47 kDa proteins in response to physiologic agonists indicates that they

are being produced. It was anticipated that there would be a defect in the phosphorylation of one, or both, of these proteins in affected platelets. The aggregation of affected platelets by phorbol ester and higher concentrations of thrombin and failure of aggregation in response to ADP and PAF suggested that the defect was located prior to activation of protein kinase C and phosphorylation of the 47 kDa protein. Because of the normal shape change, occurrence of granule secretion and generation of a contractile force, defective phosphorylation of the 20 kDa myosin light chain was considered unlikely.

The measurement of thromboxane B_2 is a direct indication of the production of thromboxane A_2 (TxA_2) by platelets. Thromboxane A_2 is an important second messenger which is essential for irreversible aggregation of platelets stimulated by weak, but physiologically important, agonists such as ADP and lower concentrations of PAF and thrombin. The complete failure of aggregation in response to the weak agonist ADP suggested that TxA_2 production would not occur, as the production of TxA_2 in response to weak agonists is dependent upon primary aggregation. However, the ability of aspirin to inhibit the ADP-induced secretion of dense granule contents from affected platelets indicated that affected platelets were able to produce TxA_2 in the absence of primary aggregation.

In conclusion, it was hypothesized that affected platelets had a defect in the generation of second messengers and transduction of signals from the exterior to the interior of the cell. Because

affected platelets bound fibrinogen in the absence of aggregation, it was assumed that the defect involves a post-fibrinogen binding process, but it was not possible to be more specific given the data available at the start of the study.

CHAPTER III: PLATELET AGGREGATION, DENSE GRANULE SECRETION AND TRANSMISSION ELECTRONMICROSCOPY

INTRODUCTION

Platelet aggregation is a critical step in normal hemostasis. Damage to the vascular endothelium and exposure of the subendothelium must lead rapidly to the formation of a hemostatic platelet plug and prevention of further blood loss. This process involves several distinct steps known as the platelet response: the **adhesion** of platelets to sites of vascular injury, **shape change**, **aggregation** of other platelets on the adherent layer and **secretion** of granule contents and liberation of arachidonic acid which aids in the recruitment of additional platelets. Activation of the coagulation system on the surface of the platelets and the formation of a meshwork of fibrin consolidates the platelet plug and completes the hemostatic process.[288,435,467]

Von Willebrand factor is essential in the adhesion of platelets to exposed subendothelium.[421] When subendothelial components such as collagen and heparin-like glycosaminoglycans are exposed in vessel injury, von Willebrand factor binds to these components and at the same time binds to the glycoprotein Ib (GPIb) of the platelet membrane. This results in a monolayer of platelets covering the injured area.

The binding of von Willebrand factor to GPIb results in platelet activation and recruitment of further platelets to the site of injury.

When platelets are stimulated, they undergo a rapid change in shape from smooth, discoid cells to spheres with pseudopods.[467] This response is very rapid, requires metabolic energy and is independent of external Ca^{2+}. Stimulation of platelets also leads to the induction of specific fibrinogen receptors on the platelet surface. The fibrinogen receptor is the integral membrane GPIIb-IIIa complex which, in activated platelets, forms a Ca^{2+}-dependent heterodimer capable of binding fibrinogen.[158,344,436] Binding of fibrinogen to platelets is essential for aggregation and is closely correlated to platelet aggregability.[379,381] Fibrinogen has a dimeric structure and is thought to form bridges between receptors on adjacent platelets. Although shape change and fibrinogen binding both occur very early in platelet activation, they are independent processes. Aggregation occurs when spherical platelets with exposed fibrinogen receptors are brought into close contact in the presence of fibrinogen and Ca^{2+} and/or Mg^{2+}.

Adhesion, shape change and binding of fibrinogen are all potentially reversible processes. However, if the stimulus is sufficiently strong, the reversible primary aggregation is followed by secondary irreversible aggregation and the platelet release response.[449,467] The platelet release response encompasses secretion of granule contents and the liberation of arachidonic acid. Secretion of alpha and dense granule contents may be induced by all known physiologic platelet agonists. Lysosomal secretion occurs only in response to a strong

agonist such as high concentrations of thrombin. Dense granules contain ions and low molecular weight compounds: Ca^{2+}, inorganic orthophosphate and pyrophosphate, adenosine triphosphate (ATP), adenosine diphosphate (ADP), serotonin and low concentrations of catecholamines. Alpha granules contain many proteins, including fibrinogen, von Willebrand factor, thrombospondin, fibronectin, platelet-derived growth factor, ß-thromboglobulin and platelet factor 4.[201,467]

Arachidonic acid may be liberated from membrane phospholipids by the hydrolytic action of phospholipases and lipases, and then oxygenated by the cyclooxygenase pathway to endoperoxides (prostaglandin G_2, prostaglandin H_2) and thromboxane A_2. These arachidonic acid products are potent platelets agonists which, along with dense granule ADP and serotonin, act to recruit further platelets into the aggregate. The formation of arachidonic acid metabolites is always associated with secretion of dense and alpha granule contents. The secretion response provides a localized high concentration of agonists, Ca^{2+} and adhesive proteins on the surface of the growing hemostatic platelet plug.

Platelet aggregation is generally studied <u>in vitro</u> using an optical aggregometer in which agonists are added to stirred platelet suspensions and the changes in light transmission recorded.[46,520] In suspension, the first response of platelets to most agonists is shape change. As platelets change from discoid to spheroid, there is a small decrease in light transmission through the suspension. All platelet agonists, except epinephrine and phorbol esters, induce platelet shape

change. Primary aggregation is reversible and not associated with secretion of granule contents or arachidonic acid metabolites. If the agonist is present in high enough concentration, primary aggregation will be followed by secondary irreversible aggregation which is mainly the result of the release reaction. As the platelets aggregate, there is an increase in light transmission through the suspension. Optical aggregometry is not a very sensitive means of detecting the initial phase of aggregation. It does not detect aggregates of less than approximately seven platelets and the change in optical density is not detectable until 80% of single platelets have aggregated. The initial stages of platelet aggregation are better measured by counting the disappearance of single platelets.[484]

Agonists are classified as "weak", "intermediate" or "strong" depending on their ability to induce the release response and irreversible aggregation.[80,449,531] Weak agonists (ADP and epinephrine) cannot induce irreversible aggregation in the absence of the release response or cyclooxygenase activity. Secretion in response to these weak agonists occurs only in association with aggregation and is abolished by treatment with agents which inhibit cyclooxygenase. In contrast, strong agonists (high concentrations of thrombin and collagen) can induce irreversible aggregation in the absence of the release response and secretion is not dependent on aggregation. Agonists with intermediate potency [platelet activating factor (PAF), vasopressin, prostaglandin and endoperoxide analogues] can induce secretion independently of prior aggregation and cyclooxygenase activity, but aggregation and secretion are inhibited if ADP is removed

and cyclooxygenase inhibited. Low concentrations of strong and intermediate agonists act as weak agonists.

In vitro, dense granule ATP or serotonin secretion can readily be measured. The most commonly used assay for detection of secreted ATP is the firefly luciferin-luciferase reaction.[130,520] The measurement of the ATP content of a sample depends on the reaction:

$$\text{luciferin} + \text{ATP} + O_2 \xrightarrow{\text{luciferase, } Mg^{2+}} \text{AMP} + \text{PP} + CO_2 + \text{oxyluciferin} + \text{light}$$

The light output is directly proportional to the ATP concentration and can be detected by a photomultiplier tube to give a continuous measurement of ATP secretion on a chart recorder. Serotonin secretion can be measured discontinuously by prelabelling platelets with radioactive serotonin and measuring the radioactivity in the supernatant at the completion of aggregation. Reuptake of the secreted serotonin must be prevented by the addition of imipramine.

The suspensions most commonly used to study platelet function in vitro are platelet rich plasma, washed platelets and gel-filtered platelets. Blood for the preparation of platelet rich plasma is usually anti-coagulated with trisodium citrate to reduce the concentration of Ca^{2+} sufficiently to prevent coagulation without inhibiting platelet aggregation. The advantage of using platelet rich plasma is that it requires minimal manipulation of the platelets and supplies the fibrinogen needed by weak agonists. However, the low concentration of Ca^{2+} does create a non-physiological situation and

potential artifacts. For example, ADP-induced secretion only occurs in a low-Ca^{2+} medium, not at physiological Ca^{2+} concentrations.[343]

For studies of platelet activation by thrombin, it is essential to transfer the platelets from plasma to a buffered balanced salt solution to prevent the formation of a fibrin clot when the thrombin is added. Platelets may be washed, but this involves multiple centrifugations, and very careful handling is required to minimize activation and damage of platelets. An alternative is to gel-filter the platelets over a Sepharose column. A morphologic study in which transmission and scanning electronmicroscopy were used to compare the methods concluded that gel-filtration appeared less harmful than washing.[165] All methods of platelet preparation for functional studies require a rest period after preparation to allow the platelets to recover.

In the investigation of the platelet defect in Basset hound hereditary thrombopathy described in the preceding chapter, ADP was the only agonist used to stimulate the platelets. At the conclusion of the study, there was no clear indication of the location of the defect. Therefore, it was felt that a broader knowledge of the aggregation and dense granule ATP secretion responses of control and affected canine platelets might help to localize the area to be investigated. In this study, a range of agonists [ADP, PAF, thrombin, calcium ionophore A23187, phorbol myristate acetate (PMA) and dithiothreitol] were used to investigate aggregation and dense granule secretion of control and affected platelets in either platelet rich plasma or gel-filtered platelets. The effect of inhibition of cyclooxygenase by aspirin

(acetylsalicylic acid) on the responses of platelets to these agonists was investigated in both groups. The aggregation studies were coupled to transmission electronmicroscopy for thrombin, PAF, PMA and A23187.

MATERIALS AND METHODS

Experimental Design

Three control Basset hounds (two females and one male) were used to establish dose-response curves for control canine platelets stimulated by the above agonists. Of the agonists listed above, ADP is the only agonist that has been commonly used to stimulate canine platelets. Four affected Basset Hounds (three female and one male) were used to investigate the responses of affected platelets. During the study, the control male Basset hound died (acute death due to a gastric torsion) and was replaced by another control male Basset hound on loan on a part-time basis from a breeder. Later, another control female Basset hound was obtained and added to this study. All dogs used in the study were housed within the Veterinary Clinical Center at Michigan State University, fed a regular diet of dry kibble and exercised regularly.

Once dose-response curves for the percentage maximum aggregation and maximum slope had been established for each agonist in the control group, dense granule ATP secretion in response to selected concentrations of each agonist was measured. From these results, concentrations of agonists were selected for the measurement of aggregation and dense granule ATP secretion in the affected group.

Reagents

Sodium salts of ADP and ATP were purchased from the Sigma Chemical Company, (St. Louis, MO) and stock solutions of 2.6 mM and 1.0 mM respectively in 20 mM Tris-saline frozen at -20°C. Dithiothreitol was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Platelet activating factor was purchased from Calbiochem Biochemicals (San Diego, CA) and a 1.0 mg/ml stock solution in ethanol stored at -20°C. The calcium ionophore A23187 was purchased from the Sigma Chemical Company and a 4.16 mM stock solution in ethanol stored at -20°C. Phorbol-12-myristate-13-acetate and 4-α-phorbol were purchased from the Sigma Chemical Company and Calbiochem Biochemicals and 1.56 mM stock in DMSO or ethanol stored at -20°C. Acetylsalicylic acid (aspirin) was purchased from the Sigma Chemical Company and fresh 1.0 M stock solution in ethanol prepared at the time of use. Thrombin and Sepharose 4B were purchased from the Sigma Chemical Company. Chrono-Lume No. 395 luciferin-luciferase reagent was purchased from the Chrono-Log Corporation (Havertown, PA). All other chemicals were of reagent grade.

Stock solutions were diluted as necessary at the time of use and kept on ice. Reagents were diluted to the required concentration with either 0.9% w/v NaCl, (ADP and dithiothreitol), distilled water (thrombin and Chrono-lume) or balanced salt solution (PAF, A23187, PMA and 4-α-phorbol) containing 136 mM NaCl, 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4 and 5.6 mM glucose, pH 7.4. Calcium ionophore A23187 was diluted and vortex-mixed immediately prior to use. Final

concentrations of ethanol or DMSO did not exceed 0.5% v/v. Because this study was conducted over a long period of time, more than one lot number of each reagent was used, adding an unavoidable source of variation.

Platelet Isolation

If unacceptable variability in results is to be avoided, canine platelets must be collected with great care from large vessels in dogs that are not excited, medicated or ill.[29] Even under optimal conditions, there is considerable variation in the responses of an individual animal over a period of time.

For the preparation of platelet rich plasma, blood was collected from the jugular vein through an 18-gauge needle into a plastic syringe containing 1.0 ml of 3.2% w/v Na_3 citrate for each 9.0 mls of blood to be collected. If the collection was interrupted in any way, the sample was discarded and a second collection made. Polyethylene or polypropylene labware was used in all platelet isolation procedures. The blood was transferred to tubes and the platelet rich plasma separated by repeated centrifugations at 1,324 x g for 90 seconds with removal of a small volume of platelet rich plasma after each centrifugation. During the period of this study, it was observed that affected platelets sediment more rapidly during centrifugation than control platelets and an improved yield of affected platelets could be obtained by centrifuging for 60 second periods rather than 90 seconds. After removal of the platelet rich plasma, the blood was centrifuged at

1,324 x g for 13 minutes to prepare platelet poor plasma. The platelets in the platelet rich plasma were counted manually using a Unopette and Neubauer hemocytometer. The autologous platelet poor plasma was used to adjust the platelet count of the platelet rich plasma to 3.0 x 10^8/ml. The platelet rich plasma was aliquoted into aggregometer cuvettes, covered with parafilm and allowed to rest at room temperature for at least 30 minutes before aggregation. When platelets were to be incubated with aspirin to inhibit cyclooxygenase, it was added to the platelet rich plasma at a final concentration of 1.0 mM before the platelet rich plasma was aliquoted and allowed to rest.

For the preparation of gel-filtered platelets, blood was collected into 3.8% w/v Na_3citrate and the platelet rich plasma separated by centrifugation, as described above. Prostaglandin E_1 was added from fresh 1.0 mM stock in ethanol to a final concentration of 1.0 μM to minimize platelet activation during the subsequent centrifugation and gel-filtration. After a 10 minute incubation at room temperature, the platelet rich plasma was centrifuged at 800 x g for 15 minutes, the supernatant discarded and the platelet pellet gently resuspended in 1.0 ml of HEPES-Tyrode's-albumin buffer (HTA) containing 130 mM NaCl, 2.6 mM KCl, 0.42 mM NaH_2PO_4, 5.5 mM dextrose, 0.01 mM HEPES and 3.0 mg/ml bovine serum albumin, pH 7.2.[179] The platelet suspension was applied to a polyethylene column (10 ml bed volume) filled with Sepharose 4B and equilibrated with HTA at room temperature. Platelets came through in the void volume and were detected visually. Two to three millliliters of gel-filtered platelets were collected and the

platelet count adjusted to 3.0 x 10^8 cells/ml with HTA. $CaCl_2$ and $MgCl_2$ were added to final concentrations of 1.0 mM and 0.8 mM respectively. The $MgCl_2$ was omitted when the platelets were to be used for ATP secretion studies as the Chrono-Lume reagent contains 10 mM $MgSO_4$. The gel-filtered platelets were transferred to aggregation cuvettes, covered with parafilm and allowed to rest for 30 minutes. Aspirin-treated platelets were prepared as described for platelet rich plasma.

Platelet Aggregation Studies

The aggregation studies were performed in a Lumi-aggregometer (Chrono-log Corporation, Havertown, PA.) at 37.5°C within four hours of collection. Aggregation cuvettes containing 0.5 ml of platelet rich plasma or gel-filtered platelets were allowed to equilibrate for at least four minutes at 37.5°C before being placed in the aggregometer. After being stirred at 900 rpm for approximately 0.5 minutes, 20 μl of agonist was added. Results were recorded on a dual-pen Houston Omniscribe Chart recorder (Houston Instrument Division of Bausch and Lombe Inc., Austin, TX.). Zero aggregation was defined as a failure to record an increase in light transmission above the zero baseline when the sample was allowed to stir in the aggregometer for five minutes after the addition of agonist. Under these conditions, the slope or rate of aggregation was also defined as zero. The time to maximum aggregation under these circumstances was designated NA ("not applicable"), as a time could not be assigned to an event which did not occur. Aggregation results recorded in the presence of the Chrono-Lume

reagent were not included in this study because of the potentiating effect of Chrono-Lume on platelet aggregation.[324] This potentiation was frequently observed, particularly at low concentrations of agonist.

ATP Secretion Studies

Storage pool adenine nucleotide secretion was monitored in the Lumi-aggregometer which simultaneously measures platelet aggregation and ATP secretion as detected by the firefly-luciferase system. Aggregometer cuvettes containing 0.45 ml of platelet rich plasma or gel-filtered platelets were allowed to equilibrate at 37.5°C for at least four minutes. The Chrono-Lume luciferin-luciferase reagent (50 μl) was added immediately prior to the addition of 20 μl of agonist to minimize the potentiating effect of the Chrono-Lume on platelet aggregation.[324] Immediately after the ATP secretion tracing on the recorder had peaked, a standard solution of ATP, final concentration 2.0 μM or 4.0 μM was added to quantitate the ATP secretion. If no measurable secretion had occurred within five minutes of the addition of agonist, secretion was defined as zero. The time to maximum secretion under these circumstances was designated NA ("not applicable") as a time could not be assigned to an event which did not occur. The exception to this rule was secretion in response to PMA which is very slow and does not occur until aggregation is almost complete.

Transmission Electronmicroscopy

Platelet rich plasma or gel-filtered platelets were prepared for aggregation, as described above. Before, or at various times after the addition of agonist, 0.5 ml of 0.1 % glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 was added and the sample allowed to stir for one minute. The samples were then centrifuged at 12,800 x g for 20 seconds, the supernatant discarded and 1.0 ml of 3.0 % glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4 added. The specimens were allowed to fix for three to six hours and then the glutaraldehyde was replaced by 0.1 M sodium cacodylate, pH 7.4 and the sample processed for electronmicroscopy as described by Mattson et. al.[313]

Statistical Analysis

All results were entered into a statistics and graphics program (Statgraphics, STSC, Rockville, MD). Because there had not been equal access to all dogs, particularly in the control group, the number of repetitions for each dog was not equal. To avoid unnecessary bias towards the results of individual dogs which had been available more frequently, the average for each dog was calculated and then used as the representative value for that animal. Although it would have been desirable to use distribution-free non-parametric tests, it was found that these were not effective for the very small sample sizes involved. Therefore, standard parametric tests for the comparison of two samples were used. Control and affected data were compared using the t-test for independent samples and pre- and post-aspirin samples were compared

using the t-test for paired samples. The arcsin transformation was applied to the percent aggregation data before analysis. Although data for the measurement of slope is not usually normally distributed, there is no transformation available. The use of parametric tests on these very small sample sizes produces a very conservative analysis. Samples from the affected group which had average and standard deviation values of zero violate the requirement of parametric tests for equal variances. Therefore, some caution should be exercised in the interpretation of the statistical analysis of the results and examination of the data itself may be of more use.

RESULTS

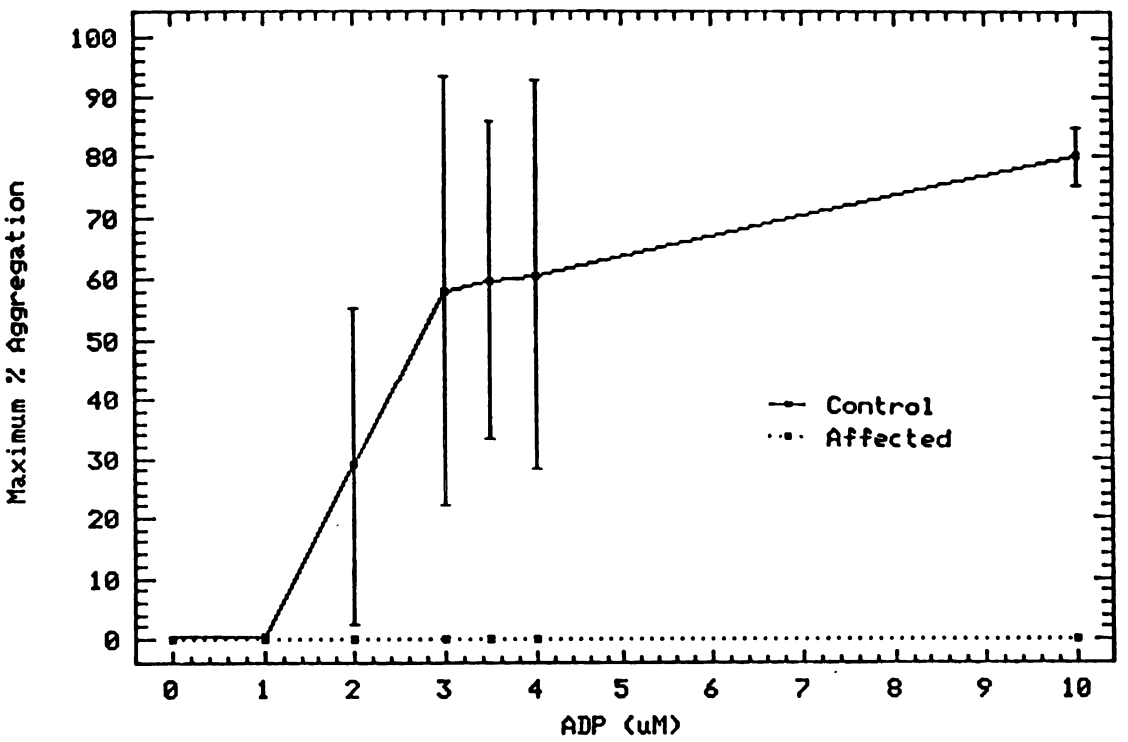
Results for each agonist are presented separately. All agonist concentrations are the final concentration of agonist in the platelet rich plasma or gel-filtered platelets. The percent aggregation is the percent increase in light transmission through the stirred platelets in the aggregometer cuvette that occurs during platelet aggregation, with the maximum being 100 %. The maximum slope of the aggregation curve is an indication of the rate of aggregation. Where appropriate, the time taken to reach maximum aggregation is presented. Dense granule secretion is described by the amount of ATP in μM measured at the peak of the secretion curve and the time in minutes taken to reach that peak.

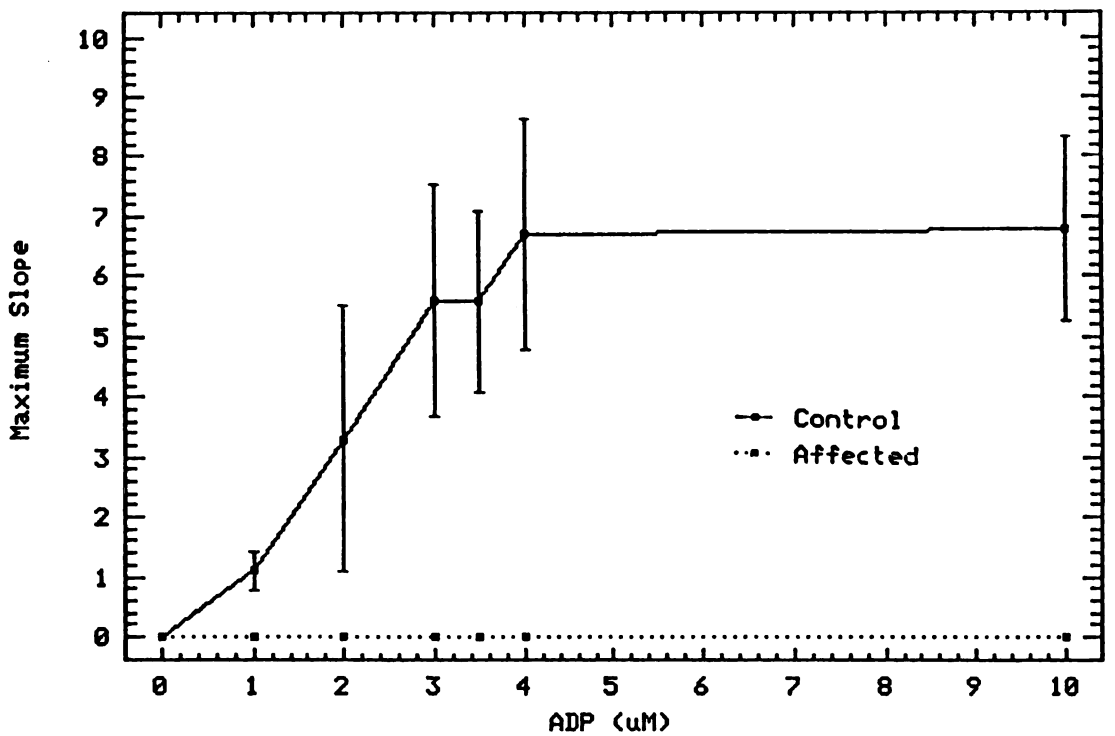
Adenosine Diphosphate

Platelet rich plasma was used for all aggregation and ATP secretion studies in response to ADP. All concentrations of ADP used caused rapid shape change, seen as an initial decrease in light transmission on the aggregation curve, of both control and affected platelets. However, in affected platelets, there was complete failure of primary aggregation in response to any concentration of ADP used. The dose response curves for aggregation and slope are illustrated in Figures 3 and 4 respectively. While low concentrations of ADP such as 2.0 μM induced a range of responses from reversible primary to irreversible secondary aggregation of control platelets, concentrations of 4.0 μM or higher induced irreversible aggregation in four of the five control dogs. The response of the fifth control dog to 4.0 μM ADP varied from irreversible to reversible aggregation. Typical aggregation curves for control and affected platelets stimulated by 10 μM ADP are illustrated in Figure 5.

Figure 3. Dose response curves for the maximum percent aggregation of control and affected platelet rich plasma stimulated by ADP.

Figure 4. Dose response curves for the maximum slope of the aggregation curves of control and affected platelet rich plasma stimulated by ADP.


Maximum % Aggregation
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
10
ADP (uM)
Control
Affected


Maximum Slope
10
9
8
7
6
5
4
3
2
1
0
0
1
2
3
4
5
6
7
8
9
10
ADP (uM)
Control
Affected

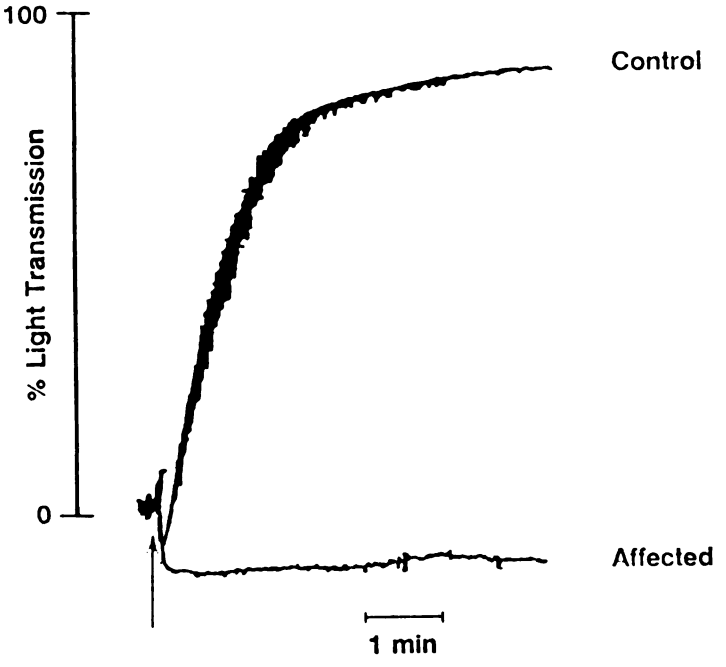


Figure 5. Typical aggregation curves for control and affected platelet rich plasma stimulated by 10 μM ADP.

Secretion of dense granule ATP by control canine platelets had a typical pattern which reached a peak an average of 2.2 minutes after the addition of 10 μM ADP. In contrast, the affected platelets had a rapid burst of ATP secretion which peaked an average of 0.4 minutes after the addition of 10 μM ADP. Control platelets secreted significantly more ATP than affected platelets. Secretion of ATP by affected platelets occurred in the complete absence of aggregation.

Control platelets incubated with 1.0 mM aspirin showed a significant reduction in the extent and rate of aggregation when stimulated by 10 μM ADP. Affected platelets did not aggregate in response to 10 μM ADP in the absence or presence of aspirin. Dense granule ATP secretion from both control and affected platelets was almost completely inhibited by aspirin. A summary of the aggregation and ATP secretion results in the absence and presence of aspirin are presented in Table 1.

Table 1. Maximum aggregation (%), slope, ATP secretion (μM) and time to maximum ATP secretion (minutes) of control and affected platelet rich plasma stimulated by 10 μM ADP in the absence and presence of 1.0 mM aspirin.

	1.0 mM Aspirin	Control	Affected
Aggregation	-	80.3 ± 4.7 (5)	0 (4)*
	+	34.8 ± 12.7 (4)#	0 (4)*
Slope	-	6.8 ± 1.5 (5)	0 (4)*
	+	4.8 ± 0.9 (4)#	0 (4)*
ATP μM	-	3.5 ± 0.7 (5)	2.1 ± 0.5 (4)*
	+	0.40 ± 0.07 (4)#	0.36 ± 0.05 (4)#
Time to Max^m Secretion	-	2.2 ± 0.4 (5)	0.41 ± 0.03 (4)*
	+	0.10 ± 0 (4)#	0.11 ± 0.01 (4)#

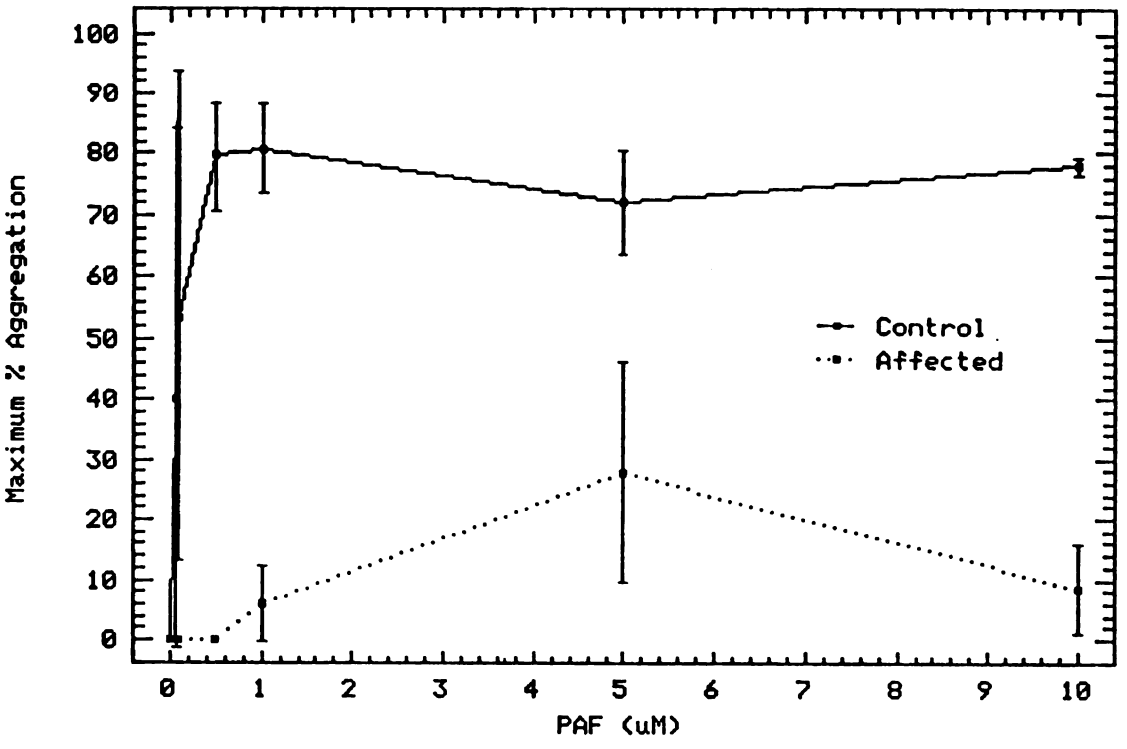
Values = mean ± SD, (n) = number of samples, * = significantly different from control platelets ($p < 0.05$), # = significantly different from non-aspirin-treated platelets ($p < 0.05$).

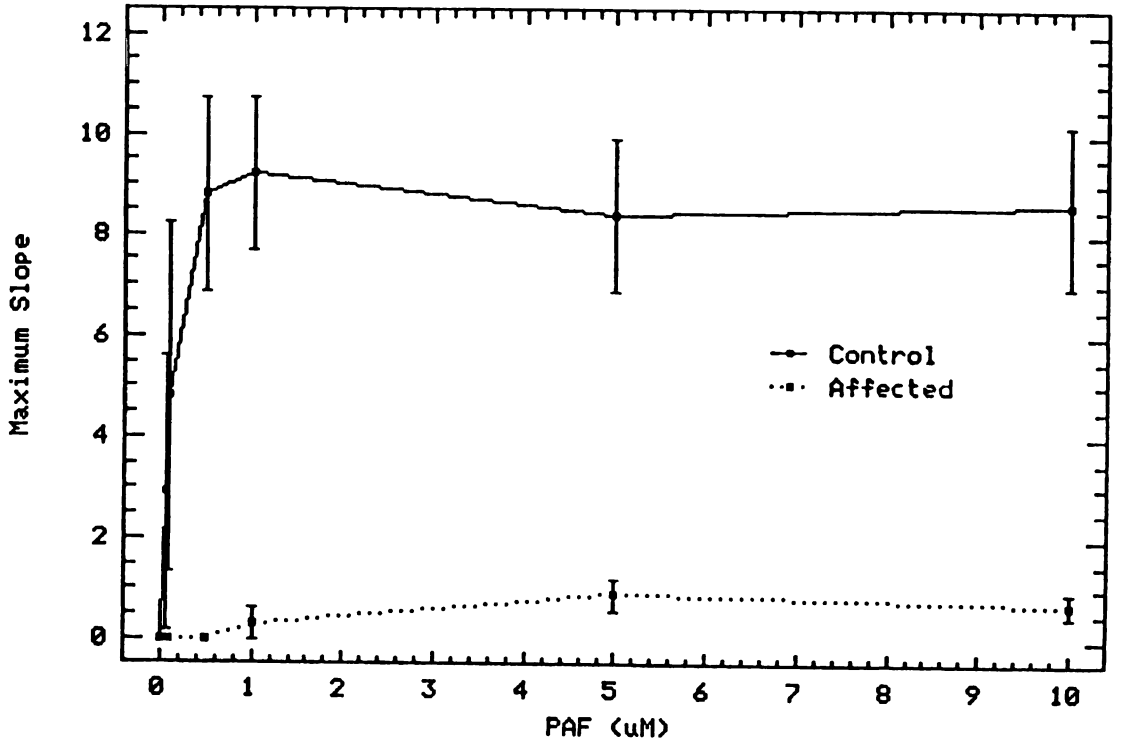
Platelet Activating Factor

Control and affected platelets in platelet rich plasma rapidly changed shape in response to all concentrations of PAF used. All control platelets aggregated irreversibly to concentrations of 0.5 μM or higher, although a biphasic response was frequently seen at the lower concentrations. The responses of control platelets to concentrations of PAF less than 0.5 μM varied considerably between individuals and ranged from irreversible to reversible aggregation. Although affected platelets did not aggregate in response to levels of PAF that caused rapid and irreversible aggregation of control platelets, at very high concentrations of PAF (5.0 and 10 μM) there was an inconsistent, moderate increase in light transmission recorded. When the cuvette was removed from the aggregometer, it was seen to contain a fine, but visible, suspension. The dose response curves for the aggregation and slope of control and affected platelets are presented in Figures 6 and 7. Typical aggregation curves are illustrated in Figure 8. Similar results were obtained for gel-filtered platelets stimulated by PAF.

Figure 6. Dose response curves for the maximum percent aggregation of control and affected platelet rich plasma stimulated by PAF.

Figure 7. Dose response curves for the maximum slope of the aggregation curves of control and affected platelet rich plasma stimulated by PAF.


Maximum % Aggregation
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
10
PAF (uM)
Control
Affected


Maximum Slope
12
10
8
6
4
2
0
0
1
2
3
4
5
6
7
8
9
10
PAF (uM)
Control
Affected

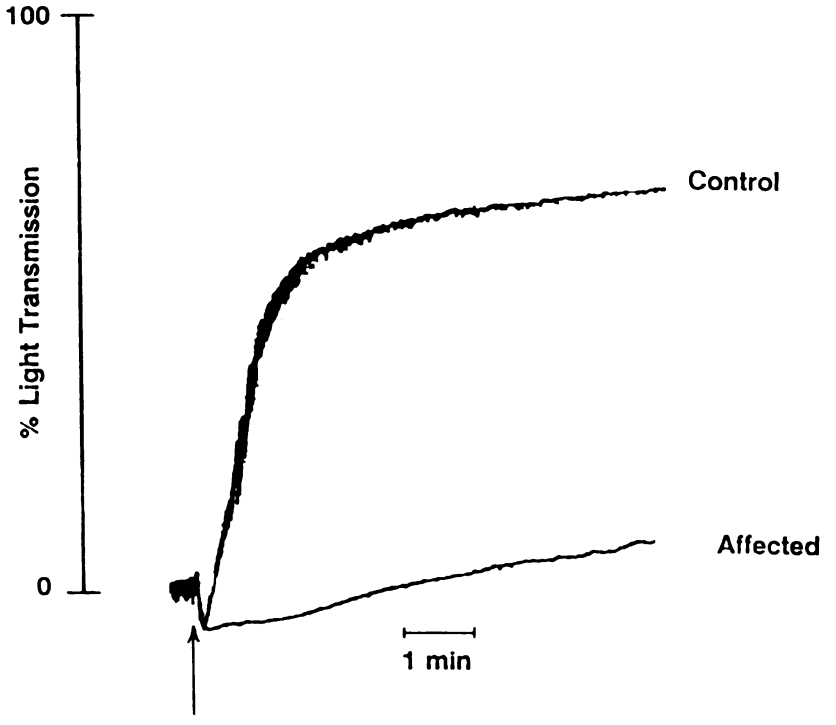


Figure 8. Typical aggregation curves for control and affected platelet rich plasma stimulated by 1.0 μM PAF.

Both control and affected platelets secreted ATP in response to concentrations of PAF of 0.5 μM or higher. At 0.1 μM, platelets from three of four control dogs aggregated and secreted ATP, while one did not aggregate and did not secrete ATP. Platelets from all four affected dogs did not secrete any ATP in response to 0.1 μM PAF. These results are summarized in Table 2.

Table 2. Dense granule ATP secretion (μM) and time to peak secretion (minutes) of control and affected platelet rich plasma stimulated by PAF.

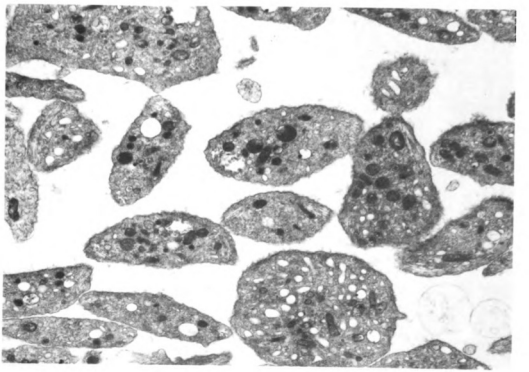
PAF	ATP (μM) Secreted		Time to Max^{m} Secretion	
(μM)	Control	Affected	Control	Affected
0.1	2.6 ± 1.7 (4)	0 (4)*	1.6 ± 0.21 (3)	NA
0.5	4.7 ± 2.8 (3)	1.2 ± 0.9 (3)	0.96 ± 0.22 (3)	0.68 ± 0.20 (3)
1.0	5.1 ± 1.3 (5)	4.2 ± 0.6 (4)	0.57 ± 0.30 (5)	0.37 ± 0.04 (4)
5.0	5.6 ± 1.8 (4)	5.6 ± 0.4 (2)	0.33 ± 0.13 (4)	0.18 ± 0.04 (2)
10	6.2 ± 2.0 (4)	8.0 ± 1.9 (3)	0.21 ± 0.06 (4)	0.13 ± 0.06 (3)

Values = mean ± SD, (n) = number of samples, NA = "not applicable", * = significantly different from control platelets ($p < 0.05$).

Transmission electronmicrographs of resting control and affected platelet rich plasma are shown in Figures 9a and 9b. Transmission electronmicrographs of control and affected platelets after stimulation by 0.1 μM PAF are shown in Figures 10a, 10b, 11a, and 11b, and 1.0 μM PAF in Figures 12a, 12b, 13a, and 13b. These electronmicrographs confirm the rapid shape change and the absence of aggregation of affected platelets consistent with the aggregometer tracings.

Figure 9a. Transmission electronmicrograph of stirred control platelet rich plasma prior to the addition of agonist (X 11,250).

Figure 9b. Transmission electronmicrograph of stirred affected platelet rich plasma prior to the addition of agonist (X 11,250).



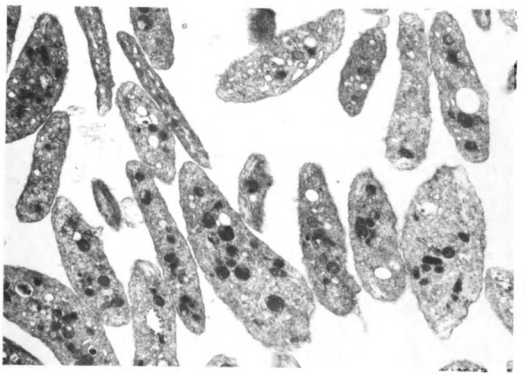
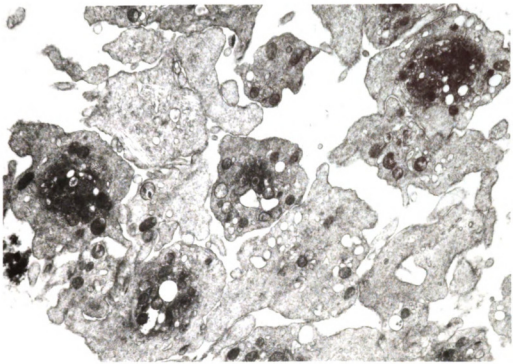


Figure 10a. Transmission electronmicrograph of control platelet rich plasma one minute after the addition of 0.1 μM PAF (X 11,250). The platelets have changed shape, centralized their granules and started to aggregate.

Figure 10b. Transmission electronmicrograph of affected platelet rich plasma one minute after the addition of 0.1 μM PAF (X 11,250). The platelets have changed shape and centralized their granules, but have not aggregated.



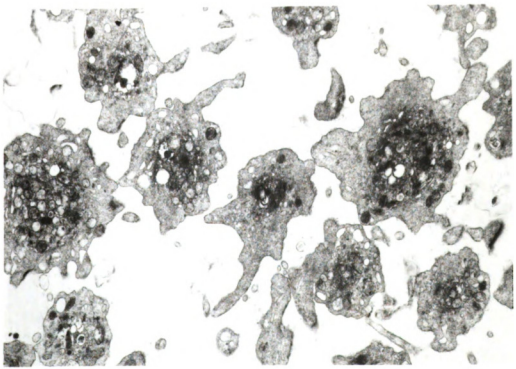
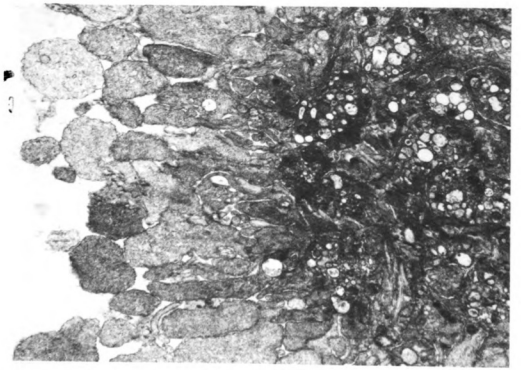


Figure 11a. Transmission electronmicrograph of control platelet rich plasma five minutes after the addition of 0.1 μM PAF (X 11,250). The platelets have aggregated fully.

Figure 11b. Transmission electronmicrograph of affected platelet rich plasma five minutes after the addition of 0.1 μM PAF (X 11,250). The platelets have changed shape, but have not released their granule contents or aggregated.



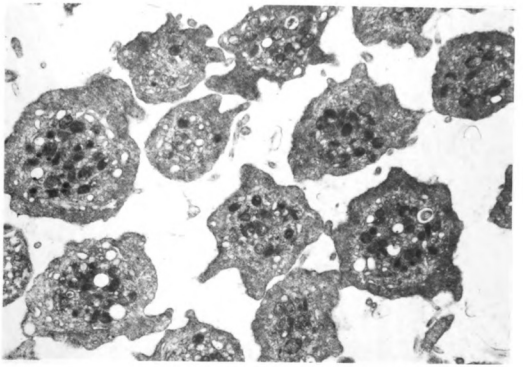
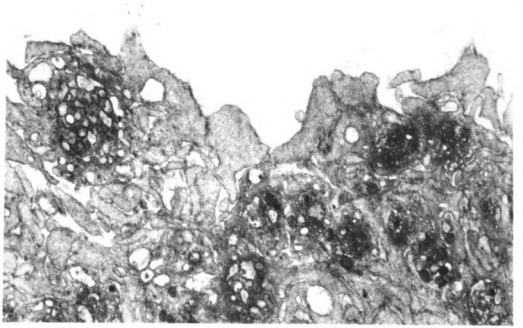


Figure 12a. Transmission electronmicrograph of control platelet rich plasma one minute after the addition of 1.0 μM PAF (X 11,250). The platelets have almost fully aggregated.

Figure 12b. Transmission electronmicrograph of affected platelet rich plasma one minute after the addition of 1.0 μM PAF (X 11,250). The platelets have changed shape and centralized their granules, but have not aggregated.



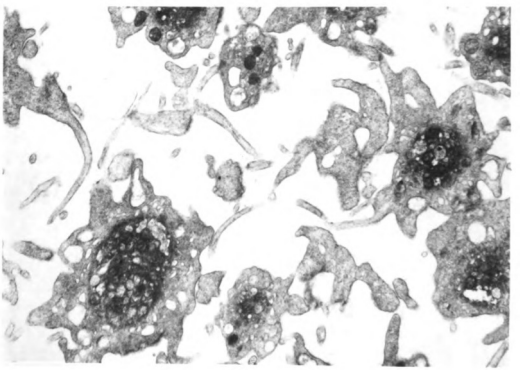
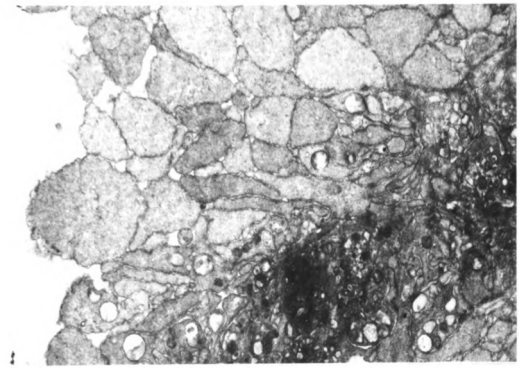
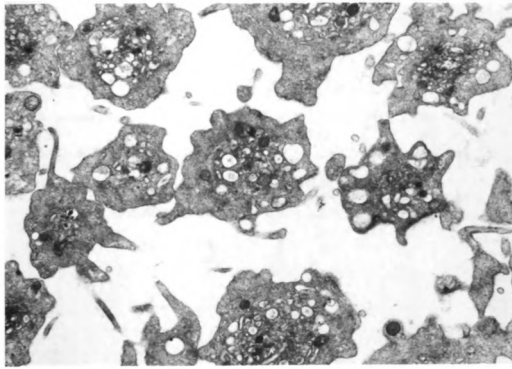


Figure 13a. Transmission electronmicrograph of control platelet rich plasma five minutes after the addition of 1.0 μM PAF (X 11,250). The platelets have aggregated fully.

Figure 13b. Transmission electronmicrograph of affected platelet rich plasma five minutes after the addition of 1.0 μM PAF (X 11,250). The platelets have changed shape and secreted some of their granules, but have not aggregated.





In additional studies, platelet rich plasma was treated with 1.0 mM aspirin and stimulated with 0.1 or 1.0 μM PAF. The percent aggregation, maximum slope, maximum ATP secretion (μM) and time to maximum secretion (minutes) were measured and compared to results obtained in the absence of aspirin. Results are shown in Tables 3 and 4.

Table 3. Maximum aggregation (%), slope, ATP secretion (μM) and time to maximum secretion (minutes) of control and affected platelet rich plasma stimulated by 0.1 μM PAF in the absence and presence of 1.0 mM aspirin.

	1.0 mM Aspirin	Control	Affected
% Aggregation	-	53.3 ± 40.1 (4)	0 (4)*
	+	41.0 ± 33.2 (4)	0 (4)*
Slope	-	4.8 ± 3.4 (4)	0 (4)*
	+	4.3 ± 2.2 (4)	0 (4)*
ATP μM	-	2.6 ± 1.7 (4)	0 (4)*
	+	0.66 ± 0.86 (4)	0 (4)*
Time to Max^m Secretion	-	1.6 ± 0.2 (3)	NA
	+	1.8 ± 0.2 (2)	NA

Values = mean ± SD, (n) = number of samples, NA = "not applicable",
* = significantly different from control platelets ($p < 0.05$).

Table 4. Maximum aggregation (%), slope, ATP secretion (μM) and time to maximum secretion (minutes) of control and affected platelet rich plasma stimulated by 1.0 μM PAF in the absence and presence of 1.0 mM aspirin.

	1.0 mM Aspirin	Control	Affected
% Aggregation	-	80.8 ± 7.0 (5)	5.9 ± 5.9 (4)*
	+	82.1 ± 13.0 (4)	0 (4)*
Slope	-	9.2 ± 1.5 (5)	0.34 ± 0.35 (4)*
	+	8.4 ± 2.1 (4)	0 (4)*
ATP μM	-	5.1 ± 1.3 (5)	4.2 ± 0.6 (4)
	+	3.4 ± 0.8 (4)#	1.1 ± 0.6 (4)*#
Time to Max^m Secretion	-	0.57 ± 0.30 (5)	0.37 ± 0.04 (4)
	+	0.68 ± 0.16 (4)	0.45 ± 0.04 (4)*

Values = mean ± SD, (n) = number of samples, * = significantly different from control platelets ($p < 0.05$), # = significantly different from non-aspirin-treated platelets ($p < 0.05$).

Aggregation of control platelets by either concentration of PAF was not significantly affected by aspirin. Secretion from control platelets in response to 0.1 μM PAF was inhibited, but not significantly. Secretion of ATP from control and affected platelets was significantly inhibited by aspirin at 1.0 μM PAF. The inhibition of ATP secretion by affected platelets stimulated by 1.0 μM PAF was greater than that of control platelets. The post-aspirin ATP concentration for affected platelets was 26.2% of the pre-aspirin value, compared to 66.7% for control platelets.

Thrombin

Thrombin is a potent physiologic agonist that causes rapid shape change, aggregation and secretion of granule contents of control gel-filtered platelets in the absence of exogenous fibrinogen. Interestingly, although affected platelets failed to aggregate in response to the physiological agonists ADP and PAF, they aggregated fully in response to concentrations of 0.2 U/ml thrombin or higher. However, the initial rate of aggregation of affected platelets (as indicated by the maximum slope) was significantly slower than that of controls and they took significantly longer to reach maximum aggregation (Table 5). Dose-response curves for aggregation, maximum slope and time to reach maximum aggregation are shown in Figures 14, 15 and 16 and typical aggregation curves in Figure 17. There was considerable individual variation in the response of the control dogs to concentrations of less than 0.1 U/ml, although the responses were consistent for each dog.

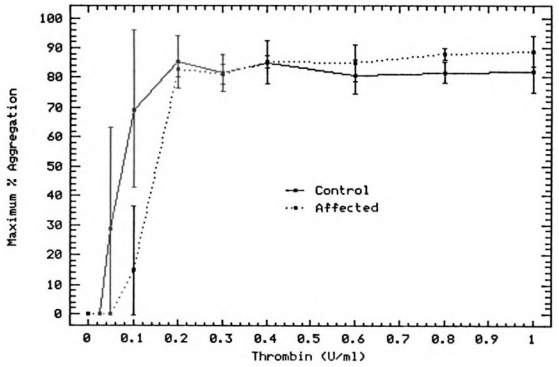
Table 5. Time (minutes) taken by control and affected gel-filtered platelets stimulated by thrombin to reach maximum aggregation.

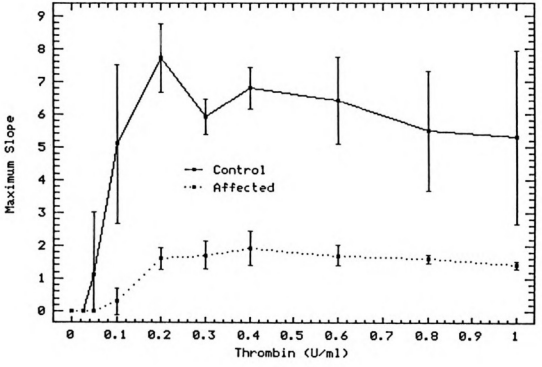
Thrombin (U/ml)	Control	Affected
0.05	6.0 ± 0.7 (3)	NA
0.1	6.5 ± 0.9 (5)	11.8 ± 1.3 (3)*
0.2	7.5 ± 1.0 (4)	11.6 ± 1.0 (3)*
0.3	6.5 ± 1.0 (5)	9.3 ± 1.3 (4)*
0.4	7.5 ± 0.6 (4)	9.7 ± 0.4 (3)*
0.6	7.7 ± 1.1 (4)	10.2 ± 0.2 (3)*
0.8	7.1 ± 1.3 (4)	10.3 ± 0.4 (3)*
1.0	7.8 ± 1.4 (4)	10.3 ± 0.6(3)*

Values = mean ± SD, (n) = number of samples, NA = "not applicable", * = significantly different from control platelets ($p < 0.05$).

Figure 14. Dose response curves for the maximum percent aggregation of control and affected gel-filtered platelets stimulated by thrombin.

Figure 15. Dose response curves for the maximum slope of the aggregation curves of control and affected gel-filtered platelets stimulated by thrombin.


Maximum % Aggregation
100
90
80
70
60
50
40
30
20
10
0
Control
Affected
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
Thrombin (U/ml)


Maximum Slope
9
8
7
6
5
4
3
2
1
0
Control
Affected
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
Thrombin (U/ml)

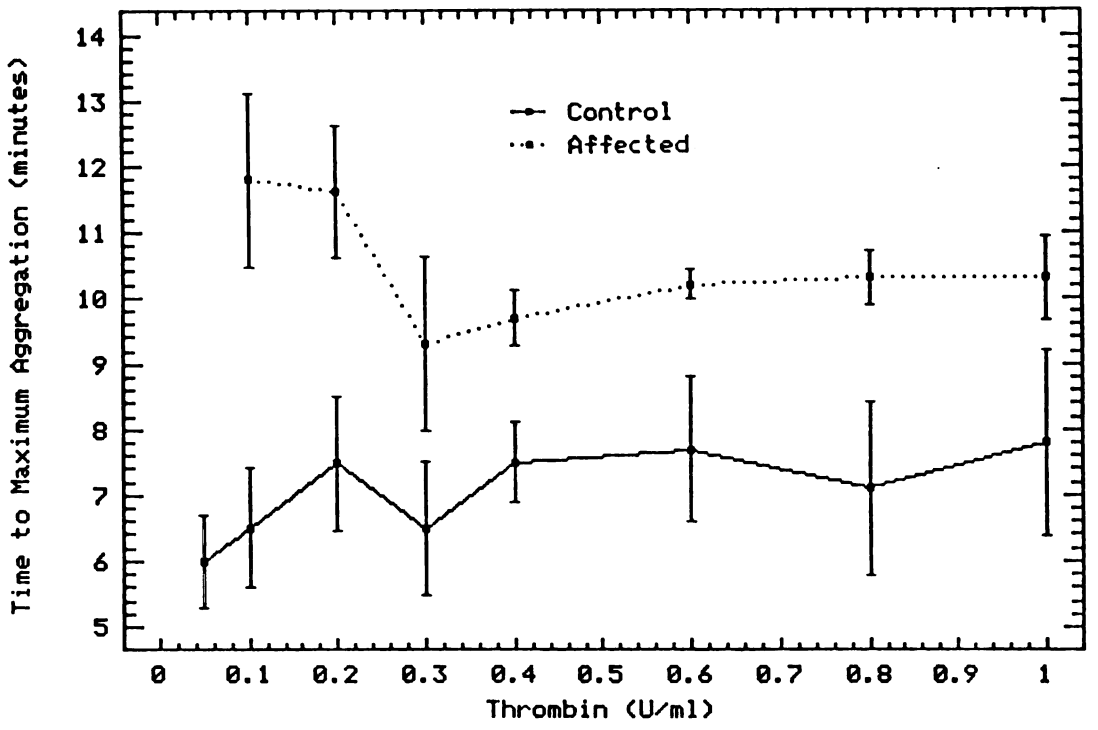


Figure 16. Time taken (minutes) by control and affected gel-filtered platelets to reach maximum percent aggregation when stimulated by thrombin.

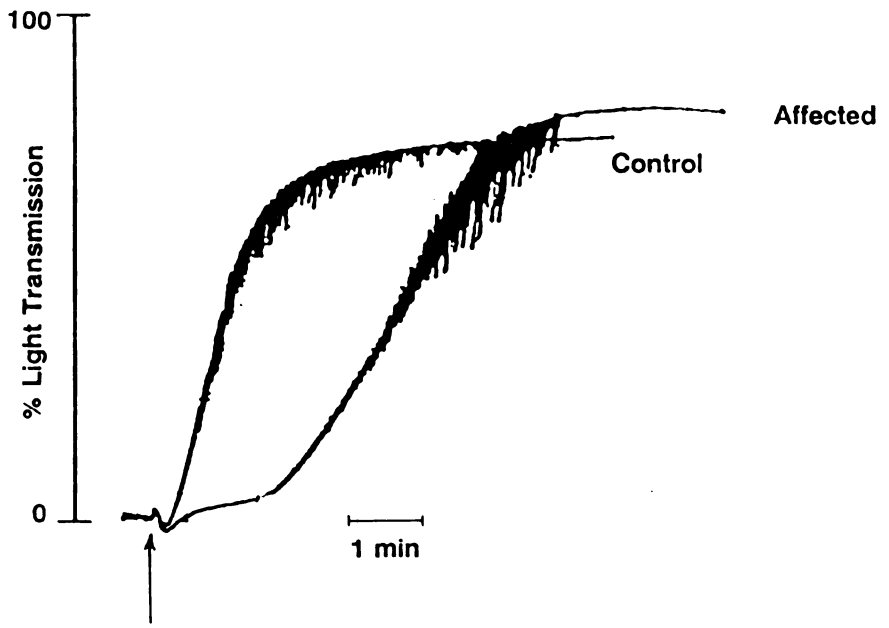
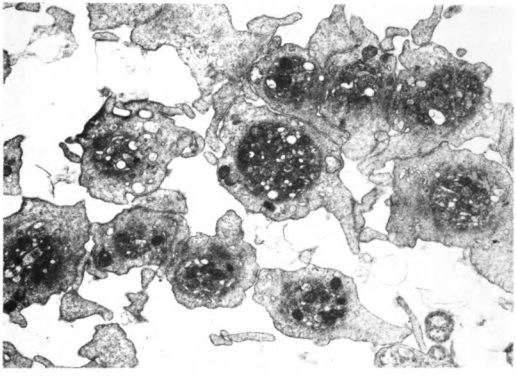


Figure 17. Typical aggregation curves for control and affected gel-filtered platelets stimulated by 0.3 U/ml thrombin.

To further investigate the different initial rates of aggregation in the two groups, aggregation studies were coupled to transmission electronmicroscopy (Figures 18a, 18b, 19a and 19b). Thirty seconds after the addition of 0.3 U/ml thrombin, control platelets had changed shape, centralized their granules and were starting to aggregate. In contrast, affected platelets had changed shape and centralized their granules, but there was no evidence of aggregation. By eight minutes, both control and affected platelets were fully aggregated and there is no apparent morphological difference between the two groups.

Figure 18a. Transmission electronmicrograph of control gel-filtered platelets 30 seconds after the addition of 0.3 U/ml thrombin (X 11,250). The platelets have changed shape, centralized their granules and started to aggregate.

Figure 18b. Transmission electronmicrograph of affected gel-filtered platelets 30 seconds after the addition of 0.3 U/ml thrombin (X 11,250). The platelets have changed shape and centralized their granules, but have not aggregated.



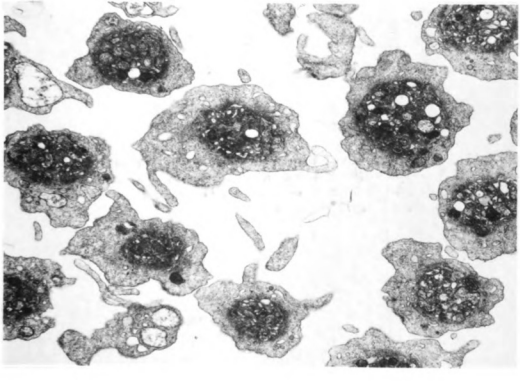
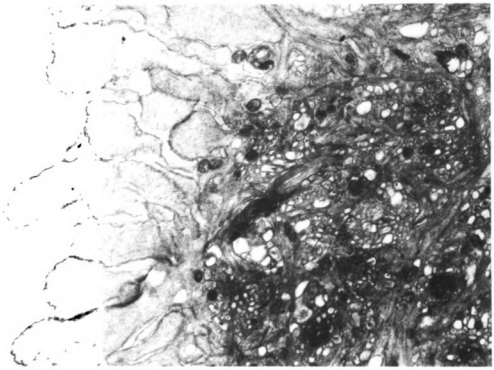
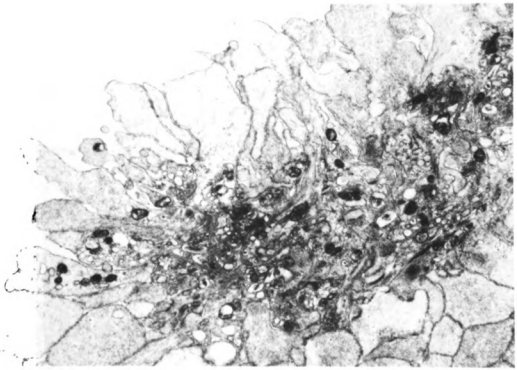


Figure 19a. Transmission electronmicrograph of control gel-filtered platelets eight minutes after the addition of 0.3 U/ml thrombin (X 11,250). The platelets have aggregated fully.

Figure 19b. Transmission electronmicrograph of affected gel-filtered platelets eight minutes after the addition of 0.3 U/ml thrombin (X 11,250). The platelets have aggregated fully.





Both control and affected platelets released ATP in response to all concentrations of thrombin used. Even very low concentrations of thrombin (0.05 U/ml) caused the platelets of some affected individuals to release a small quantity of ATP, although aggregation did not occur. There were no significant differences between the two groups in either the amount of ATP released or the time to maximum secretion. The results are summarized in Table 6.

Table 6. Dense granule ATP secretion (μM) and time to peak secretion (minutes) by control and affected gel-filtered platelets stimulated by thrombin.

Thrombin	ATP Secreted		Time to Max[a] Secretion	
(U/ml)	Control	Affected	Control	Affected
0.05	1.4 ± 1.2 (4)	0.57 ± 0.74 (3)	3.5 ± 1.0 (4)	3.6 ± 0.5 (2)
0.1	2.9 ± 0.6 (5)	1.8 ± 1.6 (4)	2.7 ± 0.5 (5)	3.7 ± 1.3 (3)
0.2	4.1 ± 0.3 (4)	2.8 ± 2.4 (3)	1.8 ± 0.7 (4)	2.0 ± 1.1 (3)
0.3	5.0 ± 0.9 (5)	4.5 ± 0.4 (4)	1.1 ± 0.5 (5)	1.4 ± 0.6 (4)
0.4	5.1 ± 0.5 (5)	5.0 ± 0.6 (3)	1.1 ± 0.5 (4)	1.4 ± 0.2 (3)
0.6	4.8 ± 0.6 (4)	4.9 ± 0.3 (3)	0.9 ± 0.1 (4)	1.1 ± 0.4 (3)
0.8	4.8 ± 1.0 (4)	5.7 ± 0.7 (3)	1.1 ± 0.5 (4)	0.60 ± 0.10 (3)
1.0	5.4 ± 0.8 (4)	5.9 ± 1.2 (3)	0.65 ± 0.06 (4)	0.57 ± 0.21 (3)

Values = mean ± SD, (n) = number of samples.

When gel-filtered platelets were incubated with 1.0 mM aspirin and stimulated by 0.3 U/ml thrombin, the extent and rate of aggregation of control platelets were slightly, but not significantly, inhibited. In contrast, the extent and rate of maximum aggregation of affected platelets were inhibited significantly while the time to maximum aggregation was markedly and significantly prolonged. The secretion of ATP from control gel-filtered platelets stimulated with 0.3 U/ml thrombin was slightly, but significantly, inhibited by 1.0 mM aspirin while the inhibition of secretion from affected platelets was also inhibited slightly, although not significantly. Aspirin prolonged the time to maximum secretion in both groups, although the change was significant only in the affected group. The effects of aspirin on the responses of control and affected platelets to 0.3 U/ml thrombin are summarized in Table 7.

Table 7. Maximum aggregation (%), slope, time to maximum aggregation (minutes), ATP secretion (μM) and time to maximum secretion (minutes) of control and affected gel-filtered platelets stimulated by 0.3 U/ml thrombin in the absence and presence of 1.0 mM aspirin.

	1.0 mM Aspirin	Control	Affected
% Aggregation	-	81.4 ± 6.0 (5)	82.7 ± 4.4 (4)
	+	72.5 ± 3.0 (3)	57.1 ± 10.0 (4)*#
Slope	-	5.9 ± 0.5 (5)	1.7 ± 0.4 (4)*
	+	5.4 ± 0.4 (3)	0.84 ± 0.53 (4)*#
Time to Max^{m} Aggreg^{n}	+	6.5 ± 1.0 (5)	9.3 ± 1.3 (4)*
	-	6.4 ± 1.0 (3)	14.0 ± 3.3 (4)*#
ATP μM	-	5.0 ± 0.9 (5)	4.5 ± 0.4 (4)
	+	3.8 ± 0.7 (3)#	3.8 ± 0.3 (4)#
Time to Max^{m} Secretion	-	1.1 ± 0.5 (5)	1.4 ± 0.6 (4)
	+	1.9 ± 0.7 (3)	2.0 ± 0.6 (4)#

Values = mean ± SD, (n) = number of samples, * = significantly different from control platelets ($p < 0.05$), # = significantly different from non-aspirin-treated platelets ($p < 0.05$).

The extent and rate of aggregation of control and affected platelets stimulated by 0.1 U/ml of thrombin was inhibited slightly, but not significantly, by 1.0 mM aspirin. Although the effect of aspirin on control platelets was not statistically significant, it should be noted that the variable response of affected platelets to 0.1 U/ml thrombin was completely eliminated by aspirin. All affected platelets failed to aggregate in response to 0.1 U/ml thrombin after incubation with aspirin. Although secretion of ATP was slightly inhibited by aspirin in both groups, these changes were not significant. The effects of aspirin on the responses of control and affected platelets stimulated by 0.1 U/ml thrombin are summarized in Table 8.

Table 8. Maximum aggregation (%), slope, time to maximum aggregation (minutes), ATP secretion (μM) and time to maximum secretion (minutes) of control and affected gel-filtered platelets stimulated by 0.1 U/ml thrombin in the absence and presence of 1.0 mM aspirin.

	1.0 mM Aspirin	Control	Affected
% Aggregation	-	69.3 ± 26.1 (5)	14.8 ± 21.4 (4)*
	+	45.4 ± 32.3 (3)	0 (4)*
Slope	-	5.1 ± 2.4 (5)	0.28 ± 0.39 (4)*
	+	3.3 ± 2.6 (3)	0 (4)*
Time to Max^m Aggreg^n	+	6.5 ± 0.9 (5)	11.8 ± 1.3 (2)*
	-	5.9 ± 1.1 (3)	NA
ATP μM	-	2.9 ± 0.6 (5)	1.8 ± 1.6 (4)
	+	2.4 ± 0.9 (3)	0.95 ± 1.24 (4)
Time to Max^m Secretion	-	2.7 ± 0.5 (5)	3.7 ± 1.3 (3)
	+	3.1 ± 1.2 (3)	3.4 ± 0.2 (2)

Values = mean ± SD, (n) = number of samples, NA = "not applicable", * = significantly different from control platelets ($p < 0.05$).

Table 9. Maximum aggregation (%), slope, time to maximum aggregation (minutes), ATP secretion (μM) and time to maximum secretion (minutes) of control and affected gel-filtered platelets stimulated by 0.05 U/ml thrombin in the absence and presence of 1.0 mM aspirin.

	1.0 mM Aspirin	Control	Affected
% Aggregation	-	28.4 ± 34.8 (5)	0 (4)*
	+	19.6 ± 34.0 (3)	0 (4)*
Slope	-	1.5 ± 1.9 (5)	0 (4)*
	+	1.1 ± 1.9 (3)	0 (4)*
Time to Max^m Aggreg^n	+	6.0 ± 0.7 (3)	NA
	-	5.5 ± 0 (1)	NA
ATP μM	-	1.4 ± 1.2 (4)	0.57 ± 0.74 (3)
	+	0.2 ± 0.17 (3)	0.09 ± 0.18 (2)
Time to Max^m Secretion	-	3.5 ± 1.0 (4)	3.6 ± 0.5 (2)
	+	5.0 ± 1.3 (2)	2.4 ± 3.4 (2)

Values = mean ± SD, (n) = number of samples, NA = "not applicable", * = significantly different from control platelets ($p < 0.05$).

Although the extent and rate of aggregation of control platelets stimulated by 0.05 U/ml thrombin were inhibited by aspirin, this was not significant. At this concentration there was marked individual variation in the responses of the control dogs, creating a large standard deviation in both the non- and aspirin-treated groups. The affected platelets did not aggregate in response to this concentration of thrombin. Secretion of ATP from control platelets stimulated by 0.05 U/ml thrombin was reduced by treatment with aspirin and the time to maximum secretion prolonged, but neither if these changes were

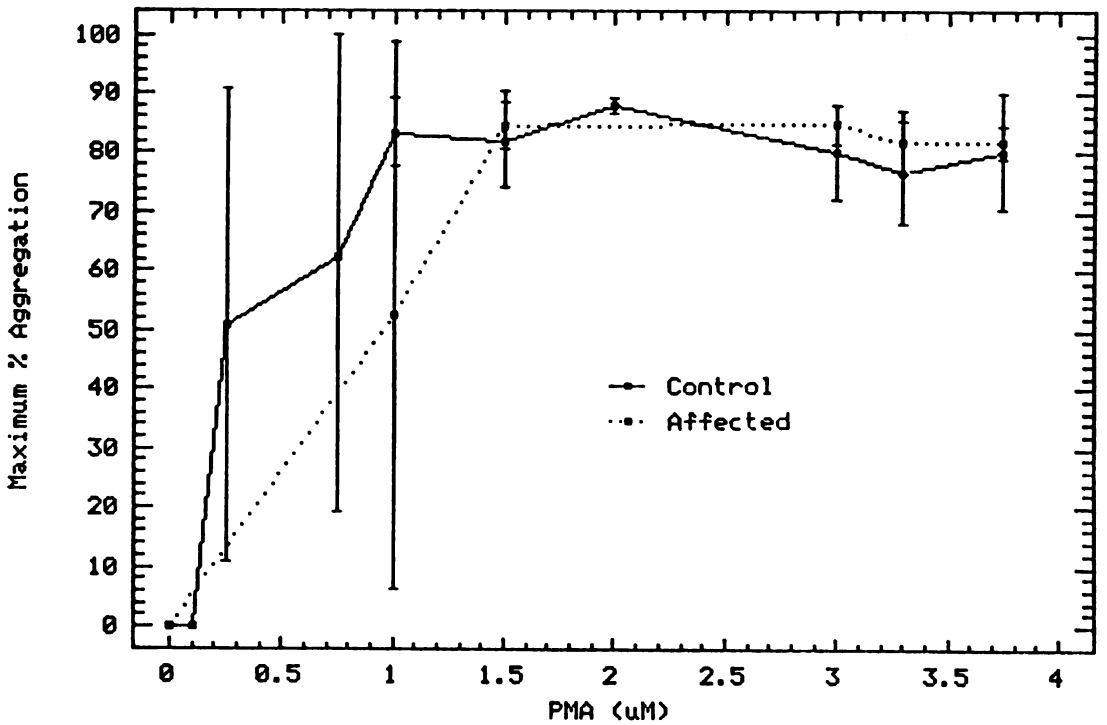
statistically significant. The small amount of ATP secretion by affected platelets in response to 0.05 U/ml thrombin was further reduced by treatment with aspirin. The effects of aspirin on the responses of control and affected platelets stimulated by 0.1 U/ml thrombin are summarized above in Table 9.

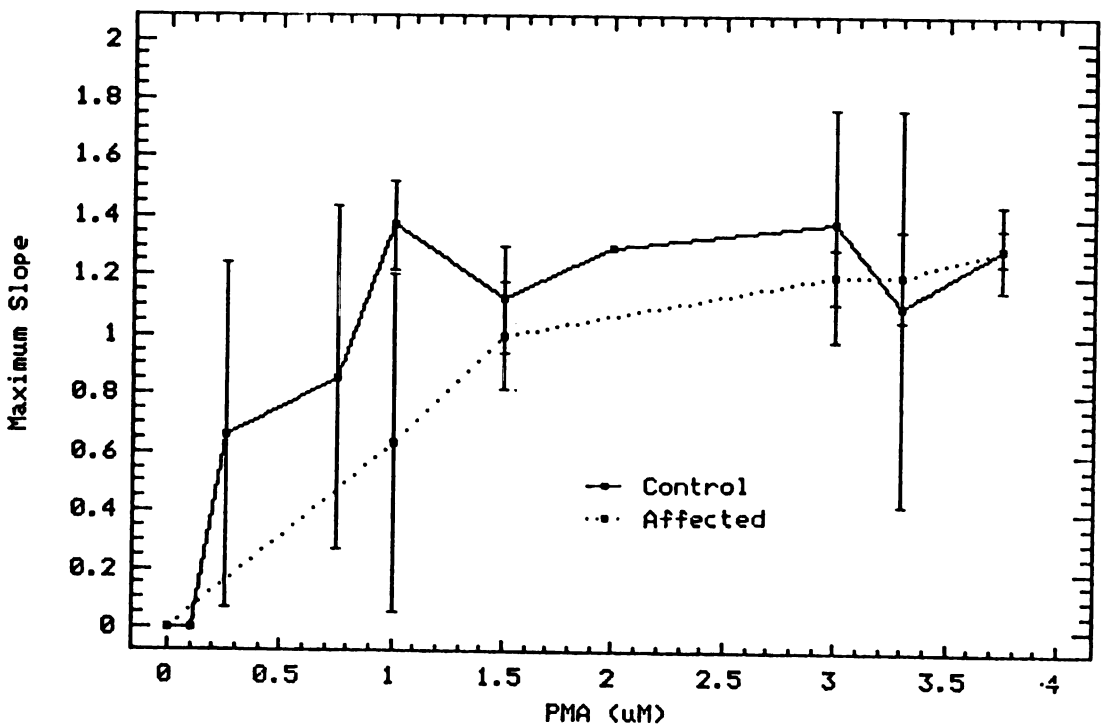
Phorbol Myristate Acetate

Phorbol myristate acetate is a non-physiologic platelet agonist which aggregates platelets in a manner that is dependent on ionized calcium and binding of fibrinogen, but independent of cyclooxygenase products.[341,438,529] Control canine platelets in platelet rich plasma did not change shape, but aggregated slowly and irreversibly in response to concentrations of PMA of 0.25 μM or higher. The affected platelets also aggregated irreversibly, although more slowly than control platelets. Dose response curves for the percent aggregation, maximum slope and time to maximum aggregation are shown in Figures 20, 21 and 22. Affected platelets took longer to reach maximum aggregation in response to all concentrations of PMA used, although the difference was significant only in response to 1.0 and 3.0 μM PMA (Table 10). Typical aggregation curves are illustrated in Figure 23. 4-α-Phorbol is an inactive analogue of PMA. Neither control or affected platelets stimulated by 4-α-phorbol changed shape, aggregated or released any granule contents.

Figure 20. Dose response curves for the maximum percent aggregation of control and affected platelet rich plasma stimulated by PMA.

Figure 21. Dose response curves for the maximum slope of the aggregation curves of control and affected platelet rich plasma stimulated by PMA.


Maximum % Aggregation
100
90
80
70
60
50
40
30
20
10
0
0
0.5
1
1.5
2
2.5
3
3.5
4
PMA (uM)
Control
Affected


Maximum Slope
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
0.5
1
1.5
2
2.5
3
3.5
4
PMA (uM)
Control
Affected

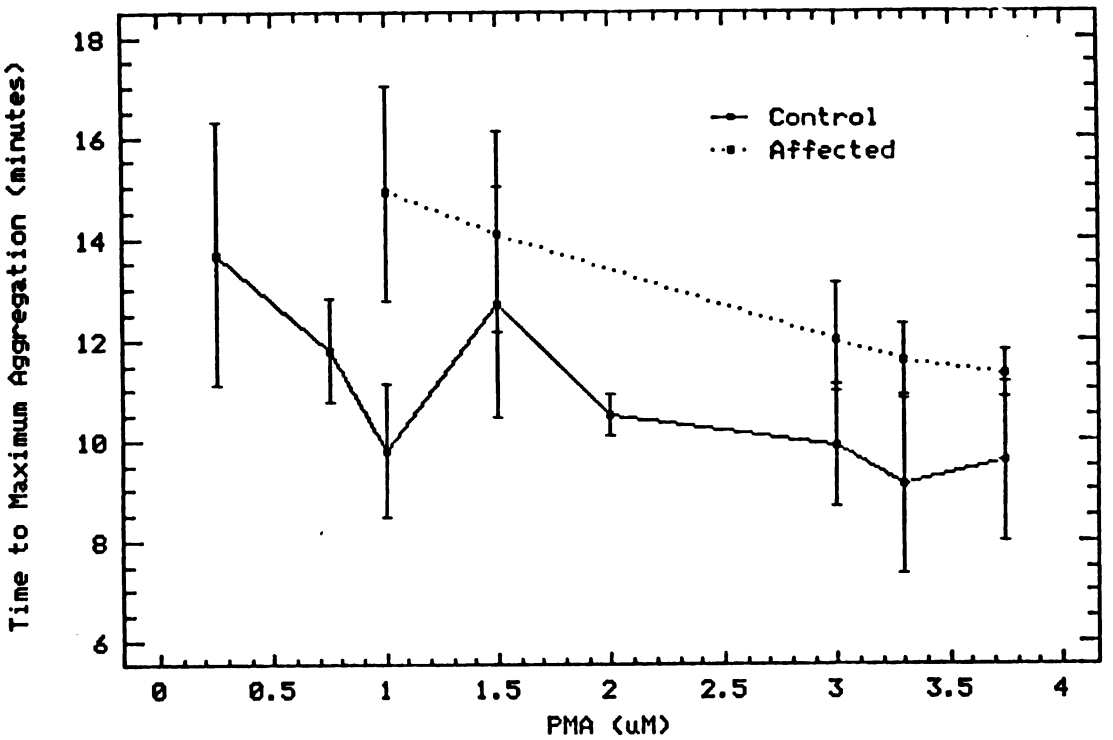


Figure 22. Dose response curves for the time taken (minutes) by control and affected platelet rich plasma to reach maximum percent aggregation when stimulated by PMA.

Table 10. Time taken (minutes) by control and affected platelet rich plasma stimulated by PMA to reach maximum aggregation.

PMA (μM)	Control	Affected
1.0	9.8 ± 1.3 (3)	14.9 ± 2.1 (2)*
1.5	12.7 ± 2.2 (4)	14.1 ± 2.0 (3)
3.0	9.9 ± 1.2 (5)	12.0 ± 1.1 (4)*
3.75	9.6 ± 1.6 (4)	11.3 ± 0.5 (3)

Values = mean ± SD, (n) = number of samples, * = significantly different from control platelets ($p < 0.05$).

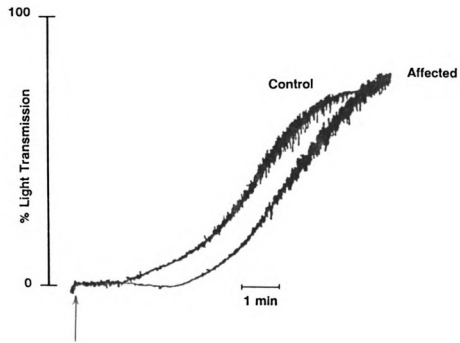
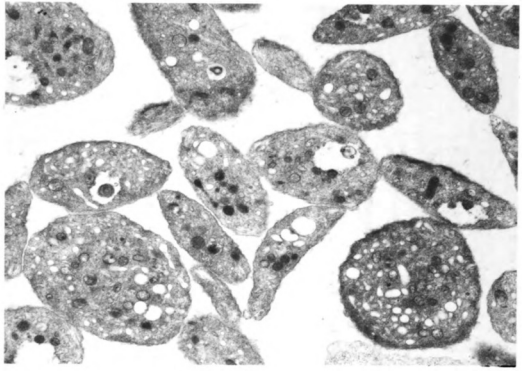


Figure 23. Typical aggregation curves for control and affected platelet rich plasma stimulated by 3.0 μM PMA.

The aggregation studies were coupled to transmission electronmicroscopy with representative results being shown in Figures 24a, 24b, 25a and 25b. There was no apparent shape change or aggregation of platelets in either group two minutes after the addition of 3.0 μM PMA. By ten minutes, aggregation of control and affected platelets appeared complete. Both control and affected platelets appeared vacuolated.

Figure 24a. Transmission electronmicrograph of control platelet rich plasma two minutes after the addition of 3.0 μM PMA (X 11,250). The platelets have not changed shape, centralized their granules or aggregated.

Figure 24b. Transmission electronmicrograph of affected platelet rich plasma two minutes after the addition of 3.0 μM PMA (X 11,250). The platelets have not changed shape, centralized their granules or aggregated.



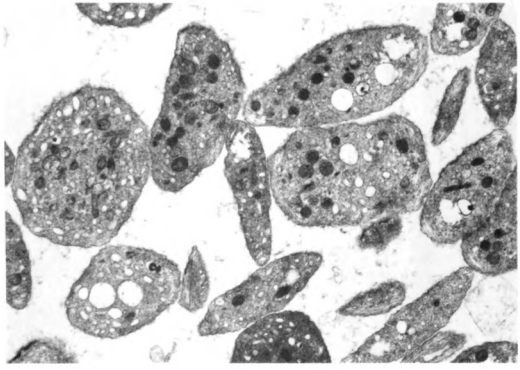
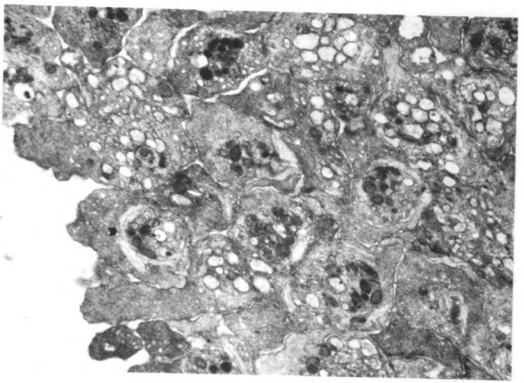
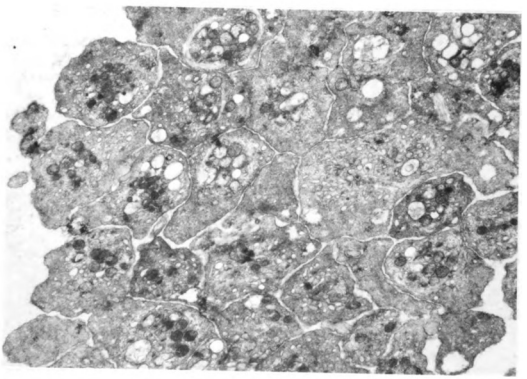


Figure 25a. Transmission electronmicrograph of control platelet rich plasma ten minutes after the addition of 3.0 μM PMA (X 11,250). The platelets have aggregated fully.

Figure 25b. Transmission electronmicrograph of affected platelet rich plasma ten minutes after the addition of 3.0 μM PMA (X 11,250). The platelets have aggregated fully.





Secretion of dense granule contents from control and affected platelets in response to PMA was very slow, occurring when aggregation was near or at its maximum on the aggregometer tracing. Affected platelets aggregated more slowly than control platelets and also secreted more slowly, although this was only significant in response to 3.0 μM PMA. Overall, the release response of both groups of platelets was less consistent, both in the amount of ATP and the time to reach peak secretion, than the responses of the agonists discussed above. The luminescence channel gain was set considerably higher than for other agonists because the luciferin-luciferase substrate-enzyme system has a temperature optimum of 23°C and is steadily inactivated at 37.5°C, reducing the sensitivity of the ATP detection system by the time PMA-stimulated secretion occurred.[165] At high gain settings there was more background noise and variation in the results. Results are summarized in Table 11.

Table 11. Dense granule ATP secretion (μM) and time to reach peak secretion (minutes) by control and affected platelet rich plasma stimulated by PMA.

PMA	ATP Secreted		Time to Max[a] Secretion	
(μM)	Control	Affected	Control	Affected
1.5	1.4 ± 0.7 (4)	1.4 ± 1.0 (3)	9.7 ± 2.5 (4)	9.0 ± 3.1 (3)
3.0	1.8 ± 0.6 (5)	2.5 ± 0.9 (4)	7.9 ± 1.1 (5)	10.7 ± 1.2 (4)*
3.75	3.1 ± 1.6 (3)	2.0 ± 0.9 (3)	8.4 ± 1.3 (3)	10.4 ± 0.8 (3)

Values = mean ± SD, (n) = number of samples, * = significantly different from control platelets ($p < 0.05$).

Neither the extent, rate or time to reach maximum aggregation of control or affected platelet rich plasma stimulated with 3.0 μM PMA was affected by 1.0 mM aspirin (Table 12). This was not unexpected, as it has been reported that PMA-induced aggregation of platelets is independent of cyclooxygenase products.[341,529] However, the secretion of ATP was slightly, but significantly, inhibited by aspirin in control platelets. The real significance of this change is doubtful.

Table 12. Maximum aggregation (%), slope, time to maximum aggregation (minutes), ATP secreted (μM) and time to maximum secretion (minutes) of control and affected platelet rich plasma stimulated by 3.0 μM PMA in the absence and presence of 1.0 mM aspirin.

	1.0 mM Aspirin	Control	Affected
% Aggregation	-	80.0 ± 7.3 (5)	80.0 ± 3.2 (4)
	+	80.5 ± 14.7 (4)	84.8 ± 12.1 (4)
Slope	-	1.4 ± 0.4 (5)	1.2 ± 0.1 (4)
	+	1.3 ± 0.2 (4)	1.2 ± 0.2 (4)
Time to Max^{m} Aggreg^{n}	+	9.9 ± 1.2 (5)	12.0 ± 1.1 (4)*
	-	10.1 ± 1.7 (4)	12.5 ± 2.2 (4)
ATP μM	-	1.8 ± 0.6 (5)	2.5 ± 0.9 (4)
	+	2.2 ± 1.3 (4)	1.6 ± 0.5 (4)
Time to Max^{m} Secretion	-	7.9 ± 1.1 (5)	10.7 ± 1.2 (4)*
	+	9.2 ± 1.3 (4)#	11.0 ± 0.9 (4)

Values = mean ± SD, (n) = number of samples, * = significantly different from control platelets ($p < 0.05$), # = significantly different from non-aspirin-treated platelets ($p < 0.05$).

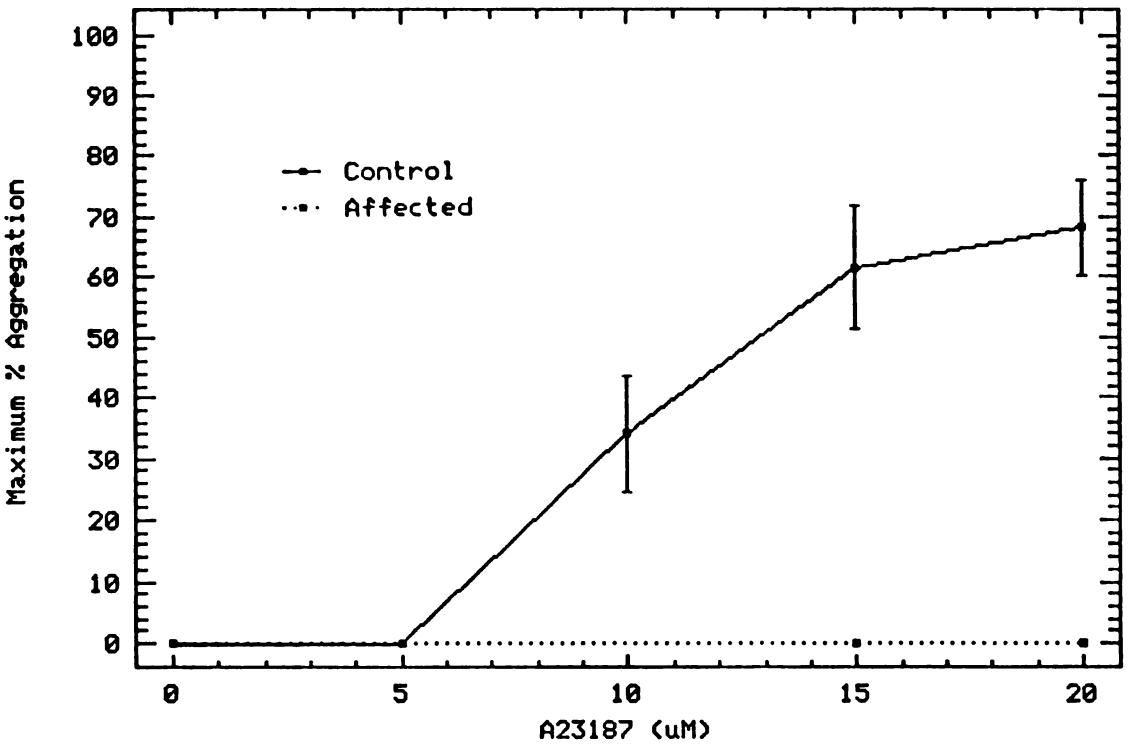
Calcium Ionophore A23187

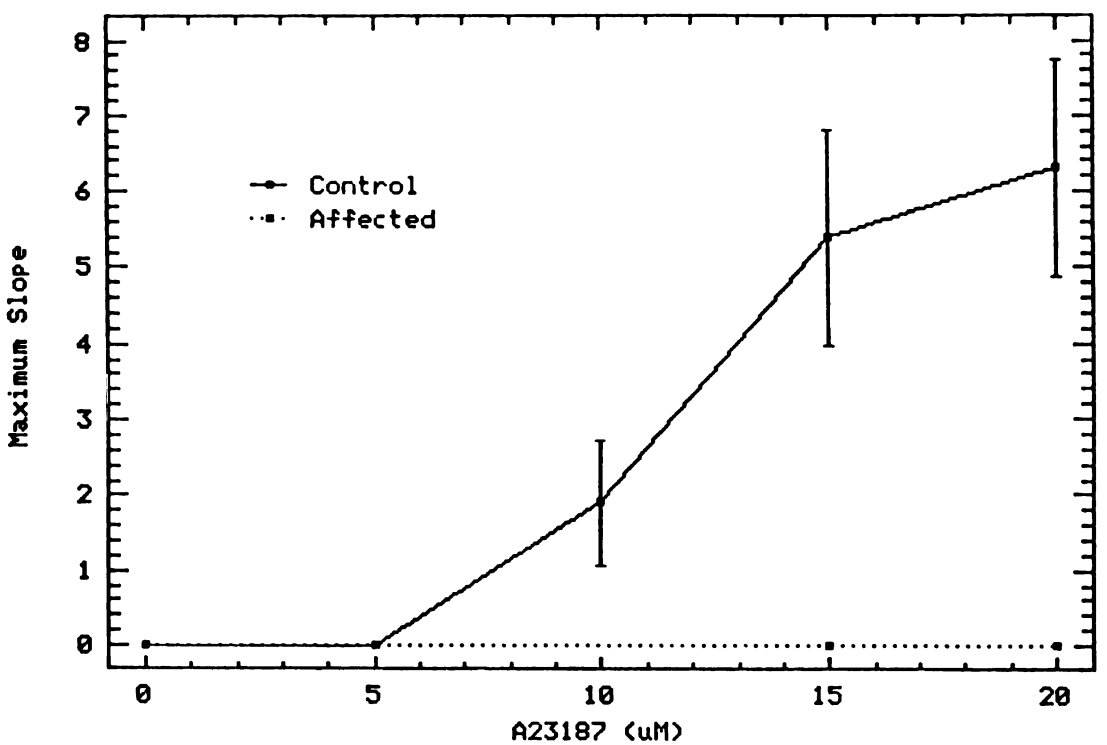
Calcium ionophores such as A23187 are non-physiologic agonists which activate platelets by their ability to mobilize calcium across membranes, increase intracytoplasmic calcium concentrations and generate endoperoxides (prostaglandin G_2 and prostaglandin H_2) and thromboxane A_2.

Affected platelets changed shape, but did not aggregate in response to A23187. In control platelet rich plasma, A23187 caused shape change, aggregation and dense granule ATP secretion. Similar results were obtained in control gel-filtered platelets in the absence of added exogenous fibrinogen. It was observed that occasionally A23187 would fail to elicit aggregation in a cuvette, even although the rest of the sample from the individual aggregated fully. This occurred more frequently at lower concentrations of A23187 and accounts for the larger standard deviations seen with this agonist. Dose-response curves for aggregation and maximum slope of control and affected platelets in platelet rich plasma are shown in Figures 26 and 27 respectively and representative aggregation curves in Figure 28.

Figure 26. Dose response curves for the maximum percent aggregation of control and affected platelet rich plasma stimulated by A23187.

Figure 27. Dose response curves for the maximum slope of the aggregation curves of control and affected platelet rich plasma stimulated by A23187.


Maximum % Aggregation
100
90
80
70
60
50
40
30
20
10
0
Control
Affected
0
5
10
15
20
A23187 (uM)


Maximum Slope
8
7
6
5
4
3
2
1
0
Control
Affected
0
5
10
15
20
A23187 (uM)

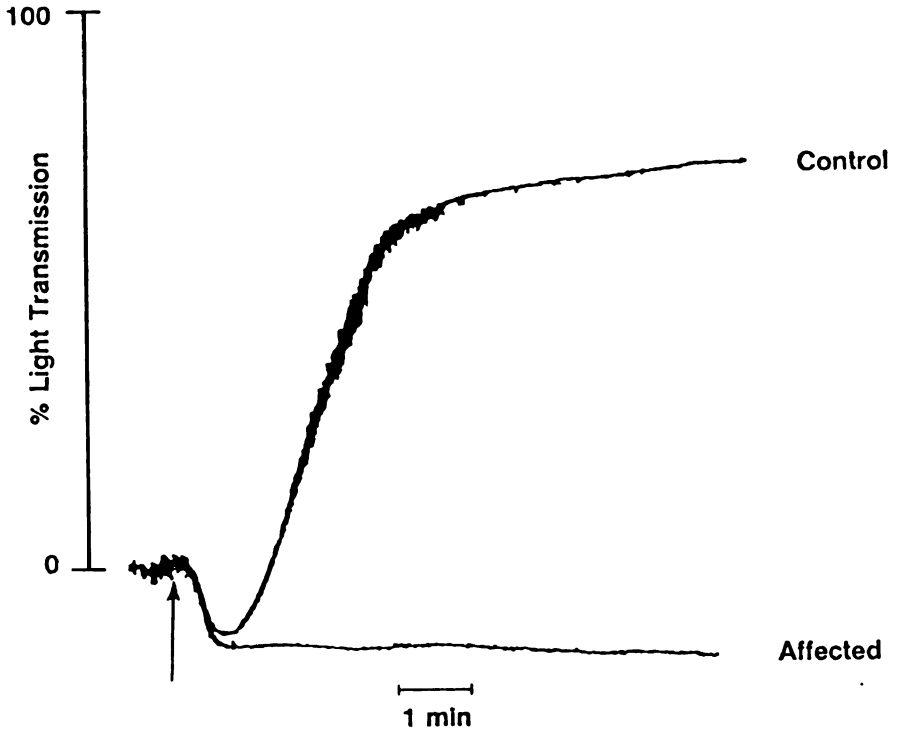


Figure 28. Typical aggregation curves for control and affected platelet rich plasma stimulated by 20 μM A23187.

Control platelets in platelet rich plasma released ATP in response to all concentrations of A23187 used. Secretion of ATP from affected platelets either did not occur or was greatly reduced compared to controls. These results are summarized in Table 13.

Table 13. Dense granule ATP secretion (μM) and time to reach peak secretion (minutes) by control and affected platelet rich plasma stimulated by the calcium ionophore A23187.

A23187	ATP Secreted		Time to Max^{m} Secretion	
(μM)	Control	Affected	Control	Affected
10	3.6 ± 1.9 (3)	ND	2.1 ± 0.5 (3)	ND
15	4.3 ± 0.9 (5)	0.21 ± 0.08 (4)*	2.8 ± 1.2 (5)	1.4 ± 0.6 (4)
20	4.7 ± 0.3 (5)	0.27 ± 0.13 (4)*	1.9 ± 0.3 (5)	1.0 ± 0.4 (4)*

Values = mean ± SD, (n) = number of samples, ND = "not done",
* = significantly different from control platelets ($p < 0.05$).

Figures 29a, 29b, 30a and 30b show transmission electronmicrographs of control and affected gel-filtered platelets one and six minutes after the addition of 7.5 μM A23187. Gel-filtered platelets, rather than platelet rich plasma, were used in this study to demonstrate the lytic effect of a high effective concentration of A23187 in buffer with a low protein content.

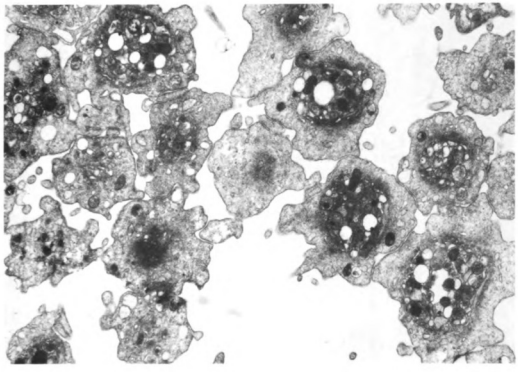
At one minute, control platelets stimulated by 7.5 μM A23187 had changed shape, centralized their granules and were starting to aggregate. The affected platelets at this time had also changed shape and centralized their granules, but there was no evidence of aggregation. By six minutes, control platelets had completely aggregated. Affected platelets had not aggregated, but still showed

shape change and some granule centralization. Lysis was not apparent morphologically at this time. When the sections of affected platelets were scanned, a single small aggregate was found. In some areas it did appear that there was possibly some contact or aggregation between groups of two or three affected platelets. This would not be detectable by optical platelet aggregometry.

Transmission electronmicrographs of control and affected gel-filtered platelets exposed to 20 μM A23187 showed rapid lysis and slower onset of lysis as the ionophore concentration was decreased to 10 μM. Figures 31a, 31b, 32a and 32b show transmission electronmicrographs of control and affected gel-filtered platelets one and three minutes after the addition of 20 μM A23187. Figure 33 shows the corresponding aggregometer tracing.

Figure 29a. Transmission electronmicrograph of control gel-filtered platelets one minute after the addition of 7.5 μM A23187 (X 11,250). The platelets have changed shape, centralized their granules and started to aggregate.

Figure 29b. Transmission electronmicrograph of affected gel-filtered platelets one minute after the addition of 7.5 μM A23187 (X 11,250). The platelets have changed shape and centralized their granules, but have not started to aggregate



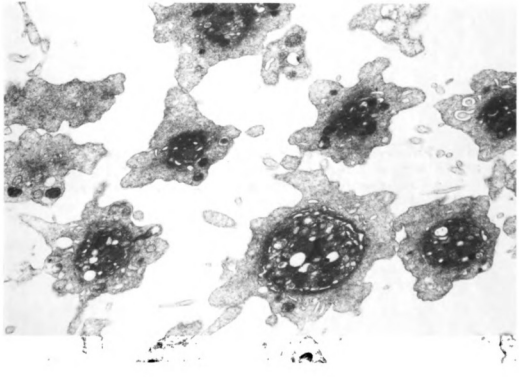
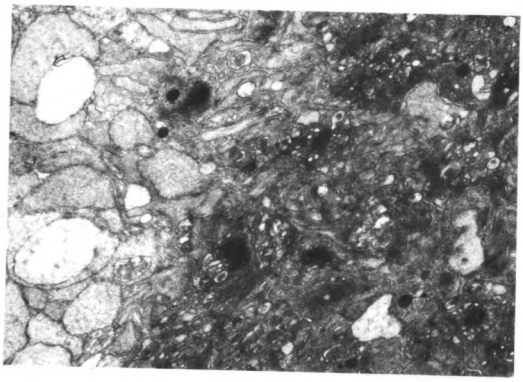


Figure 30a. Transmission electronmicrograph of control gel-filtered platelets six minutes after the addition of 7.5 μM A23187 (X 11,250). The platelets have aggregated fully.

Figure 30b. Transmission electronmicrograph of affected gel-filtered platelets six minutes after the addition of 7.5 μM A23187 (X 11,250). The platelets have changed shape and there is some centralization of granules, but they have not aggregated.



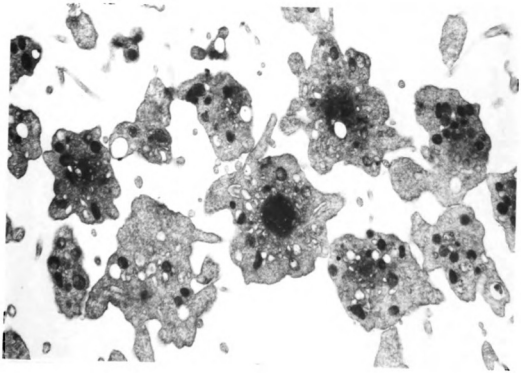
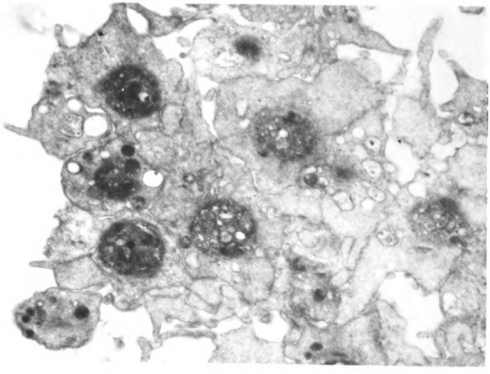


Figure 31a. Transmission electronmicrograph of control gel-filtered platelets one minute after the addition of 20 μM A23187 (X 11,250). The platelets have changed shape, centralized their granules and started to aggregate.

Figure 31b. Transmission electronmicrograph of affected gel-filtered platelets one minute after the addition of 20 μM A23187 (X 11,250). The platelets have undergone some shape change and granule centralization, but have not aggregated.



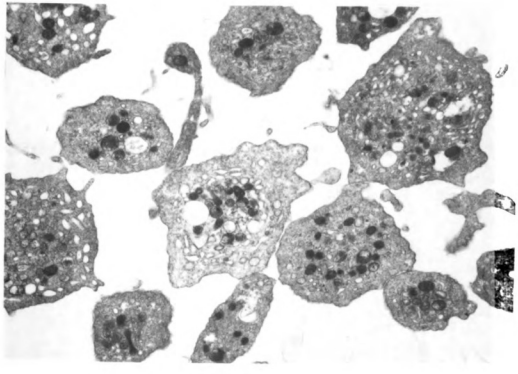
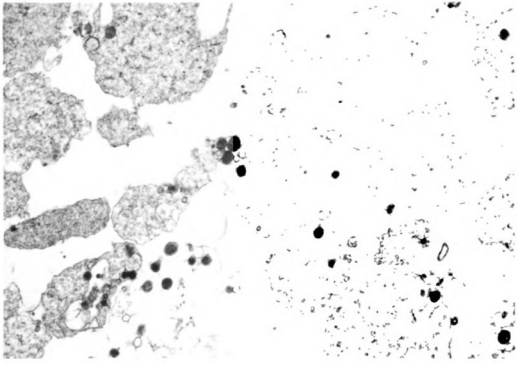
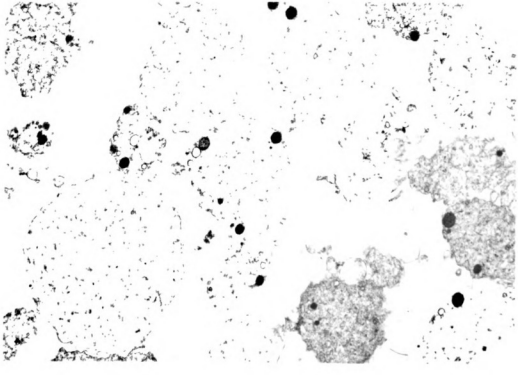


Figure 32a. Transmission electronmicrograph of control gel-filtered platelets three minutes after the addition of 20 μM A23187 (X 11,250). The platelets have undergone lysis.

Figure 32b. Transmission electronmicrograph of affected gel-filtered platelets three minutes after the addition of 20 μM A23187 (X 11,250). The platelets have undergone lysis.





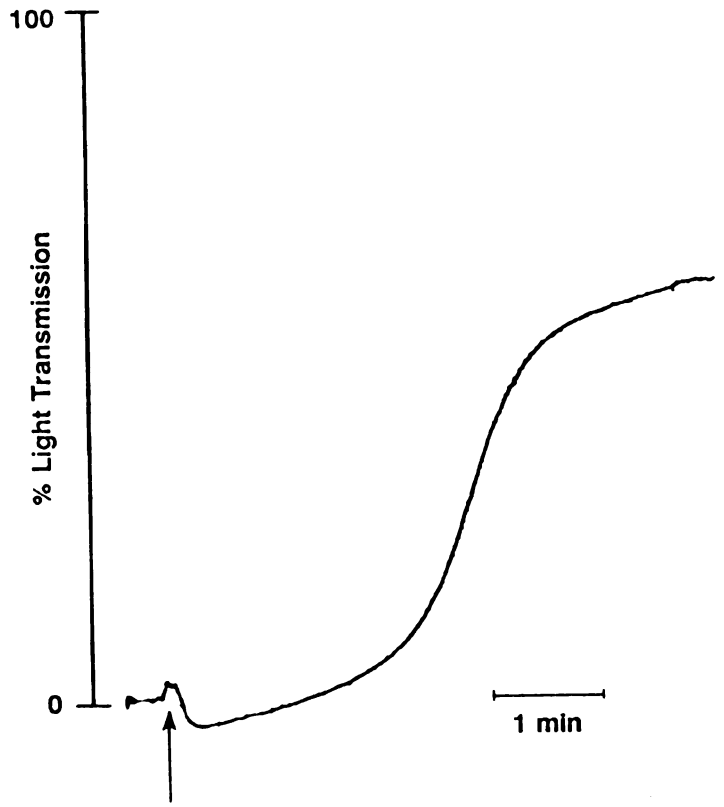


Figure 33. Aggregometer recording corresponding to the transmission electronmicrographs of affected gel-filtered platelets stimulated by 20 μM A23187 (Figures 31b and 32b).

When control platelet rich plasma was treated with 1.0 mM aspirin prior to activation with 20 μM A23187, there was moderate, but not statistically significant, inhibition of the percent aggregation. The rate of aggregation of control platelets and amount of ATP secreted were significantly inhibited while the time to maximum secretion was significantly increased. Although similar changes occurred in aspirin-treated platelets stimulated by 15 μM A23187, they were not significant. These results are summarized in Tables 14 and 15.

Table 14. Maximum aggregation (%), slope, ATP secretion (μM) and time to maximum secretion (minutes) of control and affected platelet rich plasma stimulated by 20 μM A23187 in the absence and presence of 1.0 mM aspirin.

	1.0 mM Aspirin	Control	Affected
% Aggregation	-	68.8 ± 7.8 (5)	0 (4)*
	+	39.1 ± 25.8 (4)	0 (4)*
Slope	-	6.3 ± 1.4 (5)	0 (4)*
	+	2.7 ± 2.2 (4)#	0 (4)*
ATP μM	-	4.7 ± 0.3 (5)	0.27 ± 0.13 (4)*
	+	2.7 ± 0.4 (4)#	0.08 ± 0.10 (4)*
Time to Max^m Secretion	-	1.9 ± 0.3 (5)	1.0 ± 0.4 (4)*
	+	2.9 ± 0.6 (4)#	1.3 ± 0.2 (2)*

Values = mean ± SD, (n) = number of samples, * = significantly different from control platelets ($p < 0.05$), # = significantly different from non-aspirin-treated platelets ($p < 0.05$).

Table 15. Maximum aggregation (%), slope, ATP secretion (μM) and time to maximum secretion (minutes) of control and affected platelet rich plasma stimulated by 15 μM A23187 in the absence and presence of 1.0 mM aspirin.

	1.0 mM Aspirin	Control	Affected
% Aggregation	-	61.5 ± 10.1 (4)	0 (4)*
	+	35.9 ± 21.2 (3)	0 (4)*
Slope	-	5.4 ± 1.4 (4)	0 (4)*
	+	2.2 ± 1.2 (3)	0 (4)*
ATP μM	-	4.3 ± 0.9 (5)	0.21 ± 0.08 (4)*
	+	2.1 ± 0.9 (3)	0.06 ± 0.08 (4)*
Time to Max^m Secretion	-	2.8 ± 1.2 (5)	1.4 ± 0.6 (4)
	+	3.0 ± 0.1 (3)	1.5 ± 0 (2)*

Values = mean ± SD, (n) = number of samples, * = significantly different from control platelets ($p<0.05$).

Dithiothreitol

Dithiothreitol is a reducing agent which causes platelet aggregation not associated with shape change or secretion of granule contents. Platelets treated with dithiothreitol do bind ^{125}I-labeled fibrinogen. Thus, it appears that dithiothreitol exposes the fibrinogen receptor GPIIb-IIIa, enabling the platelets to bind fibrinogen and aggregate. Control and affected platelets in platelet rich plasma showed a slow but irreversible increase in light transmission on the aggregometer recording. These results are summarized in Table 16. As has been reported for human platelets, dithiothreitol (5.0 and 10 mM) did not cause the secretion of dense granule contents in either group.[530]

Table 16. Maximum percent aggregation and slope of control and affected platelet rich plasma exposed to dithiothreitol (DTT).

DTT	% Aggregation		Maximum Slope	
(mM)	Control	Affected	Control	Affected
1.42	0 (2)	ND	0 (2)	ND
1.67	17.8 ± 6.2 (3)	ND	0.23 ± 0.06 (3)	ND
2.0	22.1 ± 7.0 (3)	ND	0.33 ± 0.15 (3)	ND
5.0	33.1 ± 4.0 (3)	35.5 ± 13.4 (3)	0.78 ± 0.13 (3)	0.73 ± 0.40 (3)
6.65	33.1 ± 3.6 (5)	ND	0.77 ± 0.06 (3)	ND
10	29.4 ± 2.8 (3)	46.2 ± 9.6 (3)*	0.90 ± 0.26 (3)	1.1 ± 0.2 (3)

Values = mean ± SD, (n) = number of samples, ND = "not done",
* = significantly different to control platelets ($p < 0.05$).

DISCUSSION

The response of affected Basset hound platelets stimulated by ADP consisted of complete failure of primary aggregation that was accompanied by concurrent secretion of dense granule ATP. Secretion occurred five to six times more rapidly than in control platelets. In an earlier study, transmission electronmicrographs of affected platelets activated with ADP verified the failure of aggregation and early secretion.[377] The morphology of resting affected platelets was normal. These findings confirm and add detail to data reported by other workers.[75]

When ADP is used to activate human or control canine platelets, it acts as a weak agonist.[80,263] It cannot cause irreversible secondary aggregation of platelets unless the secretion response occurs. The secretion response is aggregation-dependent and does not occur in the absence of primary aggregation. Secretion occurs well after aggregation has started, regardless of the concentration of ADP used, and is inhibited by inhibitors of cyclooxygenase. Control canine platelets responded to 10 μM ADP in this fashion. Secretion of dense granule contents peaked approximately two minutes after the onset of aggregation and was almost completely inhibited by aspirin. The response of affected platelets to ADP does not fit that of a weak agonist in that secretion of dense granule contents occurred in the absence of primary aggregation. However, ATP secretion was almost completely inhibited by aspirin, suggesting that ATP secretion from affected platelets is the result of production of arachidonate

metabolites in the absence of primary aggregation. This situation has not been described before and cannot be explained by current knowledge. However, it does suggest that there may be an event or events other than primary aggregation that initiate secretion of arachidonate metabolites, secretion of dense granule contents and irreversible aggregation in response to a weak agonists such as ADP.

Although there have been conflicting reports about the effect of cyclooxygenase inhibitors on PAF, it is now generally considered to be an agonist of intermediate strength.[77,174,310,470] Platelet activating factor can induce secretion in the absence of the formation of arachidonate metabolites, but the response is inhibited, particularly in response to lower concentrations of PAF. The effect of cyclooxygenase inhibitors on PAF-induced aggregation is variable: inhibition of aggregation varies from no apparent inhibition in response to high concentrations of PAF to partial reversal in response to lower concentrations. Removal of extracellular ADP from the medium with apyrase or creatine phosphate/creatinine phosphokinase inhibits irreversible aggregation. Neither removal of ADP or inhibition of cyclooxygenase prevents primary aggregation.[310]

The responses of control canine platelets were similar to those described for human platelets. Irreversible aggregation was not inhibited by aspirin, although at 0.1 μM PAF the extent of aggregation was slightly reduced. There were no ADP scavengers in the medium in this study. Dense granule secretion in response to 1.0 μM PAF still occurred after incubation with aspirin, but was significantly reduced.

The inhibition of secretion in response to 0.1 μM PAF was not significant, but these results were strongly influenced by the one control dog which did not aggregate or release ATP in response to this concentration of PAF. Examination of the individual results indicates that aspirin completely or markedly inhibited ATP secretion in response to 0.1 μM PAF. These results suggest that: (1) secretion in response to PAF is more dependent than aggregation on the production of arachidonate metabolites; and (2) inhibition of arachidonate metabolite production does not significantly affect either primary or secondary aggregation in response to the concentrations of PAF used.

Platelet activating factor did not cause affected platelets to aggregate. The slight to moderate increase in light transmission that occurred when affected platelets were stimulated by high concentrations of PAF (5.0 and 10 μM) may represent true aggregation, or it may reflect platelet agglutination. The recorder tracing generated was not that of a reversible primary aggregation, but a more gradual increase in light transmission similar to that seen with dithiothreitol. Platelet activating factor is a hydrophobic molecule which is able to interact with and penetrate the platelet membrane. It may be that, in the absence of aggregation, high concentrations of PAF alter the surface properties of the platelet such that some agglutination is able to occur.

The very small increase in light transmission seen when affected platelets were stimulated by 1.0 μM PAF was completely inhibited by aspirin, although this was not statistically significant. The more

marked inhibition by aspirin of ATP secretion from affected platelets compared to controls stimulated by 1.0 μM PAF suggests that arachidonate metabolites may play a more important role in inducing secretion in affected platelets. This difference seems unlikely to be related to effects of released ADP on secretion, as neither control nor affected aspirin-treated platelets released ATP in response to ADP. However, released ADP may contribute to the aggregation response of control aspirin-treated platelets.

While ATP secretion from control platelets stimulated by 0.1 μM PAF was markedly reduced by aspirin, affected platelets failed to secrete in the presence or absence of aspirin. If the formation of arachidonate metabolites is always accompanied by the secretion of dense and alpha granule contents, it can be proposed that affected platelets fail to liberate arachidonic acid and/or metabolize arachidonic acid to endoperoxides and thromboxane in response to 0.1 μM PAF. A similar assumption could be made for the control dog which did not aggregate or secrete in response to 0.1 μM PAF. It is presumed that this difference in the control population is a reflection of individual variation in the sensitivity to PAF, as has been reported in man.[77]

The results from this study indicate that PAF is an agonist of intermediate strength. When used at a low concentration such as 0.1 μM, secretion and, to a lesser extent, aggregation are inhibited by aspirin. The effects of released ADP were not examined in this study, but released ADP may contribute to the aggregation response of aspirin-

treated platelets. At the higher concentration of 1.0 μM, aspirin partially inhibits the secretion response, but does not inhibit aggregation.

The response of affected platelets to PAF is similar to their response to ADP. Both agonists fail to initiate primary aggregation, although higher concentrations of PAF do cause some increase in light transmission. Secretion of dense granule contents is markedly inhibited by treatment with aspirin which indicates that secretion is dependent upon the formation of arachidonate metabolites. These metabolites are formed in the absence of primary aggregation. The greater sensitivity of affected platelets to the inhibitory effects of aspirin suggest that arachidonate metabolites may play a more important role in the secretory response of affected than control platelets.

Thrombin is a strong agonist which can induce irreversible aggregation in the absence of the release response. Secretion is not dependent on aggregation. However, the exact mechanism of thrombin-induced platelet activation is not known. Thrombin has at least two mechanisms of action. One is a hormone-like binding to GPIb on the platelet membrane. Binding to this high affinity binding site is essential for platelet activation by low concentrations of thrombin.[181] Thrombin is also a serine protease which specifically cleaves GPV on the platelet surface to release a hydrolytic fragment.[383] However, the role of the proteolytic activity of thrombin in platelet activation is not known. Bernard-Soulier platelets that lack GPIb, GPV and GPIX are less sensitive to low concentrations of thrombin, but respond almost

normally to high concentrations. Similarly, platelets that have been treated with exogenous proteases such as Ca^{2+}-dependent protease or chymotrypsin to remove GPIb and GPV from the platelet surface are still able to respond to thrombin. There is a delay in the onset of the aggregation and secretion response, but the extent of aggregation is not significantly altered.[322] This suggests that GPIb and GPV may not be essential for the induction of the response of platelets to thrombin, but may play a role in accelerating or amplifying it.

After a delay of one to three minutes, affected platelets aggregated maximally in response to concentrations of thrombin of 0.2 U/ml or higher. At concentrations of less than 0.2 U/ml, aggregation was either minimal or had not started within five minutes of the addition of the thrombin. From this it can be concluded that, although affected platelets do aggregate in response to thrombin, their response is different from that of the control group in at least two ways. Firstly, affected platelets are less sensitive to lower concentrations of thrombin than control platelets. Secondly, the delayed onset, decreased rate of aggregation and increased time to reach maximum aggregation compared to controls implies that the mechanisms of activation by thrombin may be different in the two groups.

Although the rate of aggregation was significantly less in the affected platelets, the amount and rate of ATP release was not significantly different from that of control platelets. Even when aggregation failed to occur within five minutes of the addition of 0.05 U/ml of thrombin, affected platelets still secreted a small amount of

ATP. As the concentration of thrombin was decreased, the amount of ATP secreted decreased and the time to maximum secretion increased in both groups. However, at concentrations of 0.2 U/ml or less, these changes in the affected group appeared to be amplified compared to the control group. The secretion results for both groups are compatible with thrombin's function as a strong agonist which is capable of inducing secretion in the absence of aggregation.

When platelets were incubated with 1.0 mM aspirin before being stimulated with 0.3 U/ml thrombin, a concentration that is generally considered to be high, it was expected that it would have no effect on aggregation or secretion, at least in the control group. There was however a slight, but significant, reduction in the amount of ATP secreted by control platelets. The overall aggregation response was reduced slightly, although not significantly. In contrast, aspirin significantly inhibited both the extent and rate of the aggregation response of affected platelets and caused a dramatic increase in the time taken to reach maximum aggregation. The amount of ATP secreted was significantly reduced and the time taken to reach maximum secretion significantly increased. This indicates that a major difference between control and affected platelets in their responses to thrombin is the much greater dependence of affected platelets on the production of arachidonate metabolites.

Although the mean values for percent aggregation, slope, time to maximum aggregation, ATP released and time to maximum secretion were reduced in aspirin-treated control platelets stimulated by

0.1 or 0.05 U/ml thrombin, these reductions were not significant. Two of the four affected dogs aggregated weakly in response to 0.1 U/ml thrombin. This aggregation was completely inhibited by aspirin. Although the reduction in ATP secretion in aspirin-treated affected platelets was not significant, the group included one dog which did not release in the absence or presence of aspirin. Examination of the individual results indicated that aspirin markedly inhibited ATP secretion by affected platelets. Affected dogs did not aggregate in response to 0.05 U/ml thrombin in the absence or presence of aspirin. The small amount of ATP secretion was further reduced in the presence of aspirin.

The lack of significant effect of aspirin on control platelets stimulated by the lower concentrations of thrombin was not expected. There was marked individual variation in the aggregation response to 0.1 and 0.05 U/ml thrombin which made establishing statistically significant differences difficult. It seems unlikely that the thrombin concentration was not low enough as only three of five control dogs aggregated at this concentration and no control dogs aggregated in response to 0.025 U/ml thrombin.

In contrast, affected platelets appeared to be more sensitive than control platelets to the effects of aspirin when stimulated by thrombin. Similar to ADP- and PAF-stimulated affected platelets, the production of arachidonate metabolites appears to play a more important role in the responses of affected platelets to thrombin than in control platelets.

Tumor-promoting phorbol esters such as PMA are non-physiologic agonists which are able to aggregate platelets irreversibly and cause dense granule secretion. Phorbol esters are able to substitute for 1,2-diacylglycerol in activation of the Ca^{2+}-phospholipid dependent protein kinase C. 1,2-Diacylglycerol sharply increases the affinity of the enzyme for Ca^{2+} as well as for phospholipid and thus initiates its activation at physiological Ca^{2+} concentrations.[73,74,361] However, it is not known how this, or other effects of PMA, cause platelet activation and aggregation.[74] In contrast to 1,2-diacylglycerol which is formed transiently during platelet activation and activates protein kinase C in a reversible manner, phorbol ester-mediated activation of protein kinase C is sustained and not accompanied by phosphatidylinositol turnover or formation of arachidonate metabolites.

Protein kinase C phosphorylates multiple platelet proteins, the most well-recognized of which is a band with a molecular weight of 47 kDa.[238,279] The relationship of the phosphorylation of these proteins to specific events in platelet activation is not known. The aggregation of platelets by phorbol esters is dependent on the presence of Ca^{2+} and the binding of fibrinogen, but is independent of the formation of arachidonate metabolites and inhibited by agents which increase intracellular cyclic AMP levels.[341,438,529] The role of secreted ADP in phorbol ester-induced platelet activation is less clear. It has been reported that the early, very slow phase of phorbol ester-induced aggregation is independent of secreted ADP, but the more rapid phase which follows is ADP-dependent.[529] At very low concentrations of phorbol ester, this rapid phase does not occur and aggregation is not

affected by the presence of ADP scavengers in the medium. Aggregation in response to very high concentrations of phorbol ester is also not affected by ADP scavengers. Therefore, released ADP appears to affect the rate of aggregation produced by intermediate concentrations of phorbol ester. Secretion of dense granule contents occurs mainly during the latter part of the steep phase of aggregation. Aspirin has been reported to have similar effects to ADP-scavenging enzymes, although it has since been reported that phorbol-ester induced aggregation is not inhibited by cyclooxygenase inhibitors and is not accompanied by the generation of arachidonate metabolites.[341]

The aggregation of control platelets was very similar to that described for human platelets, except that the concentrations of PMA required were considerably higher.[529] At concentrations just above the threshold for aggregation (≥0.25 μM), aggregation of control platelets proceeded slowly, without a rapid phase. At higher concentrations, the initial slow phase was followed by a more rapid phase. Affected platelets also aggregated fully and followed the same general pattern, but were slightly less sensitive to lower concentrations of PMA, had a slower maximum rate of aggregation and took longer to reach maximum aggregation. Although affected platelets took longer to reach maximum aggregation in response to all concentrations of PMA used, the difference was significant only for 1.0 and 3.0 μM. The vacuolated appearance of PMA-treated platelets in the transmission electronmicrographs is due to the swelling of granules and their fusion with dilated channels of the open canalicular system.[508] Secretion of ATP in both groups was variable but occurred at the time of, or

slightly after, maximum aggregation. The slower response of affected platelets may reflect an inability to respond or be amplified by secreted ADP.

The maximum extent and rate of aggregation in response to 3.0 μM PMA were not significantly affected by 1.0 mM aspirin in either group. The time to maximum secretion was significantly increased by aspirin in control platelets, but the amount of ATP was not affected. In affected platelets, the amount of ATP was significantly reduced by aspirin, but the time to maximum secretion was not altered. It is difficult to assign a real significance to these effects of aspirin in view of the variability of the secretion of ATP by PMA.

In conclusion, the aggregation of affected platelets by PMA indicates: (1) affected platelets very probably have protein kinase C that is able to be activated by phorbol esters; (2) and the as-yet unidentified pathway involved in platelet activation by phorbol esters is intact in affected platelets.

The calcium ionophores (A23187 and ionomycin) are non-physiologic platelet agonists that are able to induce the same range of responses as physiologic agonists: shape change, the release reaction and aggregation.[129,131,312,505] They are able to cross the plasma membrane and directly mobilize Ca^{2+} from intracellular stores and increase the intracytoplasmic Ca^{2+} concentration. This activates phospholipase A_2 to release arachidonic acid which is metabolized to endoperoxides and thromboxane A_2.[275] High concentrations of A23187 are able to activate

the phosphomonoesteratic breakdown of the polyphosphoinositides.[281] The calcium ionophores do not bind to receptors, or directly induce phospholipase C activation, inositol lipid hydrolysis and formation of 1,2-diacylglycerol, phosphatidic acid or inositol phosphates. Activation of phospholipase C and stimulation of these events in ionophore-activated platelets is the result of endoperoxide and thromboxane production and is blocked by cyclooxygenase inhibitors.[281,413] However, even in the presence of cyclooxygenase inhibitors and absence of phospholipase C activation, the A23187-induced increase in cytoplasmic Ca^{2+} concentration is able to stimulate phosphorylation of the 47 kDa substrate of protein kinase C and the 20 kDa substrate of myosin light chain kinase.[281] Although cyclooxygenase inhibitors block arachidonate metabolism, platelet activation induced by A23187 is not completely blocked.[275] At lower concentrations of A23187, secretion of dense granule contents is dependent upon formation of cyclooxygenase products and the presence of ADP.[413] Higher concentrations of ionophore overcome this inhibition.

Control and affected gel-filtered platelets and platelets in platelet rich plasma changed shape in response to the concentrations of A23187 used. Shape change in control platelets was followed by a dose-dependent aggregation and release of ATP. Affected platelets did not aggregate and released only 4-6% of the amount of ATP released by control platelets.

When control platelet rich plasma was stimulated by 15 or 20 μM A23187 in the presence of 1.0 mM aspirin, the extent and rate of

aggregation were reduced. Only the reduction in the rate of aggregation in response to 20 μM A23187 was statistically significant. The amount of ATP secreted in response to 20 μM A23187 was significantly reduced and the time to maximum secretion significantly increased in aspirin-treated platelets. Although aspirin also reduced the extent and rate of aggregation of platelets stimulated by 15 μM A23187, the decrease was not statistically significant. Treatment with aspirin further reduced the small amount of ATP secreted by affected platelets.

Because A23187 binds strongly to albumin, the concentration needed to activate platelets without causing significant cell lysis depends on the albumin content of the medium. Concentrations of 30 μM in platelet rich plasma and 8.0 μM in gel-filtered platelets (0.2% albumin) are the highest concentrations of A23187 that do not cause more than 2% cell lysis.[202] Affected gel-filtered platelets in a buffer containing 0.3% albumin were rapidly lysed by 20 μM A23187. The curve generated by this lysis was not unlike an aggregation tracing, but lacked the normal oscillations generated by the shape of the platelets (Page 200). When the cuvette was removed from the aggregometer, the contents were clear, with no visible aggregate formation or turbidity. From this, it is concluded that the aggregation of affected Basset hound hereditary thrombopathy gel-filtered platelets by 40 μM A23187 that was reported by Catalfamo et al.[75,76] can be attributed to platelet lysis. Affected platelets do not have an aggregation response that is detectable by optical platelet aggregometry when stimulated by non-lytic concentrations of A23187.

The failure of affected platelets to aggregate or secrete normally in response to A23187 would suggest that arachidonic acid might not be mobilized or metabolized in response to A23187. It is also possible that A23187 fails to mobilize Ca^{2+} in affected platelets. However, the normal shape change and centralization of granules would suggest that Ca^{2+} is able to be mobilized. Preliminary work with the Ca^{2+} indicators aequorin and fura-2 also demonstrate that Ca^{2+} mobilization occurs in affected platelets stimulated by A23187.[482] A failure of release or metabolism of arachidonic acid would be in contrast to the response of affected platelets to other agonists such as thrombin, PAF and ADP where arachidonate metabolites seem to play a more important role than in control platelets.

Dithiothreitol is a reducing agent which, in the presence of fibrinogen, causes platelet aggregation that is not associated with shape change or secretion of granule contents.[530] The aggregation is not inhibited by metabolic inhibitors or prostaglandin E_1, but is inhibited at pH 6.5 or the presence of EDTA. In the presence of dithiothreitol, fibrinogen and Ca^{2+}, stirred platelets will bind fibrinogen and aggregate. The mechanism by which dithiothreitol induces fibrinogen binding is poorly understood, but it is thought that reduction of membrane disulfide bonds may be involved.[381] The ability of platelets to aggregate in response to dithiothreitol depends primarily on their ability to bind fibrinogen. Therefore, the ability of affected platelets to aggregate normally in response to dithiothreitol indicates that affected platelets are able to bind fibrinogen.

In conclusion, the patterns of aggregation and dense granule secretion of affected platelets do not fit any described genetic or acquired platelet defect or any situation created in vitro. They apparently have the ability to aggregate, if the appropriate stimulus is provided. However, it appears that either the pathway(s) involved in transmitting the signal from the agonist at the cell membrane to the cell interior is(are) defective, or that the signal is transmitted to the cell interior but is unable to be converted to the normal sequence of cellular events. Alternatively, the pathway(s) may be intact, but the mechanisms which control the pathway(s) is(are) defective. The ability of affected platelets to aggregate in response to phorbol ester and higher concentrations of thrombin indicates that at least one pathway of platelet activation is functional.

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FUNCTIONAL AND BIOCHEMICAL ABNORMALITIES IN BASSET HOUND HEREDITARY THROMBOPATHY

VOLUME II

By

Mary Frances McConnell

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DOCTOR OF PHILOSOPHY

Department of Pathology

604 1383

CHAPTER IV: PHOSPHORYLATION OF PLATELET PROTEINS

INTRODUCTION

Many extracellular signals such as physiological platelet agonists do not permeate the plasma membrane. Instead, they interact with receptors at the cell surface which then relay the signal to the interior of the cell via the production of a limited range of second messengers.[35,305] These second messengers include cyclic adenosine monophosphate (cyclic AMP) and cyclic guanosine monophosphate (cyclic GMP). Second messenger molecules derived from the phosphoinositides include 1,2-diacylglycerol (DAG), 1,4,5-inositol trisphosphate [(1,4,5)IP_3] and metabolites of arachidonic acid. Their production is initiated by specific agonists, they are potent activators of other cellular reactions and they are rapidly degraded to inactive compounds. Many eicosanoids derived from arachidonic acid have these properties of second messengers, although they tend to act upon neighboring cells rather than intracellularly.[303]

Once formed, messengers evoke a wide range of intracellular and cell-to-cell reactions which collectively make up a specific physiological response. Covalent modification of key regulatory proteins by reversible phosphorylation-dephosphorylation is a widely-used mechanism in the control of cellular processes such as secretion, contractility,

protein synthesis, cell growth and differentiation.[439] While a large number of phosphoproteins can be demonstrated in many cell types, including platelets, the function of relatively few of these is modified by reversible phosphorylation. Reversible phosphorylations are catalyzed by protein kinases and phosphatases which are selective for either serine/threonine or tyrosine residues. A single protein may be phosphorylated at multiple sites by different protein kinases with differing effects on biological activity.

Thus, the physiological response of a cell depends not only on the presence of the appropriate agonist binding to its cell surface receptor, but also on the type and distribution of protein kinases, phosphoprotein phosphatases and their cellular targets. In this way, a signal transduction system of second messengers common to many cell types is able to elicit unique biological responses in different cell types. However, the precise means by which the reactions invoked by second messenger molecules induce specific cellular responses such as platelet aggregation and the release response are not clear. Stimulation of platelets by thrombin, collagen, PAF, A23187, serotonin, arachidonic acid and endoperoxide analogues leads to the phosphorylation of a range of proteins. Phosphorylation in response to agonists is prominent in two of these protein bands. One is a 20 kDa protein that has been identified as the 20 kDa myosin light chain.[106] The other is a 47 kDa cytoplasmic protein which has not been positively identified.[183] Phosphorylation of these proteins has not been reported for canine platelets.

Phosphorylation of the 20 kDa myosin light chain is preceded by certain cellular events. Endogenous Ca^{2+} is released from the dense tubular system by the action of the second messenger (1,4,5)IP_3 which is cleaved from phosphatidylinositol bisphosphate by phospholipase C.[12,55,468] The Ca^{2+} that is released by (1,4,5)IP_3 can serve as a trigger for subsequent events, including the activation of Ca^{2+}-dependent enzymes such as the Ca^{2+}-calmodulin-dependent myosin light chain kinase.[189] When four molecules of Ca^{2+} bind to calmodulin, the complex is able to bind to myosin light chain kinase and activate it. Myosin comprises approximately 12% of platelet protein and consists of two heavy chains of M_r = 200 kDa, two light chains of M_r = 20 kDa and two light chains of M_r = 16 kDa.[150] Phosphorylation of the 20 kDa myosin light chain results in polymerization of myosin into filaments, interaction with actin, a 6-to-10-fold increase in its ATPase activity and the association of myosin with the platelet cytoskeleton. There is evidence that elevation of intracytoplasmic Ca^{2+} levels and myosin phosphorylation is associated with platelet shape change and the secretion of platelet granule contents.[105,107,185,359] The properties of phosphorylated myosin may cause the folding of the platelet surface membrane and the contractile wave centralizing the secretory granules seen during shape change. During granule secretion, myosin phosphorylation is further increased.

Phosphorylation of the 47 kDa platelet protein is mediated by the Ca^{2+}- and phospholipid-dependent protein kinase C.[219] In the resting platelet, protein kinase C is present in an inactive form in the cytosol. During platelet activation, protein kinase C binds to the

inner leaflet of the platelet membrane. Although normally almost absent from platelet membranes, DAG is transiently produced in the membrane when platelets are activated. A small amount of DAG dramatically increases the apparent affinity of protein kinase C for Ca^{2+}, fully activating the enzyme without any change in the intracytoplasmic Ca^{2+} concentration.[360] Phorbol esters are able to substitute for DAG in the activation of protein kinase C.[73] In contrast to DAG which is formed transiently during platelet activation and activates protein kinase C in a reversible manner, phorbol ester-mediated activation of protein kinase C is sustained.

Protein kinase C has a broad substrate specificity, phosphorylating the serine and threonine residues of many endogenous proteins in most tissues. The 47 kDa substrate in platelets has been purified, but its function has not been positively identified; it is most likely the IP_3 5'-phosphomonoesterase which is phosphorylated and activated by protein kinase C.[97,219] Another less likely possibility is lipocortin which, in the non-phosphorylated state, exerts an inhibitory effect on phospholipase A_2.[485]

The binding of fibrinogen and aggregation of platelets by phorbol ester suggests that protein kinase C does play a role in the induction of fibrinogen receptors.[438] It is unlikely that protein kinase C has a specific role in the induction of platelet shape change. The fact that phorbol ester is able to substitute for DAG in the activation of protein kinase C and activate platelets without causing shape change provides evidence against such a role.[529] On the other hand, several

studies suggest that phosphorylation of the 47 kDa protein is an important intermediate step in secretion, but it has also been shown that dense granule secretion may be inhibited by low concentrations of chlorpromazine without inhibiting phosphorylation of the 47 kDa protein.[183,368,443] In permeabilized platelets in which the intracellular Ca^{2+} concentration can be controlled, higher Ca^{2+} concentrations are required for optimal secretion than for optimal phosphorylation of both the 47 and 20 kDa proteins.[443] However, it has also been shown that activation of protein kinase C, plus an increase in cytoplasmic Ca^{2+}, can trigger secretion.[238,279] Therefore, the role of protein kinase C activation in platelet secretion is not clear. The phosphorylation of the 47 kDa protein by protein kinase C occurs during primary reversible aggregation induced by many platelet stimuli, but whether the relationship is causal or coincidental is not known.

Because affected Basset hound platelets had shown a unique pattern of responses to agonists, it was logical to try to relate the events of shape change, aggregation and release to the phosphorylation of the 20 and 47 kDa proteins. The agonists used were adenosine diphosphate (ADP), platelet activating factor (PAF), thrombin, calcium ionophore A23187 and phorbol myristate acetate (PMA). This group of agonists contains weak (ADP), intermediate (PAF) and strong (thrombin) physiologic agonists, as well as the non-physiologic agonists A23187 and PMA which directly elevate intracytoplasmic Ca^{2+} and activate protein kinase C, respectively.

It was anticipated that the phosphorylation of the 20 and/or 47 kDa proteins would be absent or reduced compared to that of control platelets. In particular, it was predicted that the 47 kDa protein would be phosphorylated in response to PMA, and possibly thrombin, but not in response to agonists which failed to elicit primary aggregation (ADP, PAF or A23187). It was hoped that a difference in the post-activation phosphorylation patterns of the two groups of platelets would identify the defective pathway in Basset hound hereditary thrombopathy.

MATERIALS AND METHODS

Experimental Design

The same groups of control and affected dogs described in the previous chapter were used in this study (Page 130).

Reagents

Thrombin, ADP, PAF, PMA and A23187 were purchased, stored and prepared as described in the previous chapter (Page 131). Sodium dodecyl sulfate (SDS), acrylamide, bis-acrylamide, TEMED and ammonium persulfate were purchased from Biorad (Richmond, CA). Molecular weight standards were purchased from Pharmacia (Piscataway, NJ). Bromphenol blue, Coomassie Brilliant Blue R250, Tris, glycine and 2-mercaptoethanol were purchased from the Sigma Chemical Co. (St. Louis, MO). Carrier-free (^{32}P)phosphate as orthophosphoric acid in

HCl-free water was purchased from New England Nuclear (Boston, MA) All other chemicals were of reagent quality.

Preparation of Suspensions of Labelled Platelets

This procedure was carried out based on the method described by Fox and Phillips.[143] Samples were always collected as a pair (control and affected) and were run in parallel throughout the whole procedure. For all work involving ^{32}P, plexiglass protective shielding, test tube blocks and storage containers were used.

Blood was collected from the jugular vein into a plastic syringe containing 3.2% Na_3 citrate at a ratio of 9:1. The blood was transferred to plastic tubes and the platelet rich plasma separated by repeated centrifugations at 1324 x g for 60 seconds. The platelet rich plasma was then centrifuged at 730 x g for 10 minutes to sediment the platelets. The platelet pellet was washed twice by resuspension in a buffer containing 120 mM NaCl, 13 mM Na_3 citrate, and 30 mM dextrose, pH 7.0 (buffer A). The platelets were counted manually with a Unopette and Neubauer hemocytometer after the first wash and resuspended to a final count of 2.0×10^9 /ml after the second wash in a buffer containing 150 mM NaCl, 10 mM HEPES, and 1.0 mM EDTA, pH 7.4 (buffer B). Carrier-free ^{32}P-phosphate, approximately 0.4 mCi/ml, was added and the platelet suspension incubated at 37°C for 60 minutes.

The platelets were then separated from excess radiolabel by centrifugation at 855 x g for 12 minutes, resuspended into buffer B

without ^{32}P and counted manually. They were centrifuged again and resuspended at a final concentration of 3.5 x 10^8/ml in a buffer containing 138 mM NaCl, 2.9 mM KCl, 12 mM $NaHCO_3$, 0.36 mM Na_2PO_4, 5.5 mM glucose and 1.0 mM EDTA, pH 7.4 (buffer C). This platelet suspension was divided into 0.4 ml aliquots in 1.5 ml Eppendorf centrifuge tubes and allowed to rest at room temperature for 30 minutes. In an alternative procedure tried later in the project, the platelets were separated from excess radiolabel by gel-filtration into buffer C, counted manually and further diluted to 3.5 x 10^8/ml. This procedure was found to work well and had the added advantage of involving considerably less radiation exposure.

Incubations and Sodium Dodecyl Sulfate-Polyacrylamide Electrophoresis

Aliquots of the platelet suspension were incubated at room temperature with saline or various agonists (thrombin, PAF, ADP, A23187, or PMA). After 5 seconds, 15 seconds, 30 seconds, 1 minute, 2 minutes, 5 minutes or 10 minutes, the incubation was terminated by the addition of an equal volume of solubilization buffer containing 4.0% w/v SDS, 10% v/v 2-mercaptoethanol, 20% v/v glycerol, 0.004% w/v bromphenol blue, and 125 mM Tris HCl, pH 6.8, and the sample incubated at 78°C for 15 minutes.

Samples were electrophoresed according to the method of Laemmli[267] through slab gels in a Biorad Protean II electrophoresis apparatus (Biorad, Richmond, CA). Polyacrylamide resolving gels (1.5 mm thick) containing 11% T acrylamide, 0.1% SDS, 0.375 M Tris (pH 8.8) and

polymerized with 0.05% fresh ammonium persulfate and TEMED were poured. After polymerization, stacking gels containing 3% T acrylamide, 0.1% SDS, 0.125 M Tris (pH 6.8) and polymerized with 0.05% ammonium persulfate and TEMED were poured on top of the resolving gels. These were refrigerated overnight for use the next day.

Lanes were loaded with 200 μl of sample or 10 μl of low molecular weight standard and electrophoresed at 50 mA until the bromphenol blue marker entered the resolving gel, then at 60 mA until the marker reached approximately one centimeter from the bottom of the gel. The running buffer contained 0.192 M glycine, 0.1% SDS and 0.025 M Tris, pH 8.3. The gels were then stained with 0.1% w/v Coomassie brilliant blue R250 in 10% glacial acetic acid and 50% methanol for approximately 1.5 hours and destained in 10% methanol and 10% glacial acetic acid for 36-48 hours.

For autoradiographs, labelled gels were dried onto porous cellophane in a slab gel drier and then photographed. Gels were exposed on Kodak X-OMAT AR film at -80°C for 5-24 hours in a cassette with intensifying screens and the film developed in Kodak GBX developer. Appropriate sections of the dried gel were cut out and counted for Cerenkov radiation in a Beckman LS9000 liquid scintillation counter.[190] Unlabelled gels dried between two layers of porous cellophane and autoradiographs were scanned densiometrically on a Gilford Response 11 spectrophotometer.

RESULTS

All agonist concentrations are the final concentration in the platelet incubation mixture. Results for each agonist are presented separately and are based on a representative pair of gels for that agonist. Pairs of gels were selected on the basis of quality and resolution of the original gels and autoradiographs as well as equality of radioactive intensity. Although pairs were loaded equally, there was still some variation in the radioactive intensity of some pairs. It was found that the counts per minute of the 0 lane for the 47 kDa protein was the best indicator that a pair of gels were well-matched. The absorbance measured on densiometric scanning of peaks that were not affected by activation also indicated that the pairs were well-matched.

Densiometric scans of unlabelled 11% T dried gels of solubilized non-activated control and affected platelets did not indicate any differences between the two groups. However, because there were very large numbers of bands, differences in minor bands would not likely be detected.

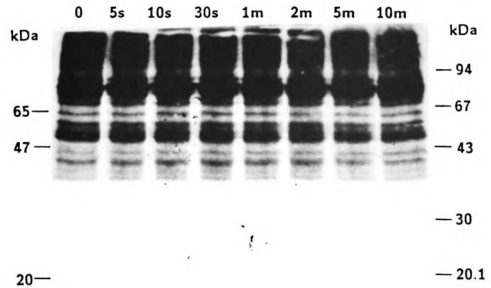
Adenosine Diphosphate

Under the non-aggregating conditions used in this study (non-stirred, with 1.0 mM EDTA and no exogenous fibrinogen in the medium) neither control or affected platelets significantly phosphorylated the 20 or 47 kDa proteins in response to 10 μM ADP. Figures 34a and 34b contain a pair of autoradiographs in which the platelets were stimulated with

0.9% NaCl (0 lane) or 10 μM ADP and the reaction stopped at 5, 15 and 30 seconds and 1, 2, 5 and 10 minutes. Densiometric scans of these autoradiographs are shown in Figures 35a, 35b, 36a, 36b, 37a and 37b. The 0, 30 seconds and 5 minute lanes were selected as being temporally representative scans of early and late activation. The appropriate bands were cut out of the labelled gels and counted by Cerenkov counting. These results are shown in Figures 38, 39 and 40. Although there appeared to be a slight increase in the absorbance of the 20 and 47 kDa protein peaks on the densiometric scans of affected platelets at 30 seconds and 5 minutes (Figures 36 and 37), this was not supported by counting the radioactivity of the sections cut out from the gels (Figures 38 and 39). The 0 lane, being an outside lane, was wider and slightly less dense compared to the inner lanes. The 20 kDa band of the affected platelets was slightly less intensely labelled than that of control platelets. There was also a noticeable difference in the density between control and affected autoradiographs of a band of approximately 65 kDa. This is apparent on the autoradiographs and densiometric scans and is reflected in a lower counts per minute for that band from the affected gel (Figures 34,35,40). This difference was apparent in the 0 lane and did not appear to be affected by stimulation by ADP.

Figure 34a. Autoradiograph of control ^{32}P-labelled platelets activated by 10 μM ADP, solubilized at various times and separated by SDS-PAGE.

Figure 34b. Autoradiograph of affected ^{32}P-labelled platelets activated by 10 μM ADP, solubilized at various times and separated by SDS-PAGE.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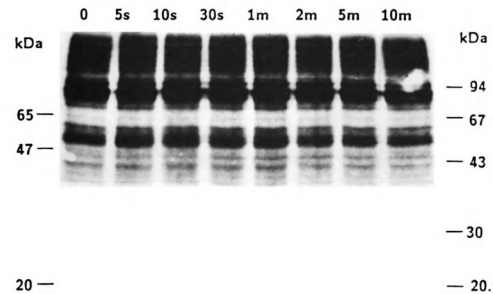
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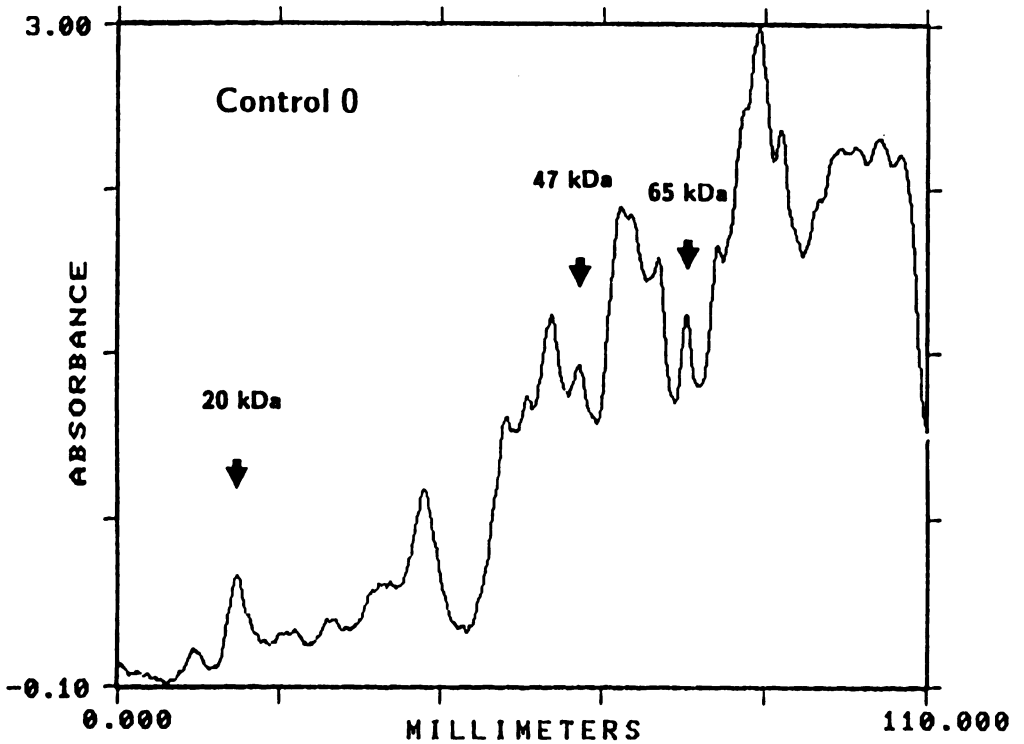
Figure 35a. Densiometric scan of an autoradiograph of resting control ^{32}P-labelled platelets solubilized and separated by SDS-PAGE.

Figure 35b. Densiometric scan of an autoradiograph of resting affected ^{32}P-labelled platelets solubilized and separated by SDS-PAGE.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Control 0
47 kDa
65 kDa
20 kDa
ABSORBANCE
-0.10
0.000
MILLIMETERS
110.000

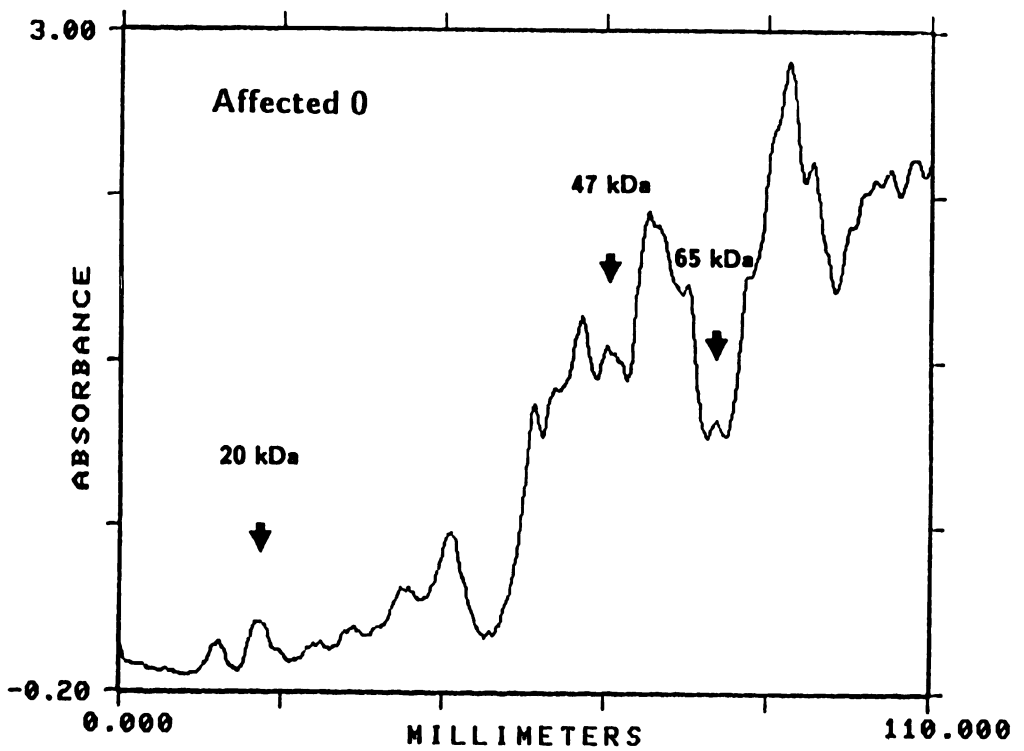
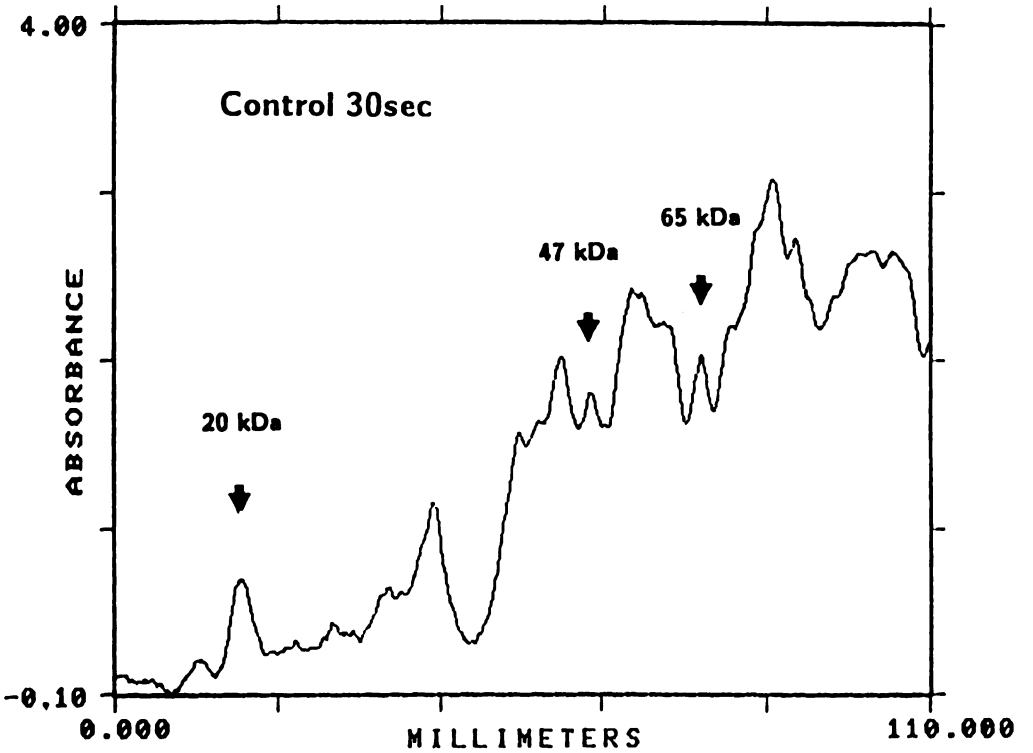

3.00
Affected 0
47 kDa
65 kDa
20 kDa
ABSORBANCE
-0.20
0.000
MILLIMETERS
110.000

Figure 36a. Densiometric scan of an autoradiograph of control ^{32}P-labelled platelets solubilized 30 seconds after the addition of 10 μM ADP and separated by SDS-PAGE.

Figure 36b. Densiometric scan of an autoradiograph of affected ^{32}P-labelled platelets solubilized 30 seconds after the addition of 10 μM ADP and separated by SDS-PAGE.


4.00
Control 30sec
65 kDa
47 kDa
20 kDa
ABSORBANCE
-0.10
0.000
MILLIMETERS
110.000

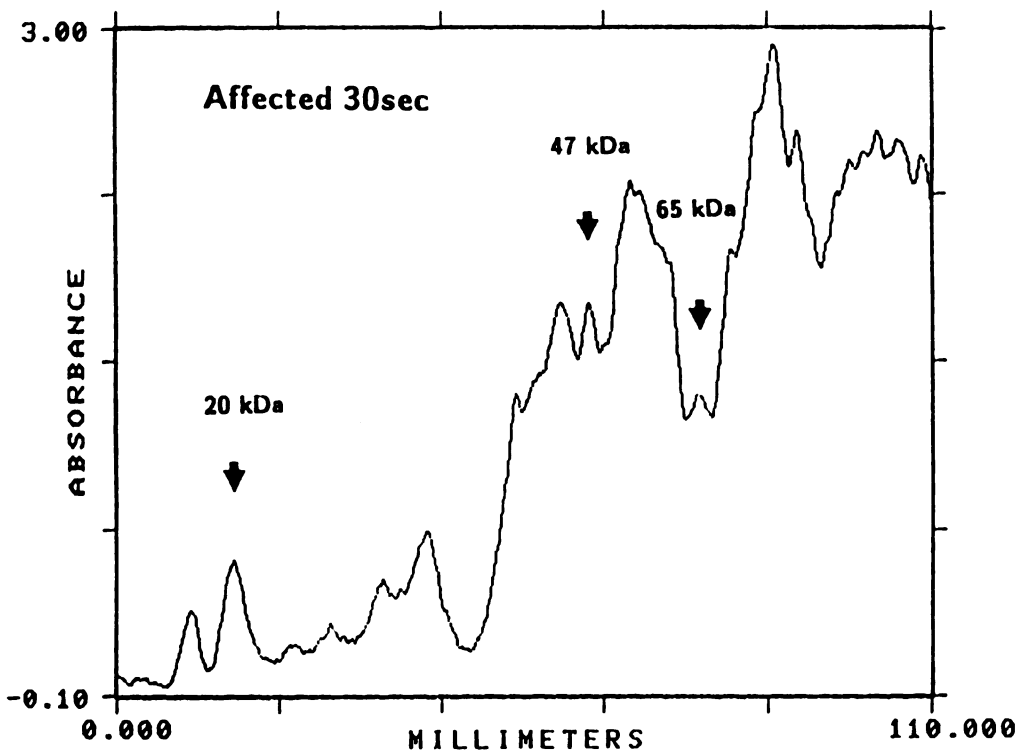
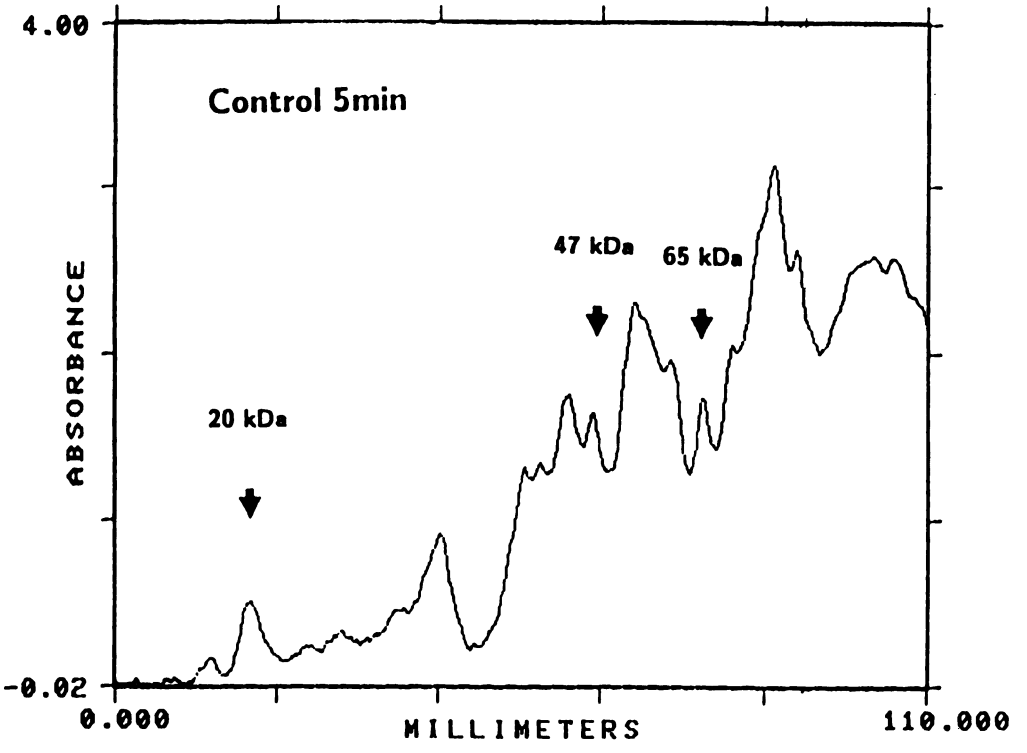

3.00
Affected 30sec
47 kDa
65 kDa
20 kDa
ABSORBANCE
-0.10
0.000
MILLIMETERS
110.000

Figure 37a. Densiometric scan of an autoradiograph of control ^{32}P-labelled platelets solubilized 5 minutes after the addition of 10 μM ADP and separated by SDS-PAGE.

Figure 37b. Densiometric scan of an autoradiograph of affected ^{32}P-labelled platelets solubilized 5 minutes after the addition of 10 μM ADP and separated by SDS-PAGE.


4.00
Control 5min
47 kDa
65 kDa
20 kDa
ABSORBANCE
-0.02
0.000
MILLIMETERS
110.000

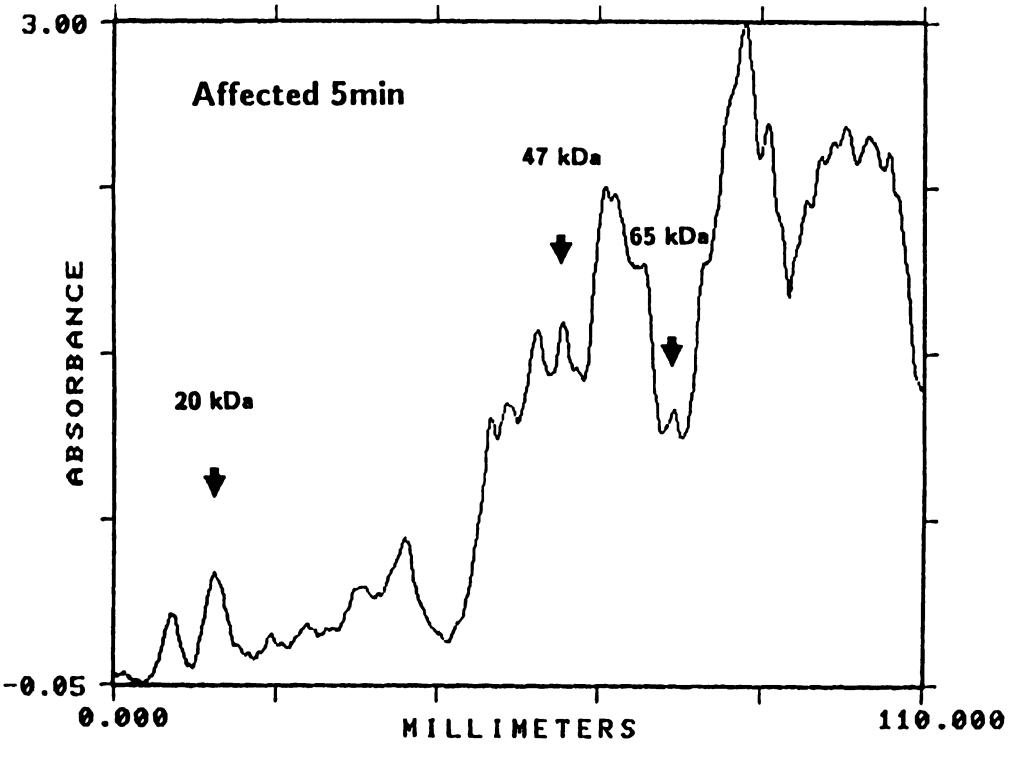
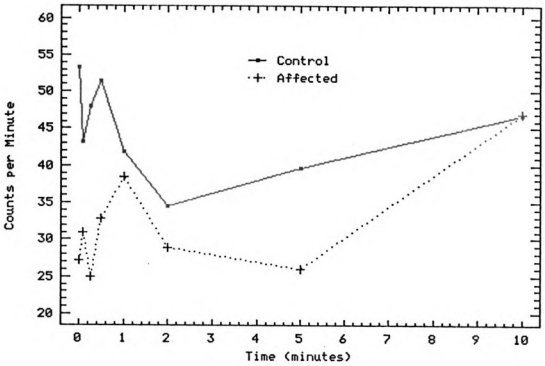
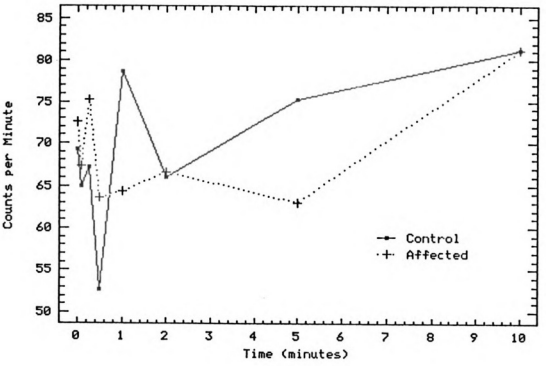

3.00
Affected 5min
47 kDa
65 kDa
20 kDa
ABSORBANCE
-0.05
0.000
MILLIMETERS
110.000

Figure 38. Phosphorylation (cpm) of the 20 kDa protein band of control and affected ^{32}P-labelled platelets solubilized after the addition of 10 μM ADP and separated by SDS-PAGE.

Figure 39. Phosphorylation (cpm) of the 47 kDa protein band of control and affected ^{32}P-labelled platelets solubilized after the addition of 10 μM ADP and separated by SDS-PAGE.


Counts per Minute
Time (minutes)
Control
Affected
60
55
50
45
40
35
30
25
20
0
1
2
3
4
5
6
7
8
9
10


Counts per Minute
Time (minutes)
Control
Affected
85
80
75
70
65
60
55
50
0
1
2
3
4
5
6
7
8
9
10

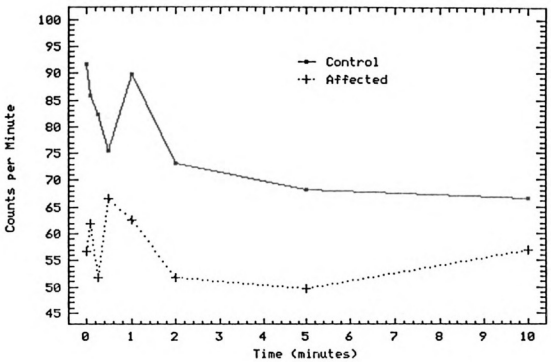


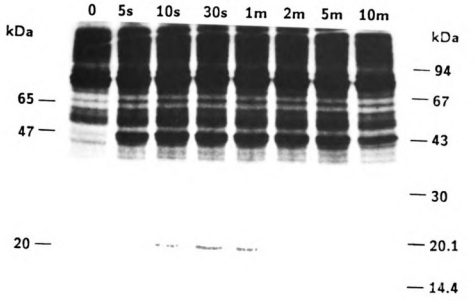
Figure 40. Phosphorylation (cpm) of the 65 kDa protein band of control and affected ^{32}P-labelled platelets solubilized after the addition of 10 μM ADP and separated by SDS-PAGE.

Platelet Activating Factor

Control and affected platelets stimulated by 1.0 μM or 0.1 μM PAF under non-aggregating conditions rapidly phosphorylated the 20 and 47 kDa proteins. The responses of control and affected platelets to both concentrations of PAF were similar and only results from platelets incubated with 0.1 μM PAF are illustrated. Figures 41a and 41b show a pair of autoradiographs in which the platelets were stimulated with 0.9% NaCl (0 lane) or 0.1 μM PAF and the reaction stopped at 5, 15 and 30 seconds and 1, 2, 5 and 10 minutes. Densiometric scans of the 0, 20 seconds and 5 minute lanes of these autoradiographs are shown in Figures 42a, 42b, 43a, 43b, 44a and 44b. The appropriate bands were cut out of the labelled gels and counted by Cerenkov counting. These results are shown in Figures 45, 46 and 47. Phosphorylation of the 20 and 47 kDa proteins was visible on the autoradiographs by five seconds and had peaked by one minute, decreasing slightly after that time (Figures 41 and 45). The phosphorylation of the 20 and 47 kDa proteins of the affected platelets declined a little more rapidly than controls, but the significance of this, if any, is not known. There was a marked decrease in the radioactive intensity of the 65 kDa band of affected platelets that was apparent on the autoradiographs, scans and by counting (Figures 41, 42 and 47). There was no obvious pattern of phosphorylation of the 65 kDa band in response to 0.1 μM PAF, although there was a slight increase in phosphorylation within one minute which then decreased. Some increased phosphorylation of the 65 kDa band was seen in other gels in which platelets were activated by 0.1 μM PAF, but it was not a consistent occurrence and is of unknown significance.

Figure 41a. Autoradiograph of control ^{32}P-labelled platelets activated by 0.1 μM PAF, solubilized at various times and separated by SDS-PAGE.

Figure 41b. Autoradiograph of affected ^{32}P-labelled platelets activated by 0.1 μM PAF, solubilized at various times and separated by SDS-PAGE.


0
5s
10s
30s
1m
2m
5m
10m
kDa
65
47
20
kDa
94
67
43
30
20.1
14.4

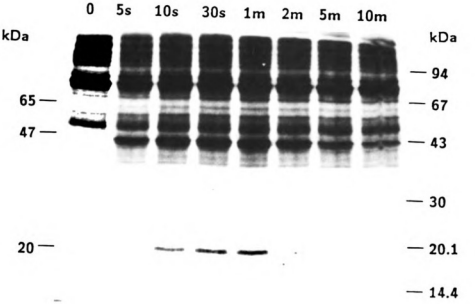
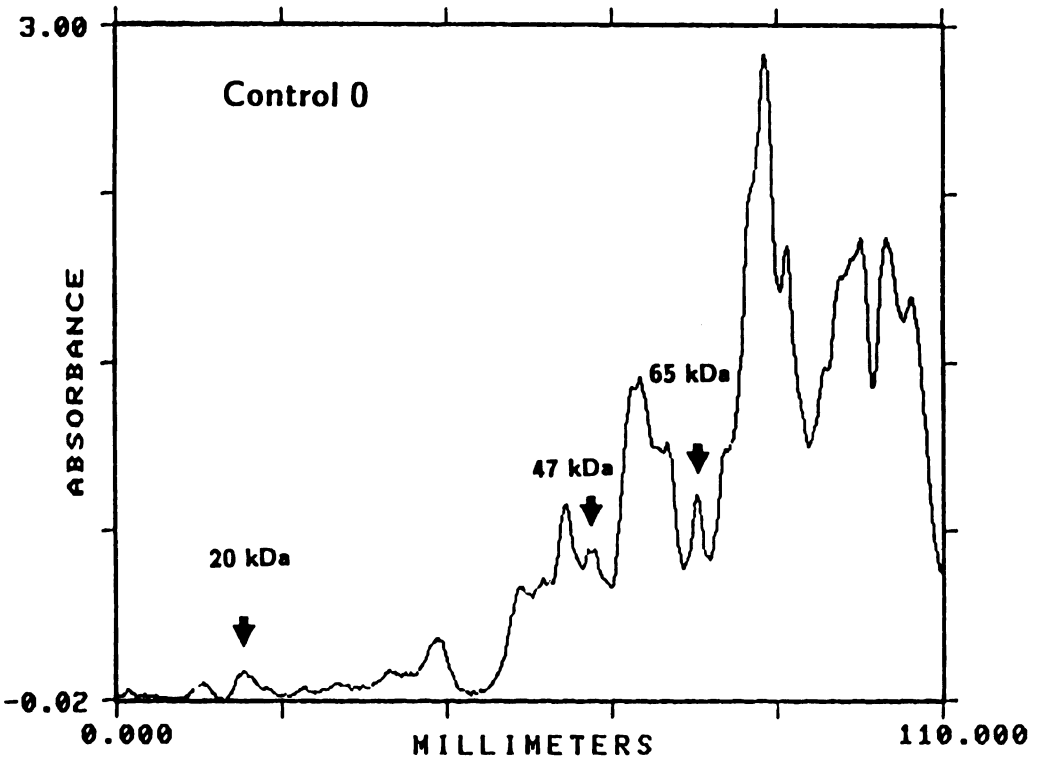

0
5s
10s
30s
1m
2m
5m
10m
kDa
65
47
20
kDa
94
67
43
30
20.1
14.4

Figure 42a. Densiometric scan of an autoradiograph of resting control ^{32}P-labelled platelets solubilized and separated by SDS-PAGE.

Figure 42b. Densiometric scan of an autoradiograph of resting affected ^{32}P-labelled platelets solubilized and separated by SDS-PAGE.


3.00
Control 0
ABSORBANCE
65 kDa
47 kDa
20 kDa
-0.02
0.000
MILLIMETERS
110.000

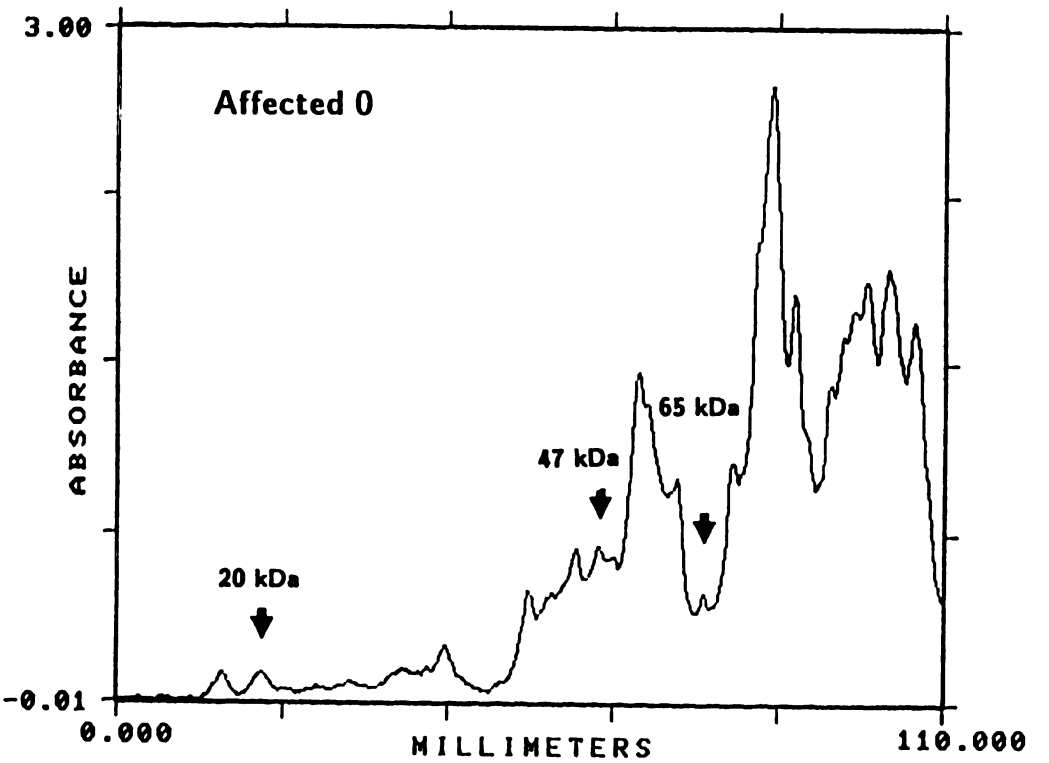
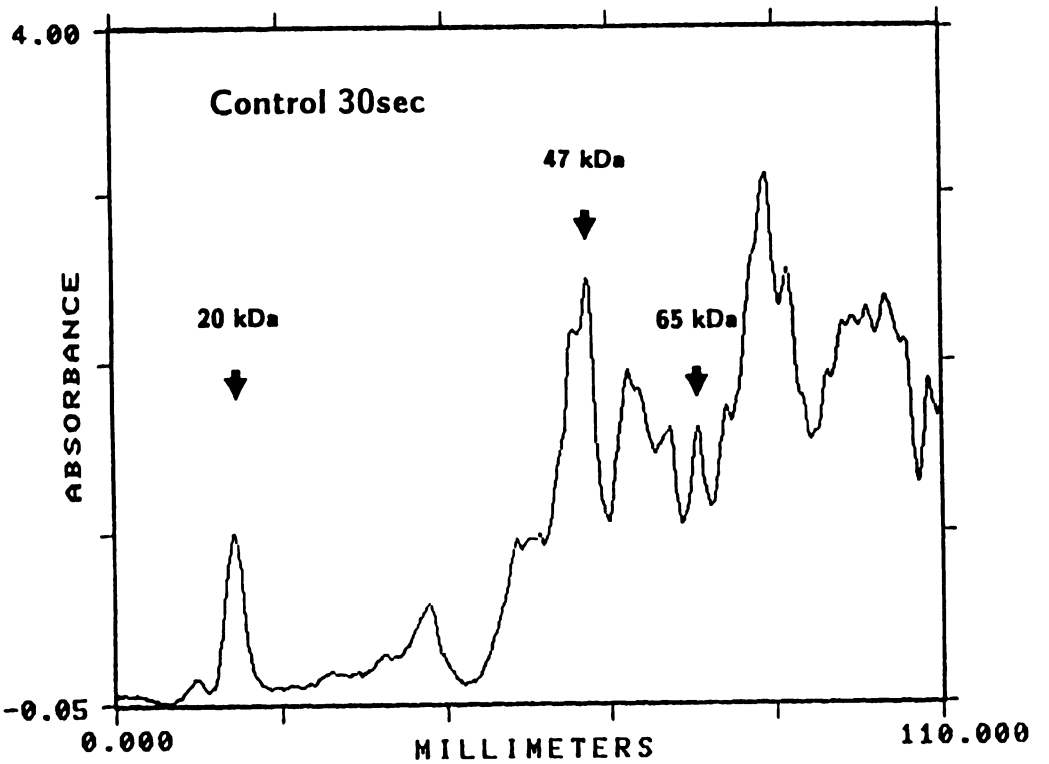

3.00
Affected 0
ABSORBANCE
65 kDa
47 kDa
20 kDa
-0.01
0.000
MILLIMETERS
110.000

Figure 43a. Densiometric scan of an autoradiograph of control ^{32}P-labelled platelets solubilized 30 seconds after the addition of 0.1 μM PAF and separated by SDS-PAGE.

Figure 43b. Densiometric scan of an autoradiograph of affected ^{32}P-labelled platelets solubilized 30 seconds after the addition of 0.1 μM PAF and separated by SDS-PAGE.


4.00
Control 30sec
47 kDa
20 kDa
65 kDa
ABSORBANCE
-0.05
0.000
MILLIMETERS
110.000

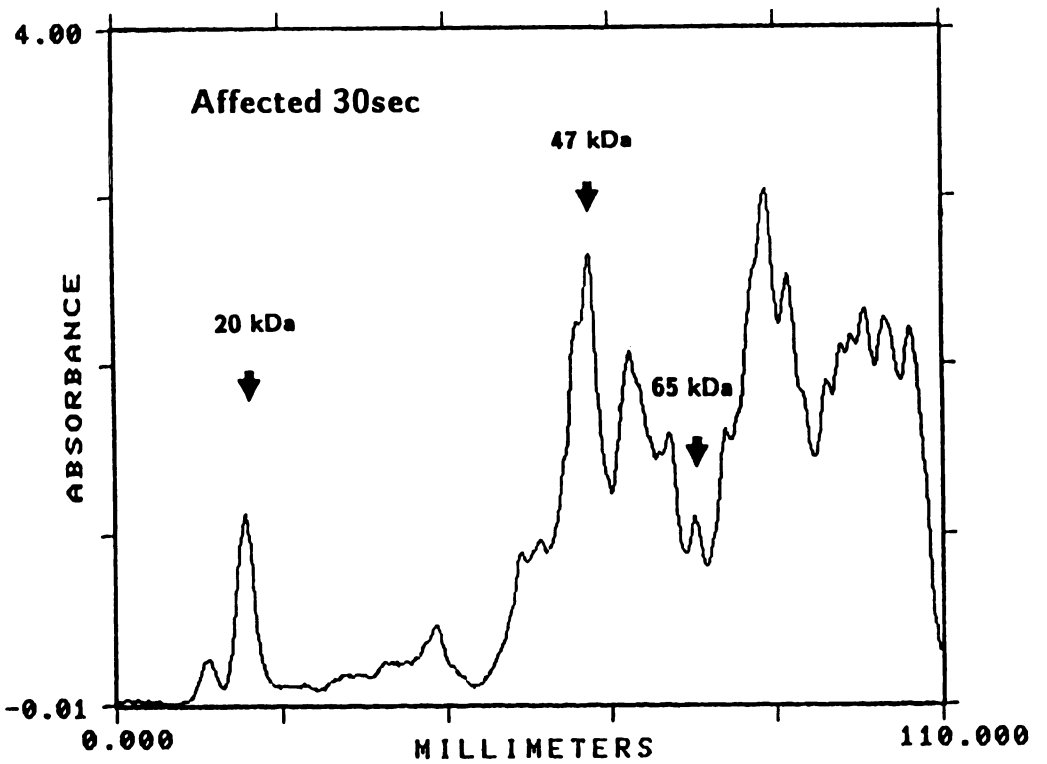
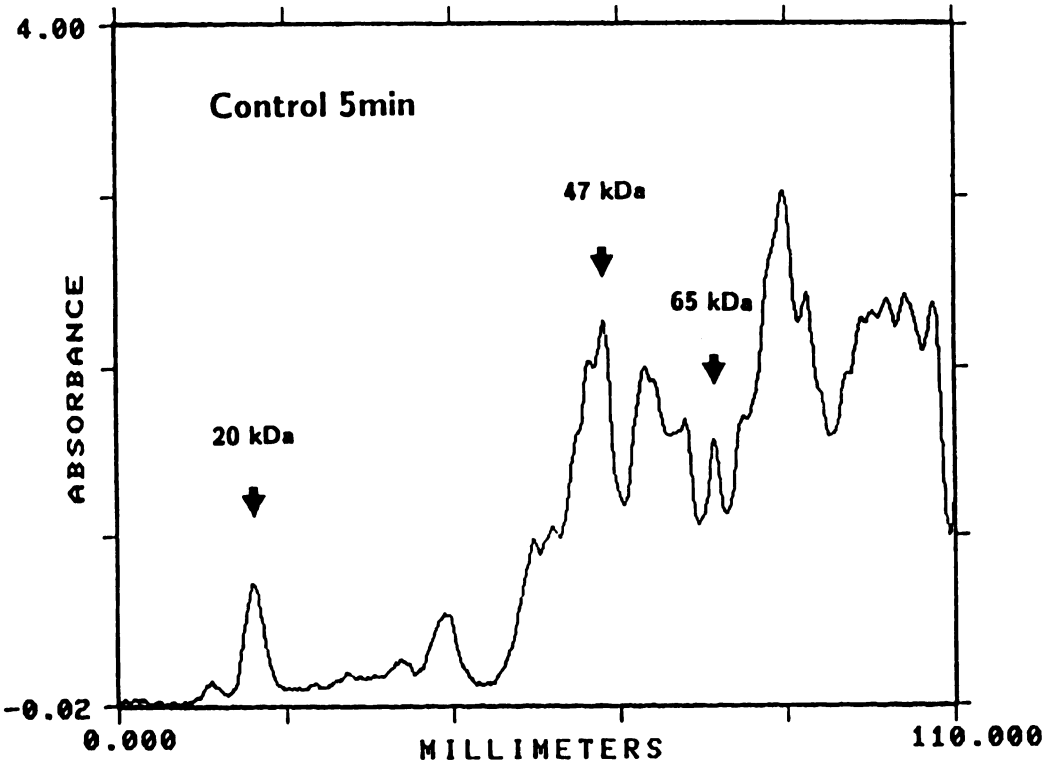

4.00
Affected 30sec
47 kDa
20 kDa
65 kDa
ABSORBANCE
-0.01
0.000
MILLIMETERS
110.000

Figure 44a. Densiometric scan of an autoradiograph of control ^{32}P-labelled platelets solubilized five minutes after the addition of 0.1 μM PAF and separated by SDS-PAGE.

Figure 44b. Densiometric scan of an autoradiograph of affected ^{32}P-labelled platelets solubilized five minutes after the addition of 0.1 μM PAF and separated by SDS-PAGE.


4.00
Control 5min
47 kDa
65 kDa
20 kDa
ABSORBANCE
-0.02
0.000
MILLIMETERS
110.000

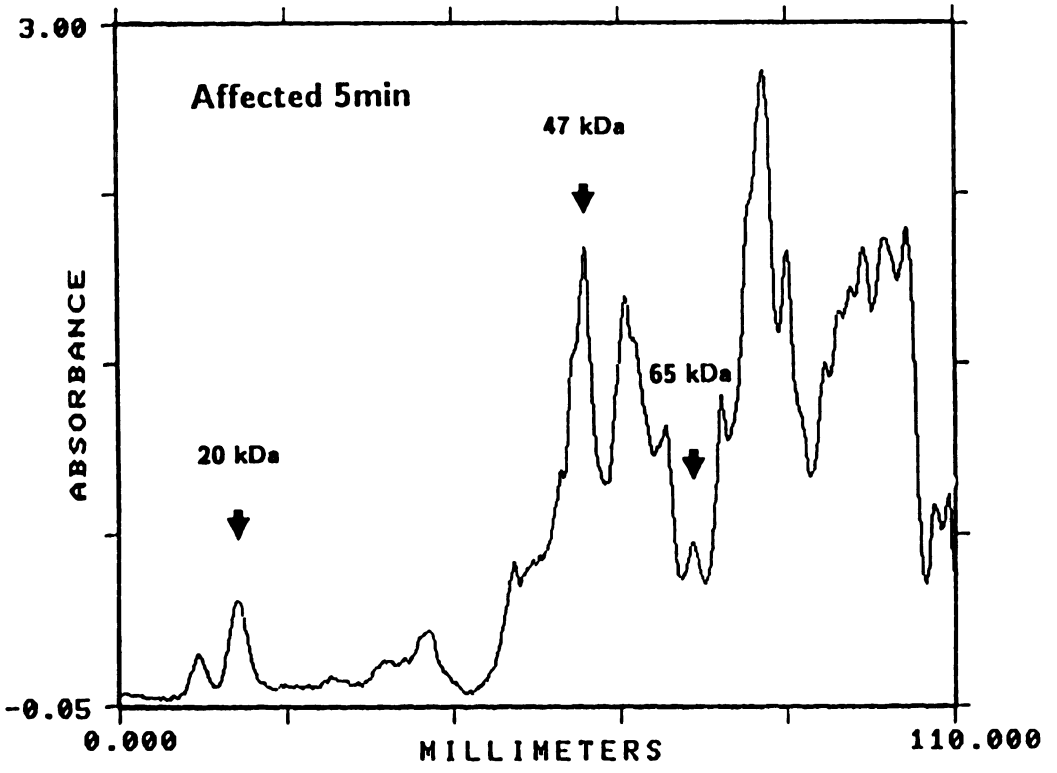
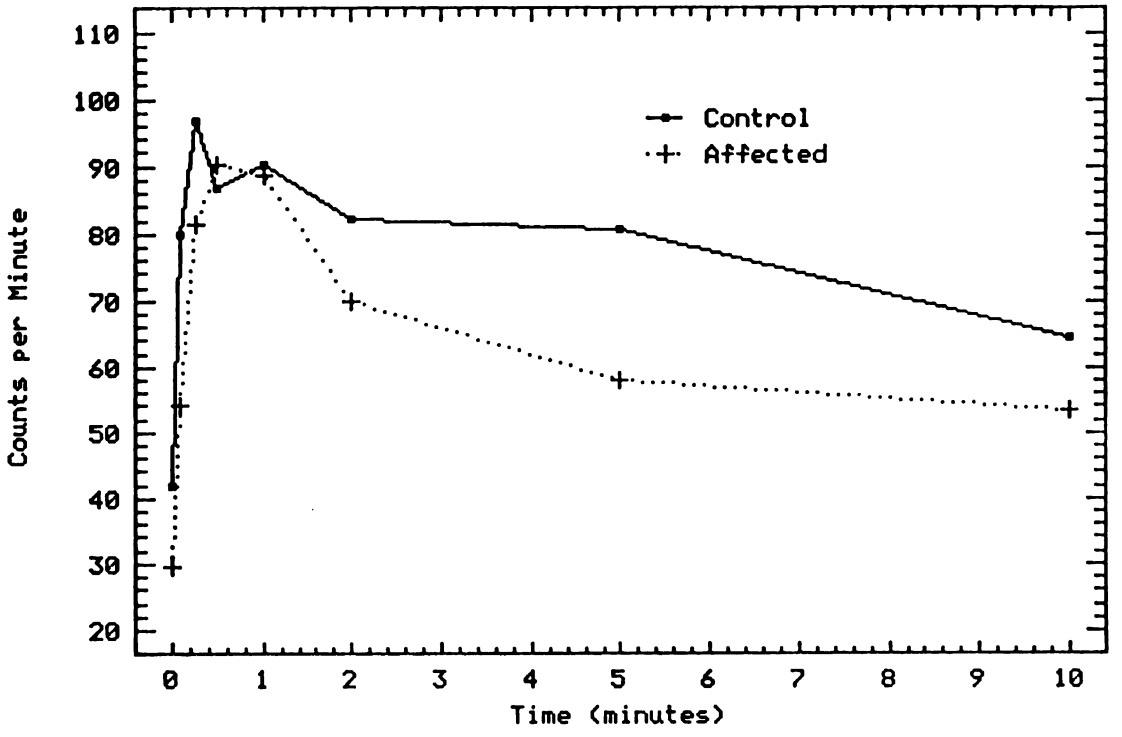
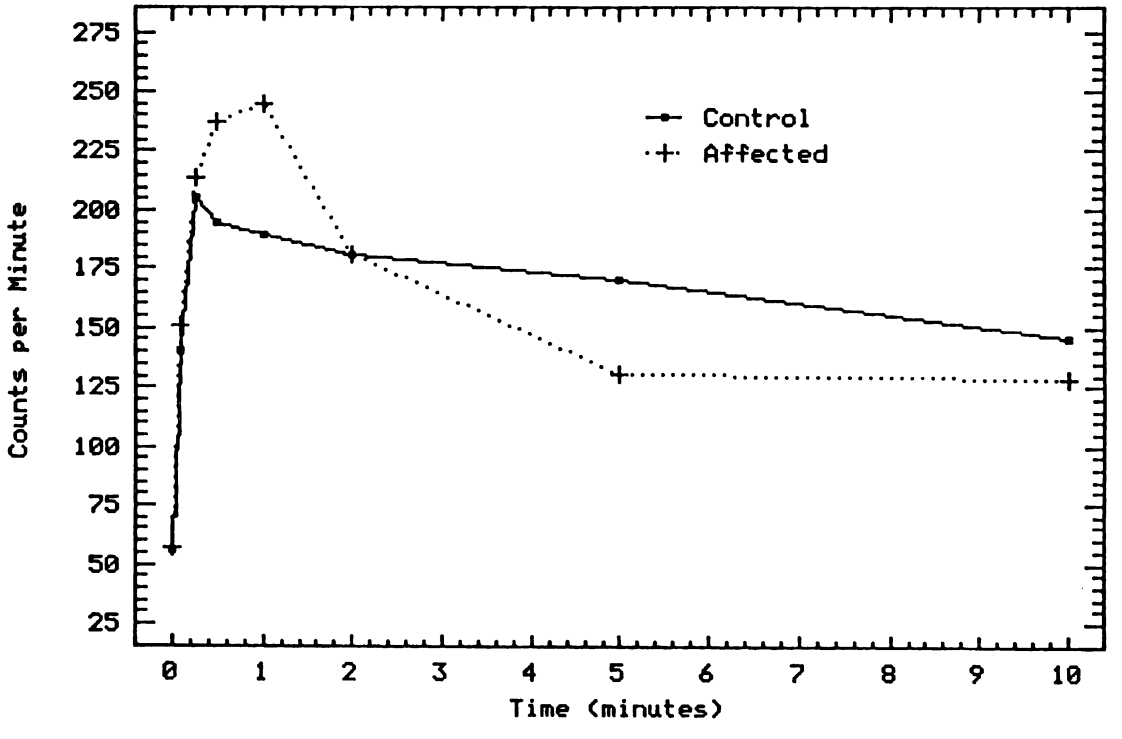

3.00
Affected 5min
47 kDa
65 kDa
20 kDa
ABSORBANCE
-0.05
0.000
MILLIMETERS
110.000

Figure 45. Phosphorylation (cpm) of the 20 kDa protein band of control and affected ^{32}P-labelled platelets solubilized after the addition of 0.1 μM PAF and separated by SDS-PAGE.

Figure 46. Phosphorylation (cpm) of the 47 kDa protein band of control and affected ^{32}P-labelled platelets solubilized after the addition of 0.1 μM PAF and separated by SDS-PAGE.


Counts per Minute
110
100
90
80
70
60
50
40
30
20
0
1
2
3
4
5
6
7
8
9
10
Time (minutes)
Control
Affected


Counts per Minute
275
250
225
200
175
150
125
100
75
50
25
0
1
2
3
4
5
6
7
8
9
10
Time (minutes)
Control
Affected

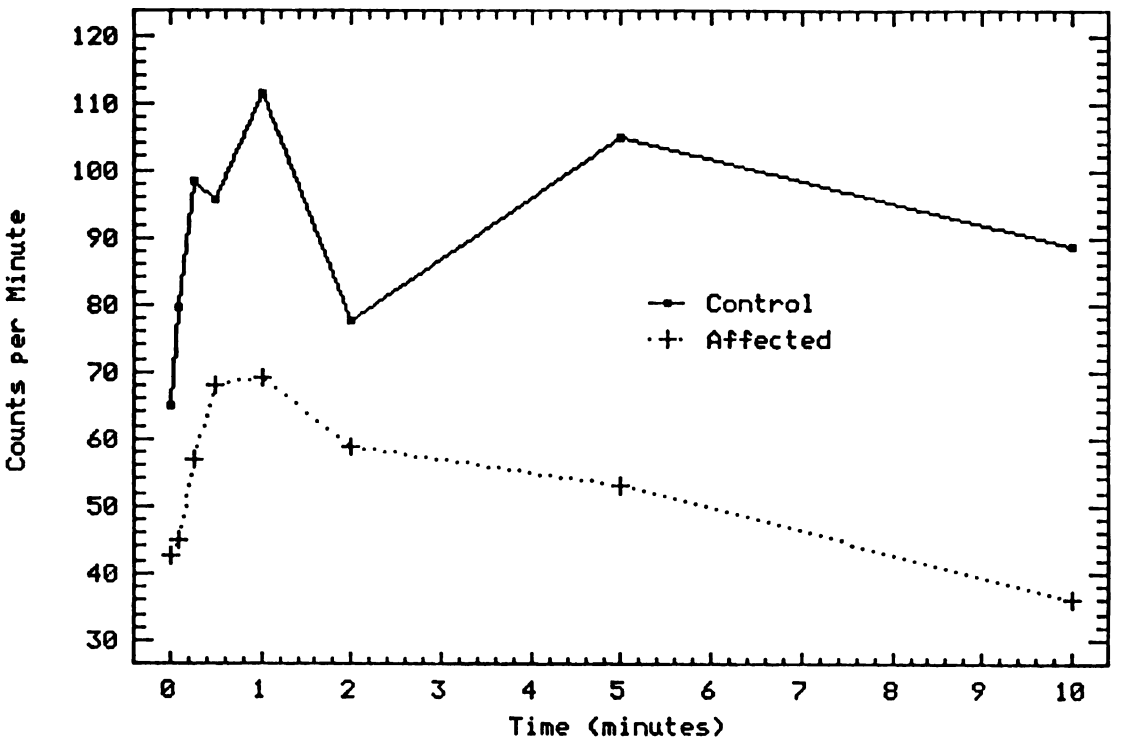


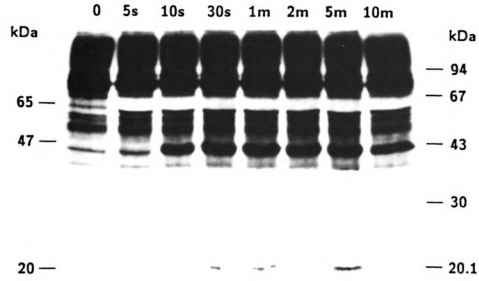
Figure 47. Phosphorylation (cpm) of the 65 kDa protein band of control and affected ^{32}P-labelled platelets solubilized after the addition of 0.1 μM PAF and separated by SDS-PAGE.

Thrombin

Control and affected platelets stimulated by 0.3 U/ml thrombin under non-aggregating conditions rapidly phosphorylated the 20 and 47 kDa proteins. Figures 48a and 48b show a pair of autoradiographs in which the platelets were stimulated with 0.9% NaCl (0 lane) or 0.3 U/ml thrombin and the reaction stopped at 5, 15 and 30 seconds and 1, 2, 5 and 10 minutes. Densiometric scans of the 0, 30 seconds and five minutes lanes of these autoradiographs are shown in Figures 49a, 40b, 50a, 50b, 51a and 51b. The appropriate bands were cut out of the labelled gels and counted by Cerenkov counting. These results are shown in Figures 52, 53 and 54. Both control and affected platelets reached peak phosphorylation of the 20 and 47 kDa bands within two minutes, but affected platelets did not phosphorylate either band quite as well as control platelets (Figures 52 and 53). There was a visible difference in the phosphorylation of the 65 kDa band in the 0 lane, but it was pushed further down into the gel after thrombin -induced activation by a heavy protein band migrating immediately above the 65 kDa band. This band was present on the stained gels, but not phosphorylated and is likely the 69.5 kDa hydrolytic fragment glycoprotein V_{f1} that is cleaved from surface membrane glycoprotein V by thrombin.[383,528] In both groups, there was an increase in phosphorylation of the 65 kDa protein in response to activation by thrombin (Figure 54). The 0 time counts per minutes value for the control was not available. This increase in phosphorylation of the 65 kDa protein band was a consistent occurrence in other gels in which control and affected platelets were incubated with thrombin.

Figure 48a. Autoradiograph of control ^{32}P-labelled platelets activated by 0.3 U/ml thrombin, solubilized at various times and separated by SDS-PAGE.

Figure 48b. Autoradiograph of affected ^{32}P-labelled platelets activated by 0.3 U/ml thrombin, solubilized at various times and separated by SDS-PAGE.


0 5s 10s 30s 1m 2m 5m 10m
kDa
kDa
65
47
20
94
67
43
30
20.1

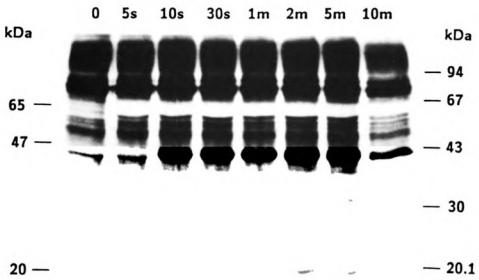
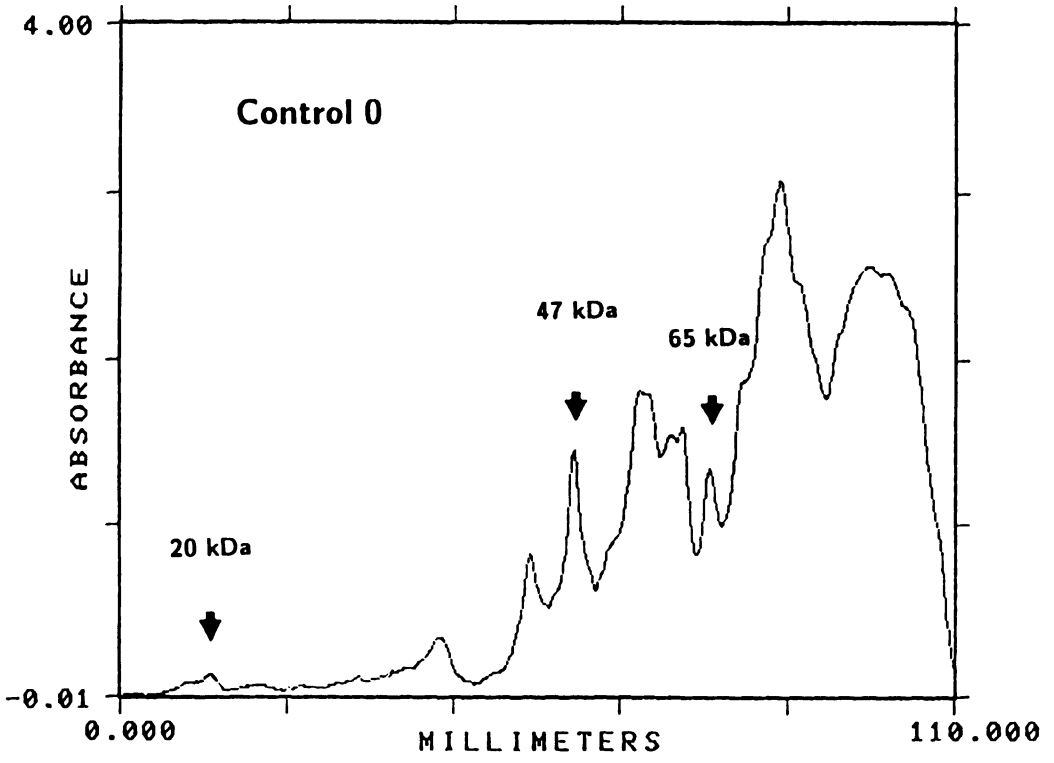

0 5s 10s 30s 1m 2m 5m 10m
kDa
kDa
65
47
20
94
67
43
30
20.1

Figure 49a. Densiometric scan of an autoradiograph of resting control ^{32}P-labelled platelets solubilized and separated by SDS-PAGE.

Figure 49b. Densiometric scan of an autoradiograph of resting affected ^{32}P-labelled platelets solubilized and separated by SDS-PAGE.


4.00
Control 0
ABSORBANCE
47 kDa
65 kDa
20 kDa
-0.01
0.000
MILLIMETERS
110.000

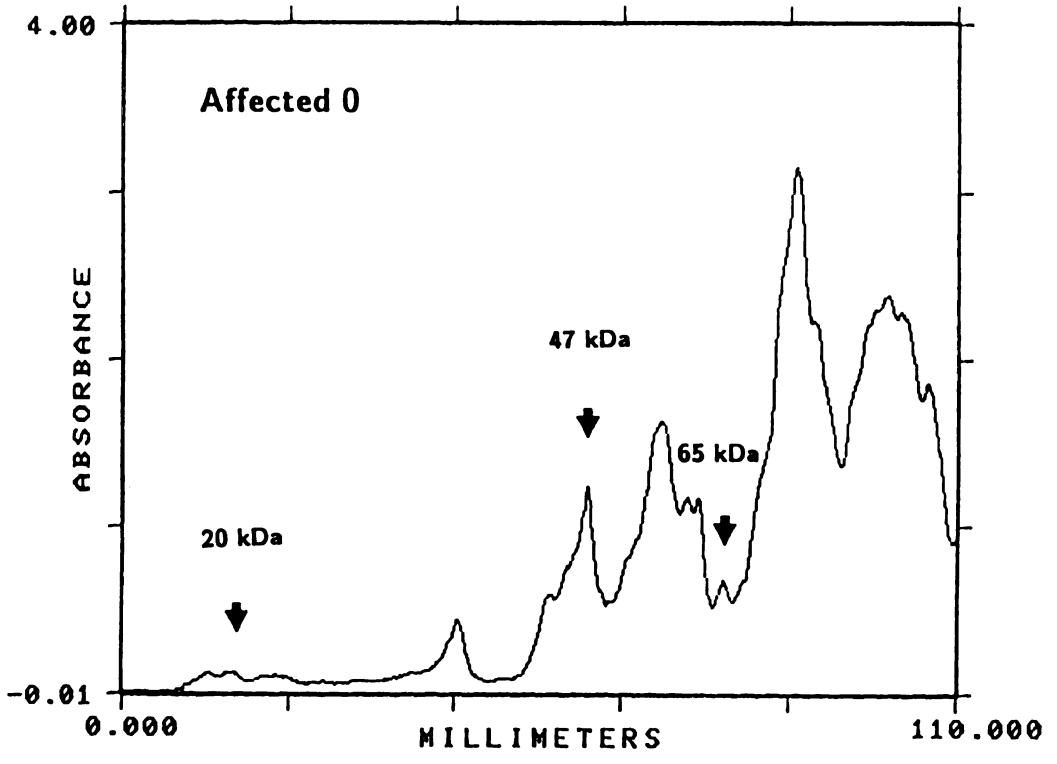
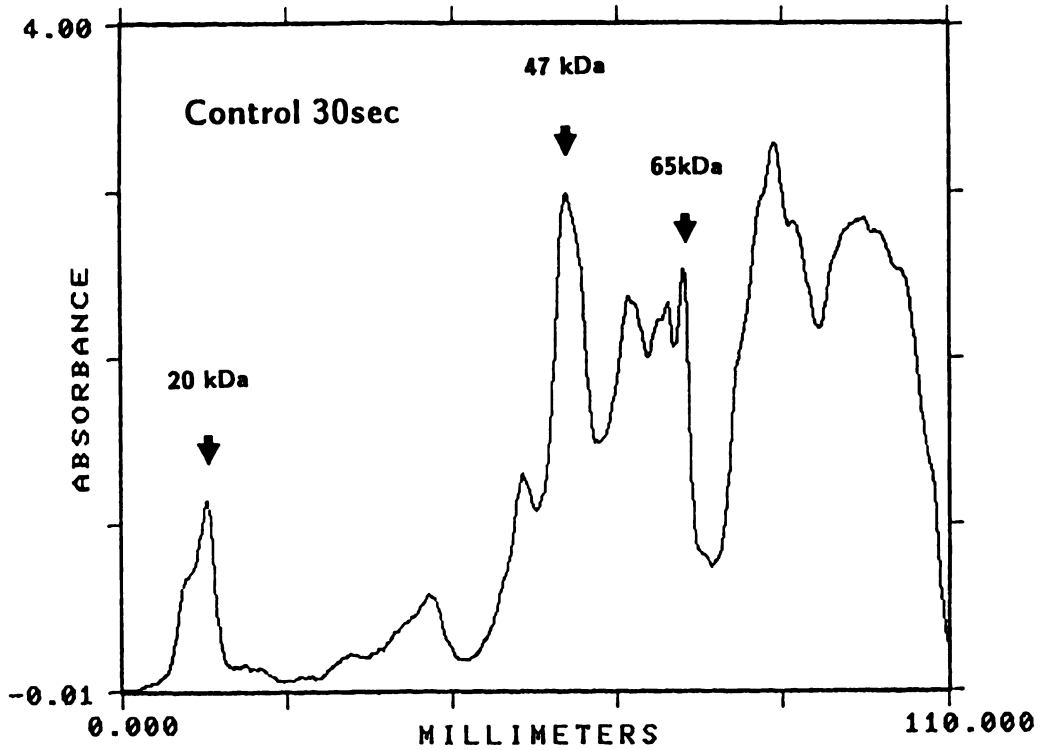

4.00
Affected 0
ABSORBANCE
47 kDa
65 kDa
20 kDa
-0.01
0.000
MILLIMETERS
110.000

Figure 50a. Densiometric scan of an autoradiograph of control ^{32}P-labelled platelets solubilized 30 seconds after the addition of 0.3 U/ml thrombin and separated by SDS-PAGE.

Figure 50b. Densiometric scan of an autoradiograph of affected ^{32}P-labelled platelets solubilized 30 seconds after the addition of 0.3 U/ml thrombin and separated by SDS-PAGE.


4.00
Control 30sec
47 kDa
65kDa
20 kDa
ABSORBANCE
-0.01
0.000
MILLIMETERS
110.000

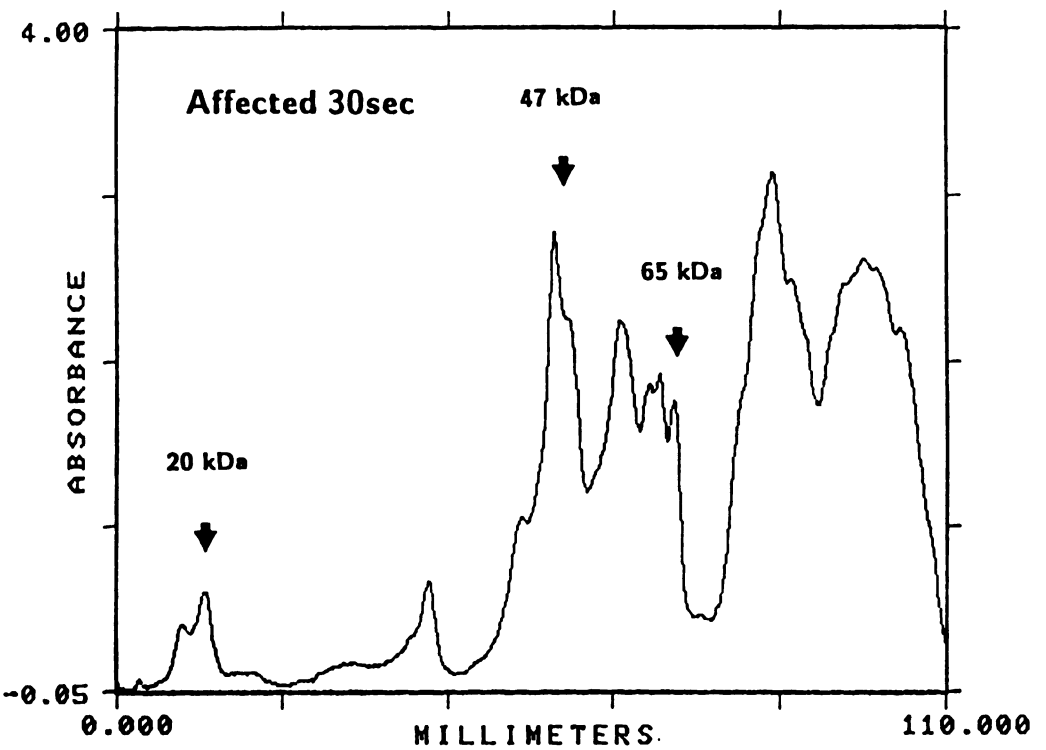
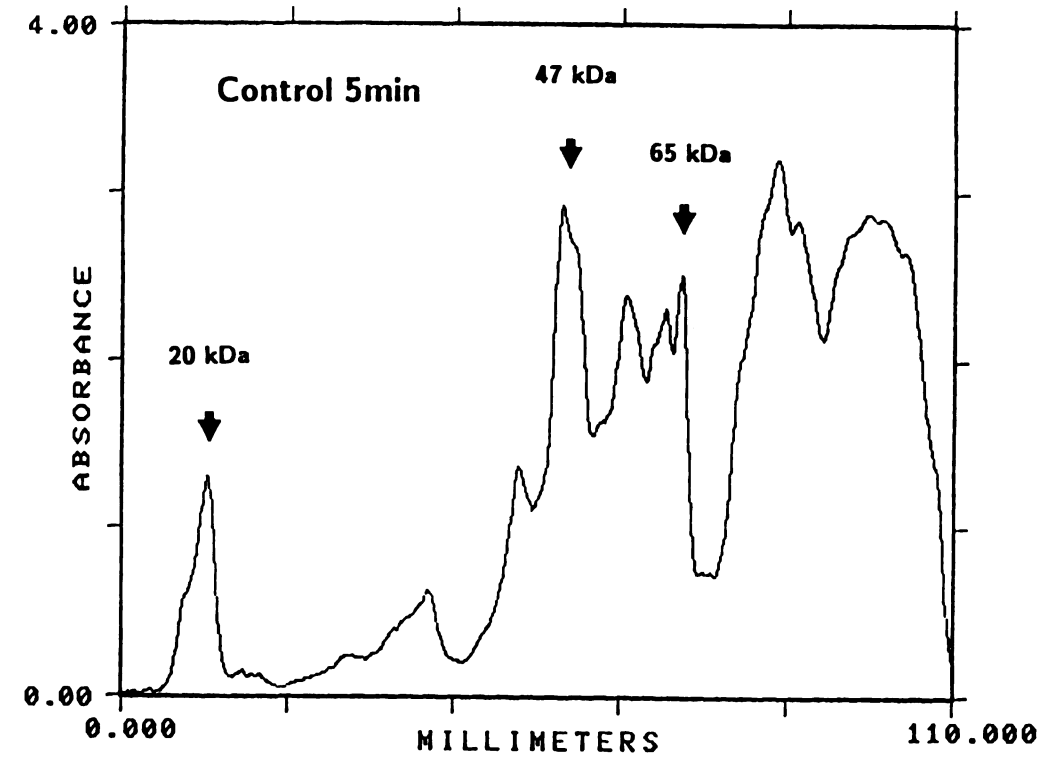

4.00
Affected 30sec
47 kDa
65 kDa
20 kDa
ABSORBANCE
-0.05
0.000
MILLIMETERS
110.000

Figure 51a. Densiometric scan of an autoradiograph of control ^{32}P-labelled platelets solubilized five minutes after the addition of 0.3 U/ml thrombin and separated by SDS-PAGE.

Figure 51b. Densiometric scan of an autoradiograph of affected ^{32}P-labelled platelets solubilized five minutes after the addition of 0.3 U/ml thrombin and separated by SDS-PAGE.


4.00
Control 5min
47 kDa
65 kDa
20 kDa
ABSORBANCE
0.00
0.000
MILLIMETERS
110.000

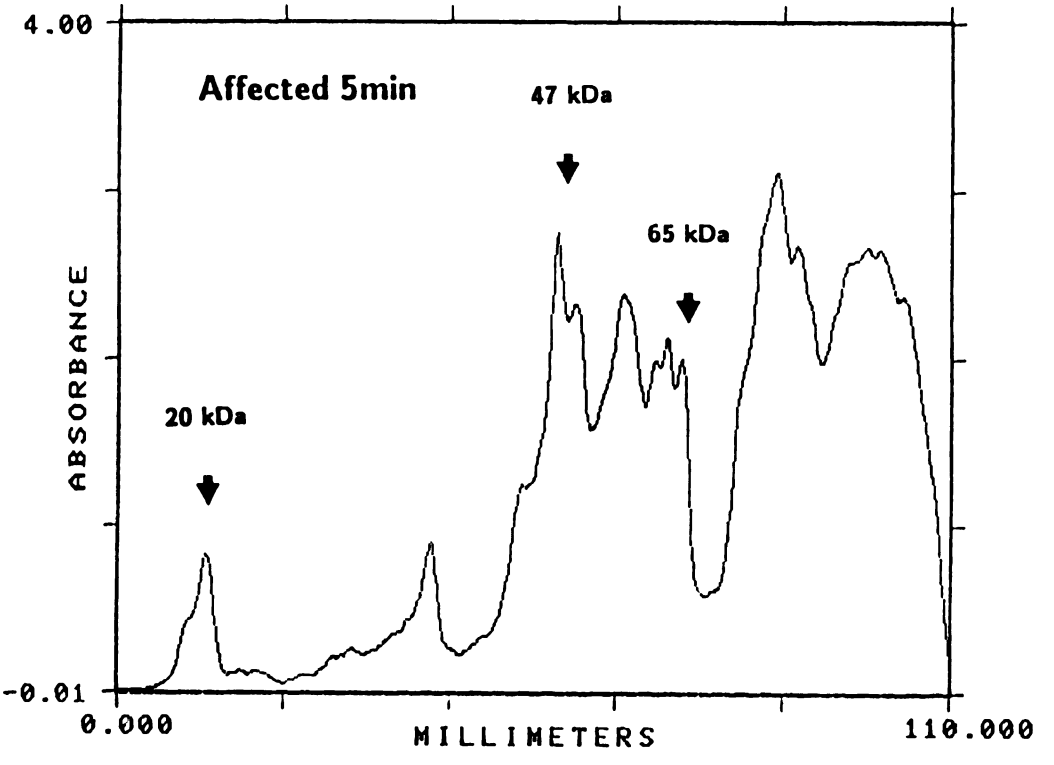
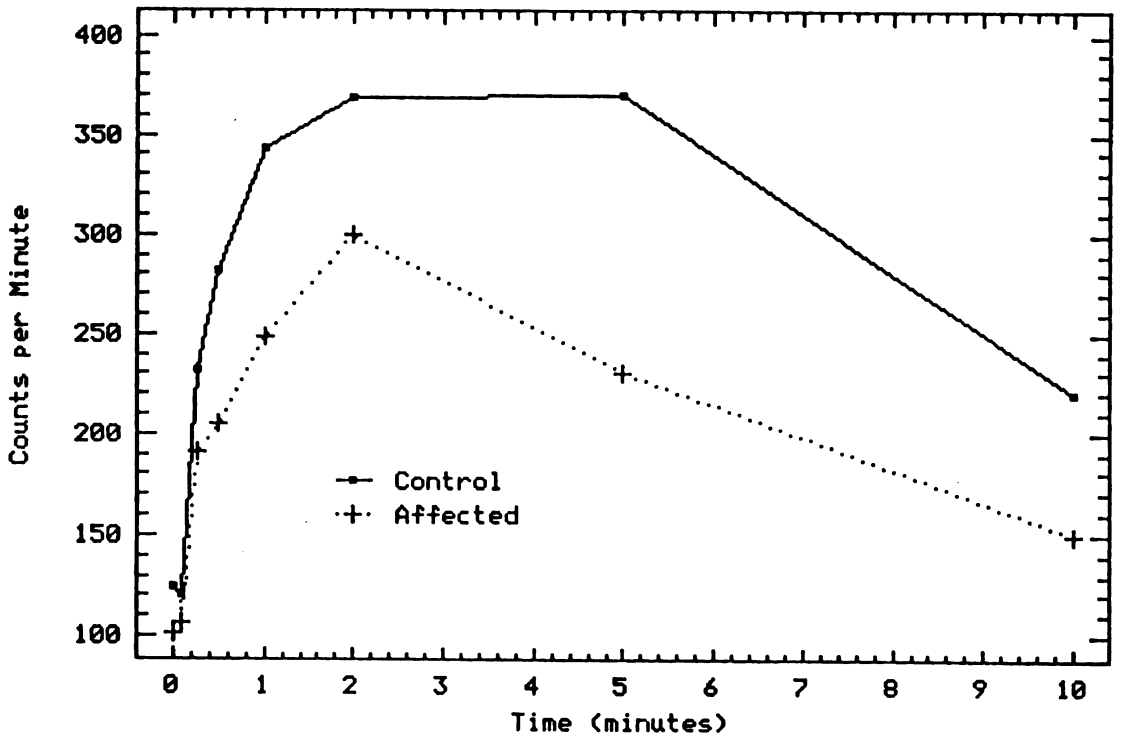
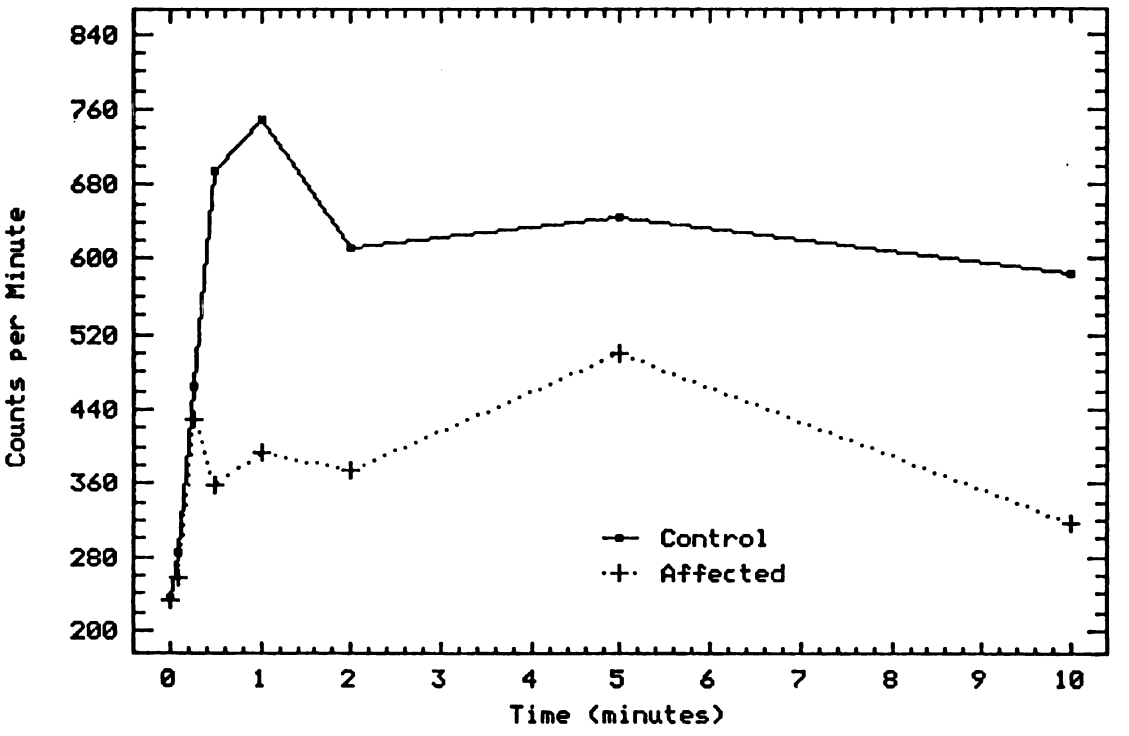

4.00
Affected 5min
47 kDa
65 kDa
20 kDa
ABSORBANCE
-0.01
0.000
MILLIMETERS
110.000

Figure 52. Phosphorylation (cpm) of the 20 kDa protein band of control and affected ^{32}P-labelled platelets solubilized after the addition of 0.3 U/ml thrombin and separated by SDS-PAGE.

Figure 53. Phosphorylation (cpm) of the 47 kDa protein band of control and affected ^{32}P-labelled platelets solubilized after the addition of 0.3 U/ml thrombin and separated by SDS-PAGE.


400
350
300
250
200
150
100
Counts per Minute
Control
Affected
0
1
2
3
4
5
6
7
8
9
10
Time (minutes)


840
760
680
600
520
440
360
280
200
Counts per Minute
Control
Affected
0
1
2
3
4
5
6
7
8
9
10
Time (minutes)

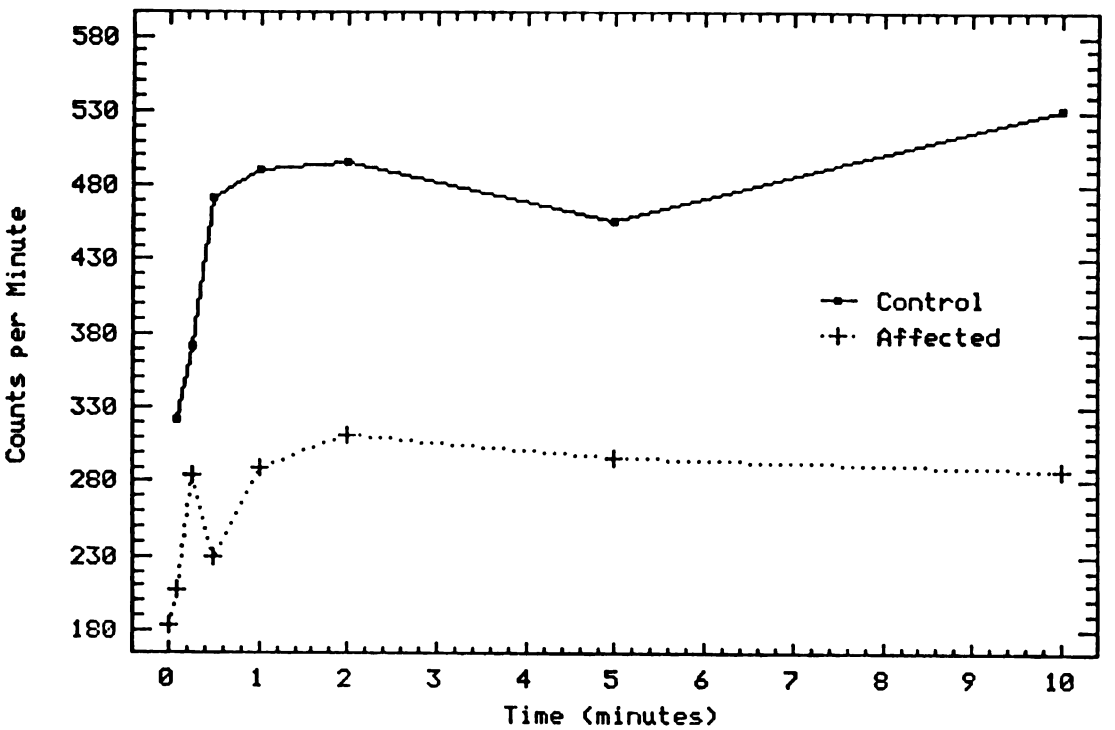


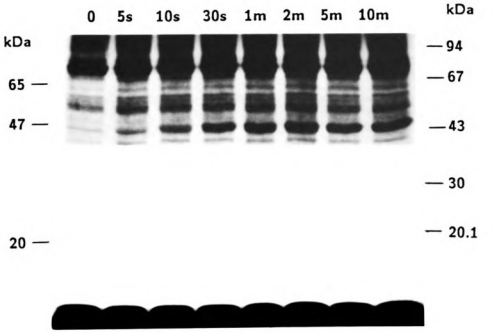
Figure 54. Phosphorylation (cpm) of the 65 kDa protein band of control and affected ^{32}P-labelled platelets solubilized after the addition of 0.3 U/ml thrombin and separated by SDS-PAGE.

Phorbol Myristate Acetate

Control and affected platelets stimulated by 3.0 μM PMA under non-aggregating conditions slowly phosphorylated the 20 kDa protein and rapidly phosphorylated the 47 kDa protein. Figures 55a and 55b show a pair of autoradiographs in which the platelets were stimulated with 9% NaCl (0 lane) or 3.0 μM PMA and the reaction stopped at 5, 15 and 30 seconds and 1, 2, 5 and 10 minutes. Densiometric scans of the 0, 30 second and five minute lanes of these autoradiographs are shown in Figures 56a, 56b, 57a, 57b, 58a and 58b. The appropriate bands were cut out of the labelled gels and counted by Cerenkov counting. These results are shown in Figures 59, 60 and 61. Control platelets slowly phosphorylated the 20 kDa protein, reaching a maximum 10 minutes after the addition of PMA. Control platelet counts per minute values were not available for the 5 second and 1 minute incubation times. Affected platelets also phosphorylated the 20 kDa protein slowly, although to a slightly lesser extent than controls (Figures 55, 56, 57, 58 and 59). There did not appear to be any difference in the pattern or extent of phosphorylation of the 47 kDa protein between the two groups (Figures 55, 56, 58 and 60). Although the 65 kDa band was phosphorylated to a lesser extent in affected platelets compared to controls, in both groups there appeared to be a time-dependent phosphorylation of this band in response to 3.0 μM PMA (Figures 55, 56, 57 and 61). This occurred in the other gels in which platelets were stimulated by PMA.

Figure 55a. Autoradiograph of control ^{32}P-labelled platelets activated by 3.0 μM PMA, solubilized at various times and separated by SDS-PAGE.

Figure 55b. Autoradiograph of affected ^{32}P-labelled platelets activated by 3.0 μM PMA, solubilized at various times and separated by SDS-PAGE.


0 5s 10s 30s 1m 2m 5m 10m
kDa
kDa
65
47
20
94
67
43
30
20.1

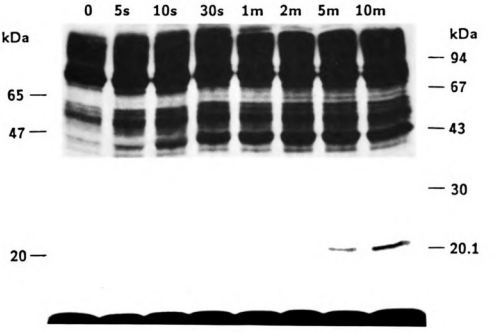
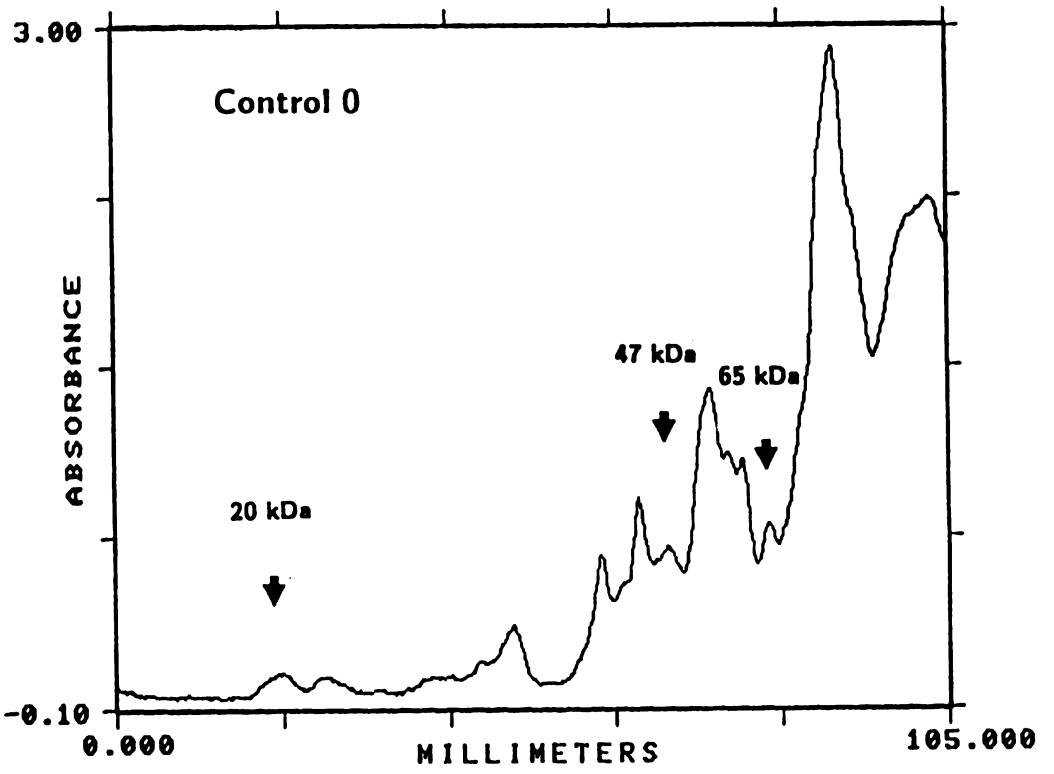

0 5s 10s 30s 1m 2m 5m 10m
kDa
kDa
65
47
20
94
67
43
30
20.1

Figure 56a. Densiometric scan of an autoradiograph of resting control ^{32}P-labelled platelets solubilized and separated by SDS-PAGE.

Figure 56b. Densiometric scan of an autoradiograph of resting affected ^{32}P-labelled platelets solubilized and separated by SDS-PAGE.


3.00
Control 0
ABSORBANCE
47 kDa
65 kDa
20 kDa
-0.10
0.000
MILLIMETERS
105.000

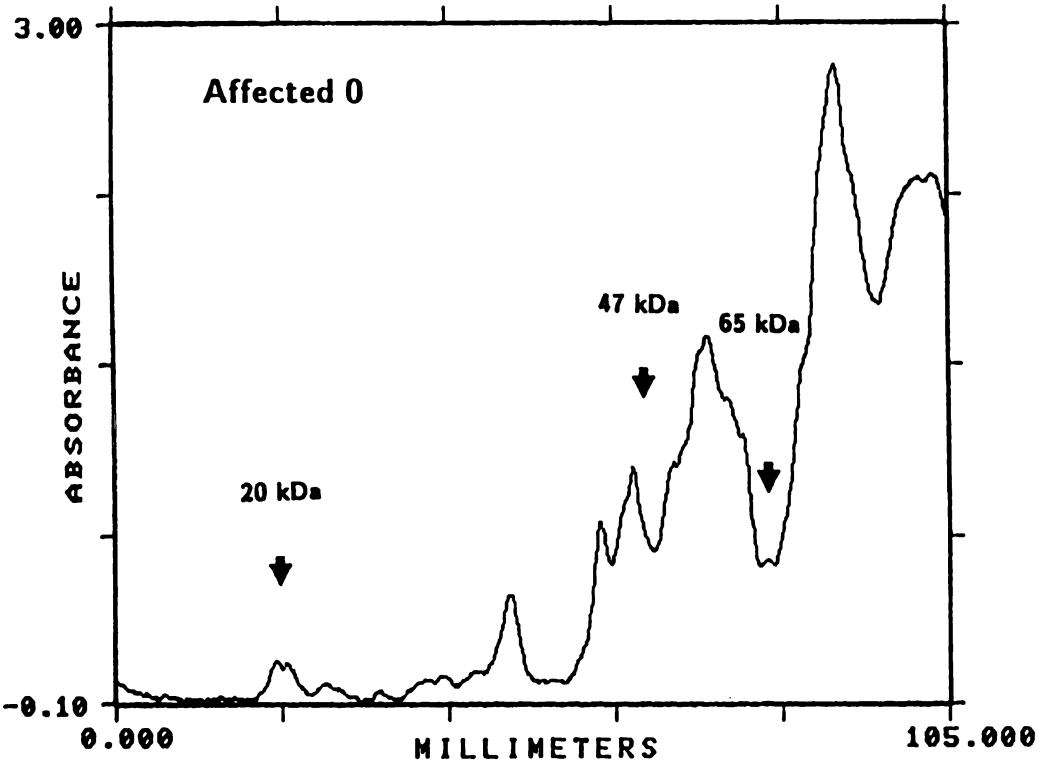
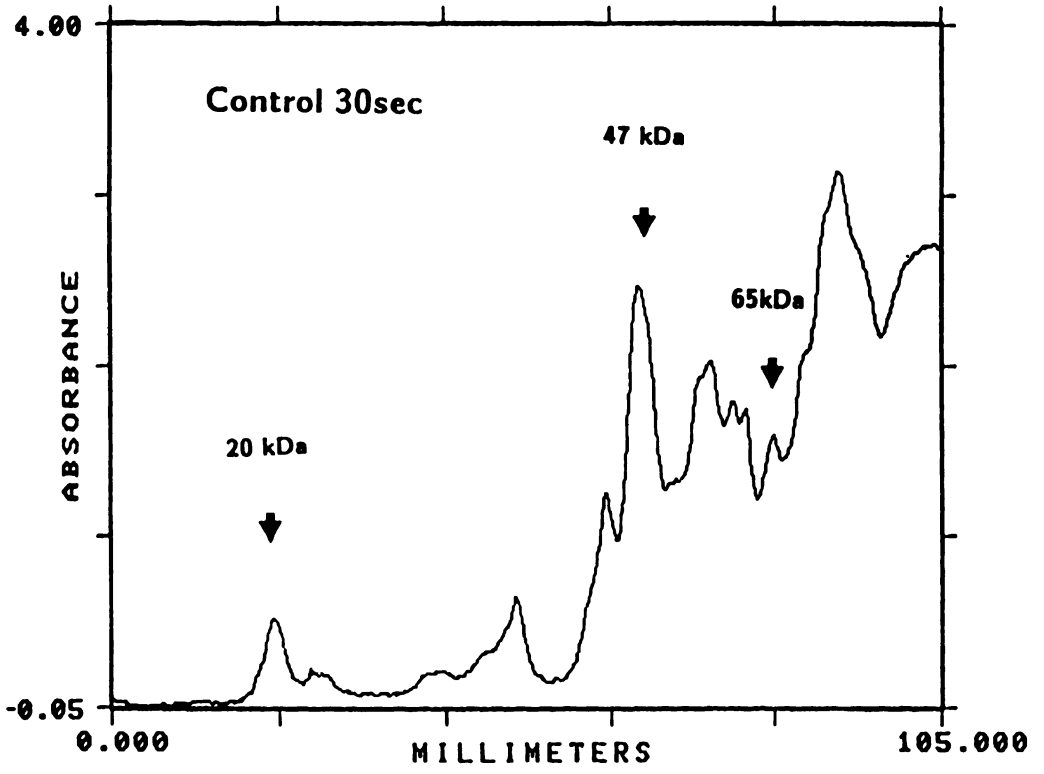

3.00
Affected 0
ABSORBANCE
47 kDa
65 kDa
20 kDa
-0.10
0.000
MILLIMETERS
105.000

Figure 57a. Densiometric scan of an autoradiograph of control ^{32}P-labelled platelets solubilized 30 seconds after the addition of 3.0 μM PMA and separated by SDS-PAGE.

Figure 57b. Densiometric scan of an autoradiograph of affected ^{32}P-labelled platelets solubilized 30 seconds after the addition of 3.0 μM PMA and separated by SDS-PAGE.


4.00
Control 30sec
47 kDa
65kDa
20 kDa
ABSORBANCE
-0.05
0.000
MILLIMETERS
105.000

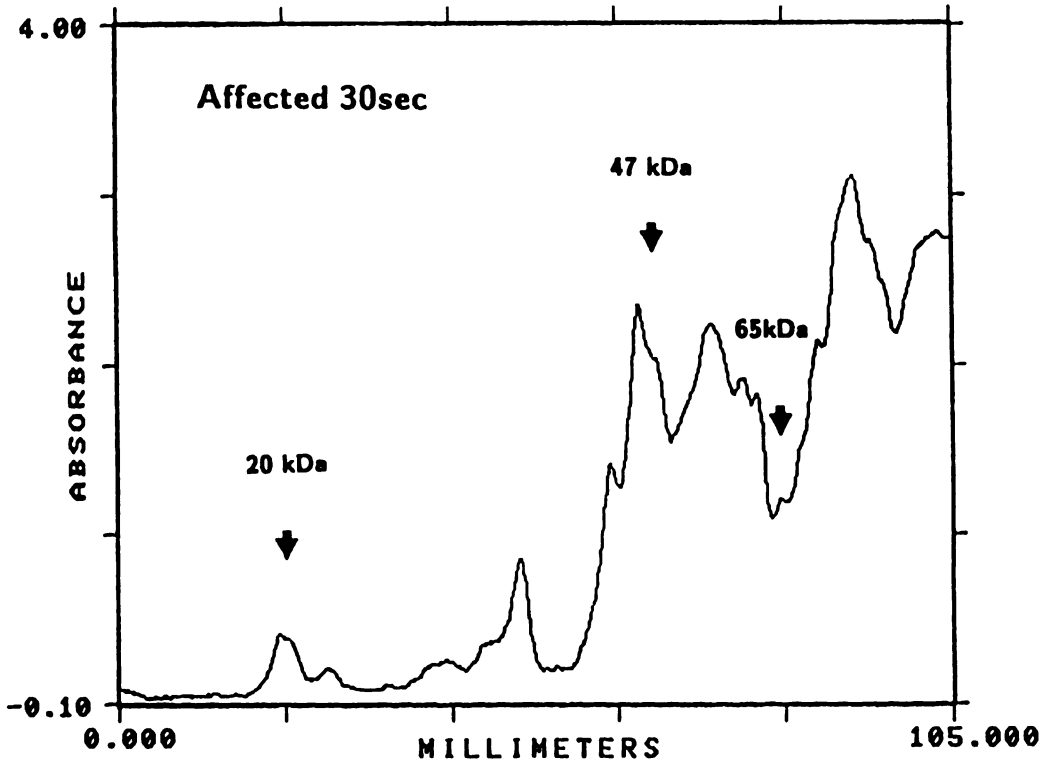
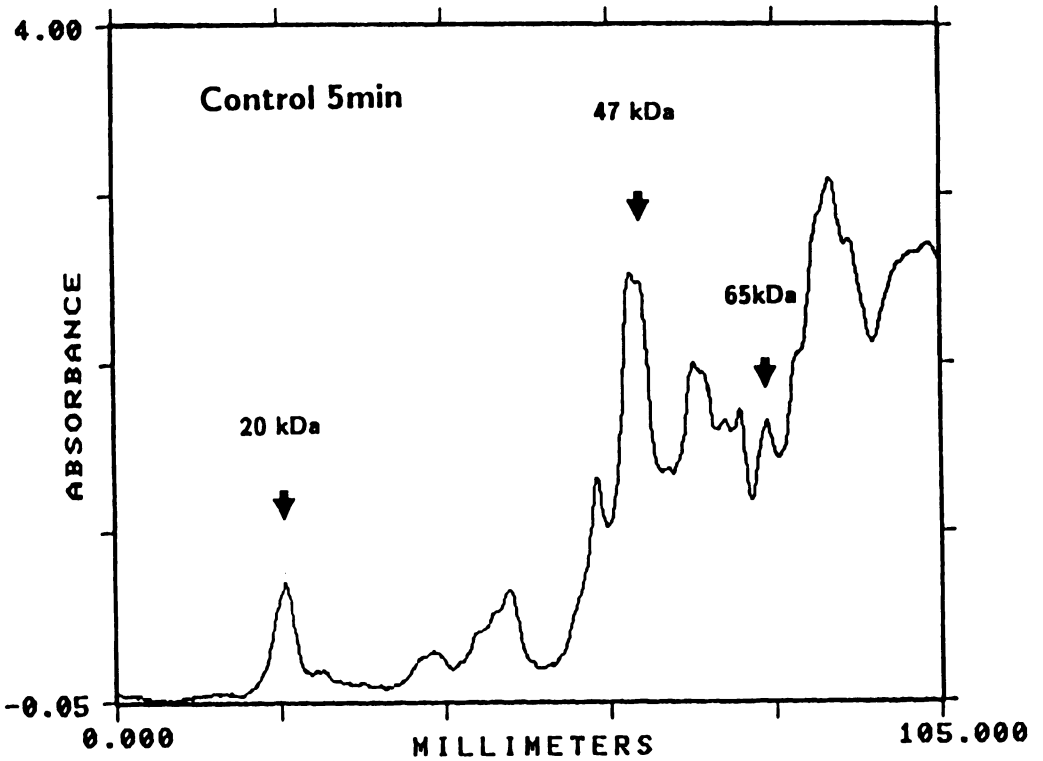

4.00
Affected 30sec
47 kDa
65kDa
20 kDa
ABSORBANCE
-0.10
0.000
MILLIMETERS
105.000

Figure 58a. Densiometric scan of an autoradiograph of control ^{32}P-labelled platelets solubilized five minutes after the addition of 3.0 μM PMA and separated by SDS-PAGE.

Figure 58b. Densiometric scan of an autoradiograph of affected ^{32}P-labelled platelets solubilized five minutes after the addition of 3.0 μM PMA and separated by SDS-PAGE.


4.00
Control 5min
47 kDa
65kDa
20 kDa
ABSORBANCE
-0.05
0.000
MILLIMETERS
105.000

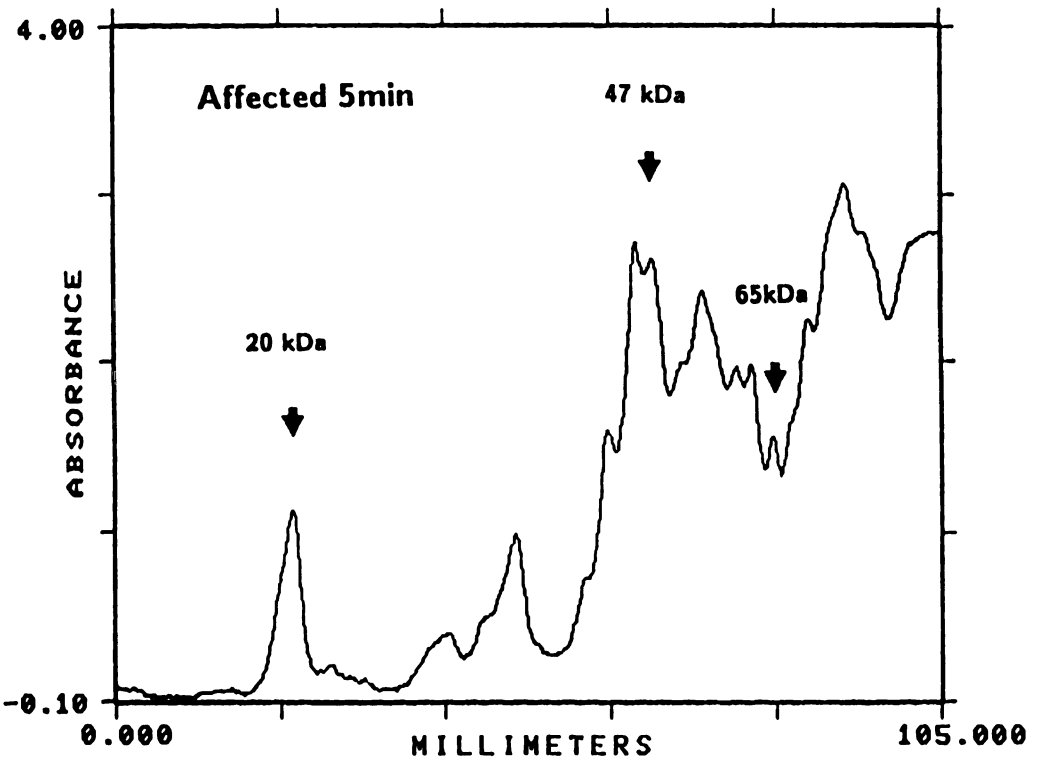
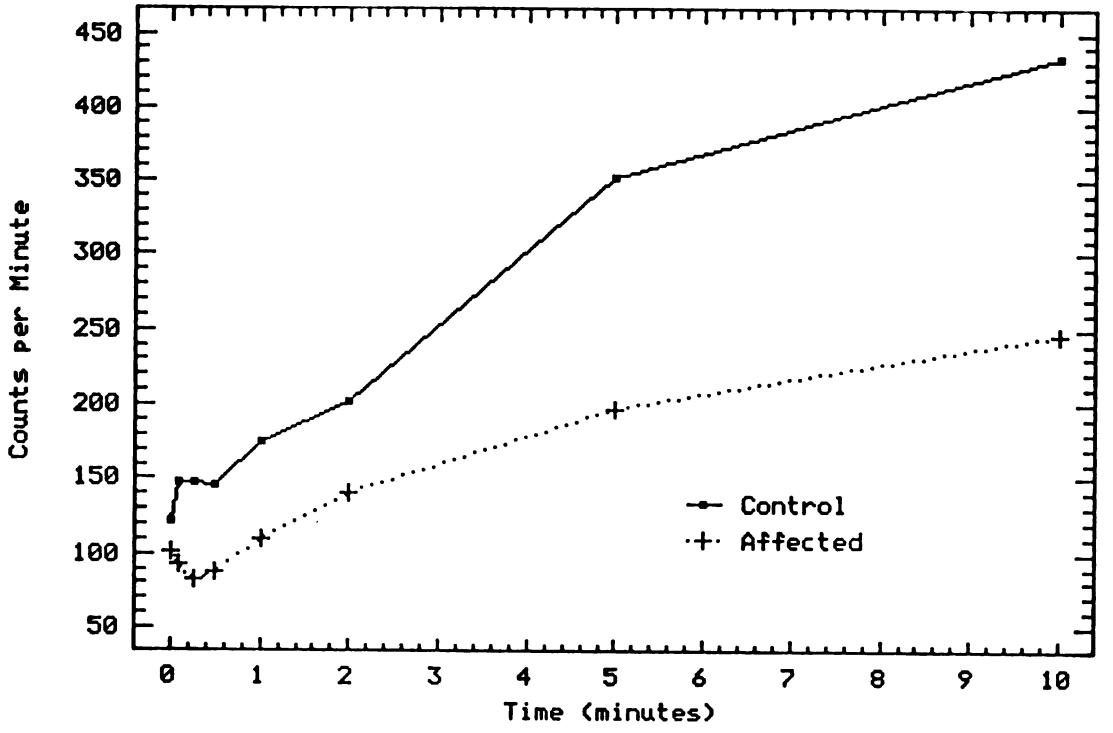
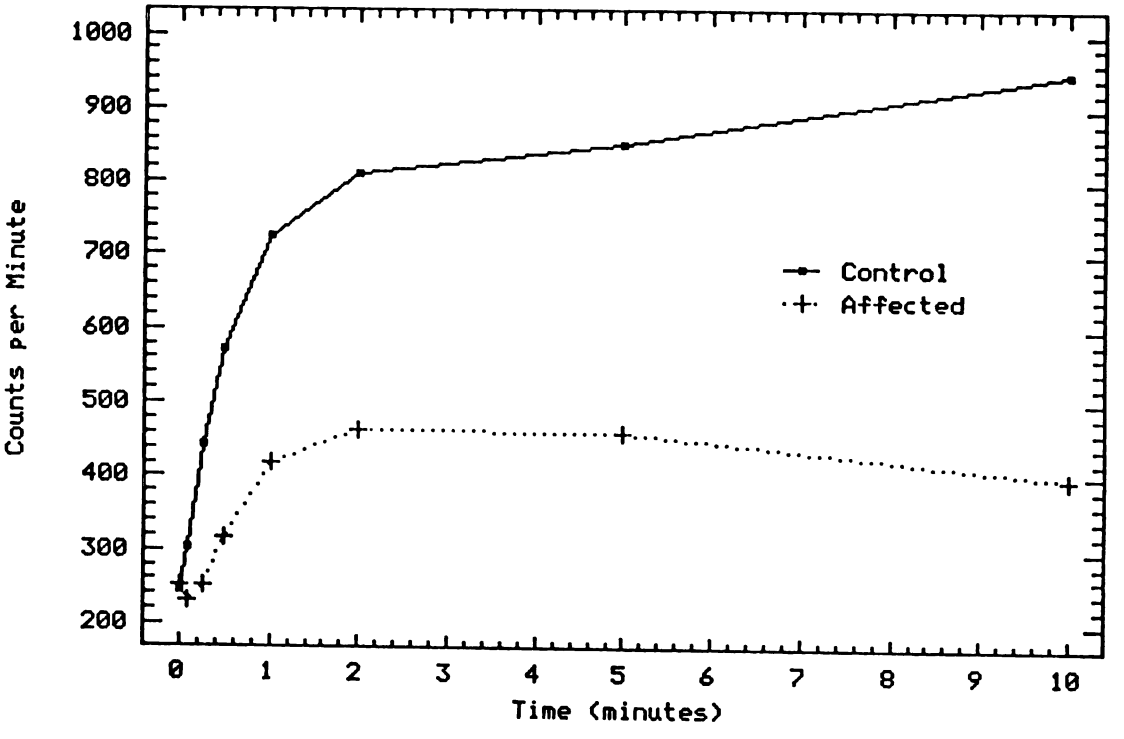

4.00
Affected 5min
47 kDa
65kDa
20 kDa
ABSORBANCE
-0.10
0.000
MILLIMETERS
105.000

Figure 59. Phosphorylation (cpm) of the 20 kDa protein band of control and affected ^{32}P-labelled platelets solubilized after the addition of 3.0 μM PMA and separated by SDS-PAGE.

Figure 60. Phosphorylation (cpm) of the 47 kDa protein band of control and affected ^{32}P-labelled platelets solubilized after the addition of 3.0 μM PMA and separated by SDS-PAGE.


Counts per Minute
450
400
350
300
250
200
150
100
50
0
1
2
3
4
5
6
7
8
9
10
Time (minutes)
Control
Affected


Counts per Minute
1000
900
800
700
600
500
400
300
200
0
1
2
3
4
5
6
7
8
9
10
Time (minutes)
Control
Affected

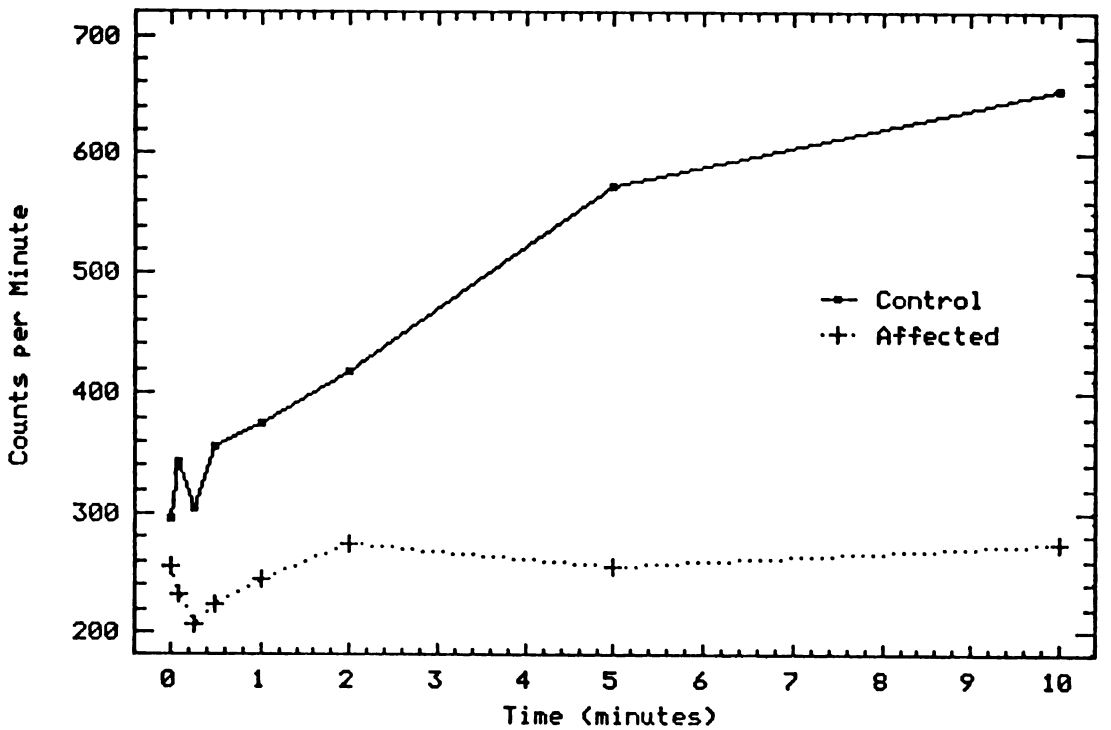


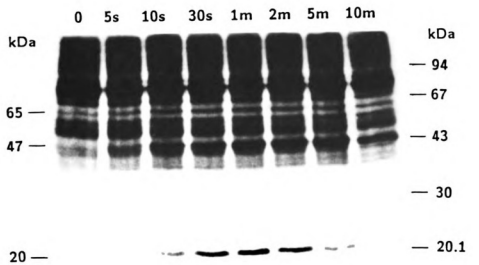
Figure 61. Phosphorylation (cpm) of the 65 kDa protein band of control and affected ^{32}P-labelled platelets solubilized after the addition of 3.0 μM PMA and separated by SDS-PAGE.

Calcium Ionophore A23187

Control and affected platelets stimulated by 1.0 μM A23187 under non-aggregating conditions rapidly phosphorylated the 20 kDa protein and more slowly phosphorylated the 47 kDa protein. Figures 62a and 62b show a pair of autoradiographs in which the platelets were stimulated with 0.9% NaCl (0 lane) or 1.0 μM A23187 and the reaction stopped at 5, 15 and 30 seconds and 1, 2, 5 and 10 minutes. Densiometric scans of the 0, 30 second and five minutes lanes of these autoradiographs are shown in Figures 63a, 63b, 64a, 64b, 65a and 65b. The appropriate bands were cut out of the labelled gels and counted by Cerenkov counting. These results are shown in Figures 66, 67 and 68. Both groups phosphorylated the 20 kDa protein band rapidly, with maximum phosphorylation occurring 0.5 to 2 minutes after the addition of A23187 (Figures 62, 63, 64, 65 and 66). The 47 kDa protein was phosphorylated in both groups, although more slowly than the 20 kDa band, with maximum phosphorylation occurring at five minutes (Figures 62, 63, 64, 65 and 67). The degree of phosphorylation of the 20 and 47 kDa proteins in affected platelets is slightly less than that of control platelets. This was also seen in other gels in which the platelets were activated by A23187. Baseline phosphorylation of the 65 kDa band was markedly less intense in affected platelets compared to controls. Phosphorylation of the 65 kDa band did not appear to be affected significantly by A23187 in either group (Figures 62, 63, 64, 65 and 68).

Figure 62a. Autoradiograph of control ^{32}P-labelled platelets activated by 1.0 μM A23187, solubilized at various times and separated by SDS-PAGE.

Figure 62b. Autoradiograph of affected ^{32}P-labelled platelets activated by 1.0 μM A23187, solubilized at various times and separated by SDS-PAGE.


0
5s
10s
30s
1m
2m
5m
10m
kDa
65
47
20
kDa
94
67
43
30
20.1

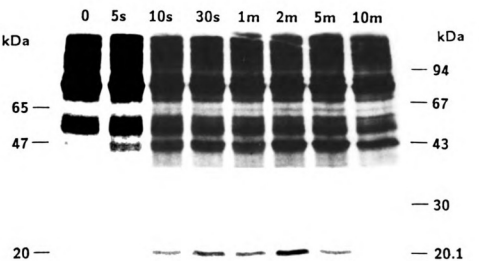
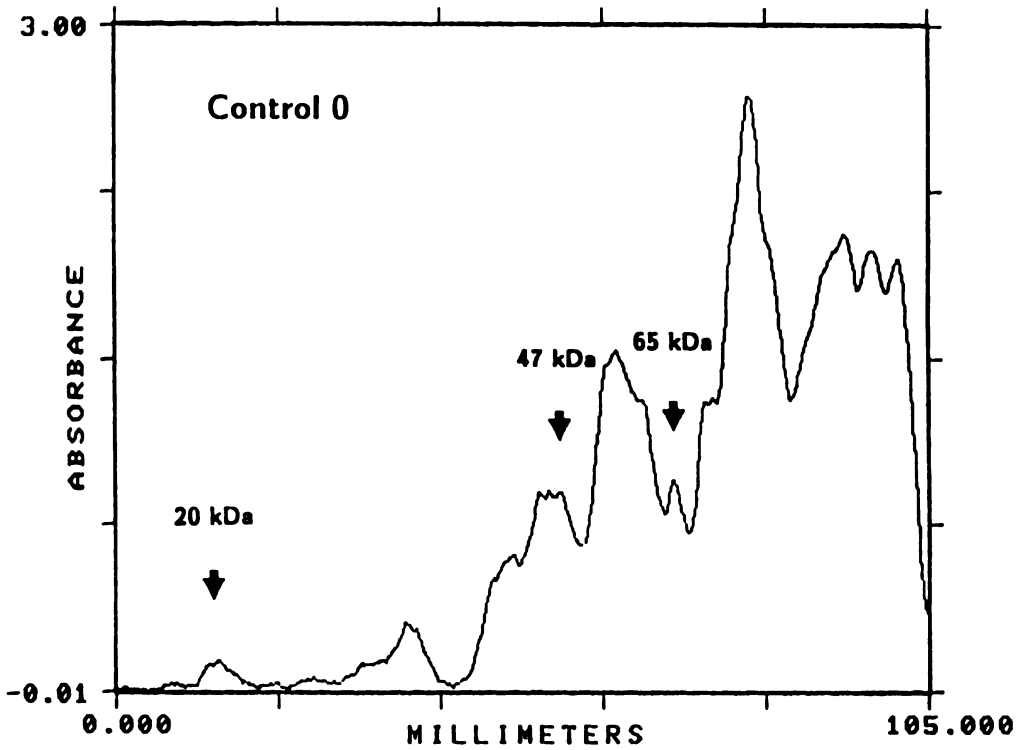

0
5s
10s
30s
1m
2m
5m
10m
kDa
65
47
20
kDa
94
67
43
30
20.1

Figure 63a. Densiometric scan of an autoradiograph of resting control ^{32}P-labelled platelets solubilized and separated by SDS-PAGE.

Figure 63b. Densiometric scan of an autoradiograph of resting affected ^{32}P-labelled platelets solubilized and separated by SDS-PAGE.


3.00
Control 0
ABSORBANCE
47 kDa
65 kDa
20 kDa
-0.01
0.000
MILLIMETERS
105.000

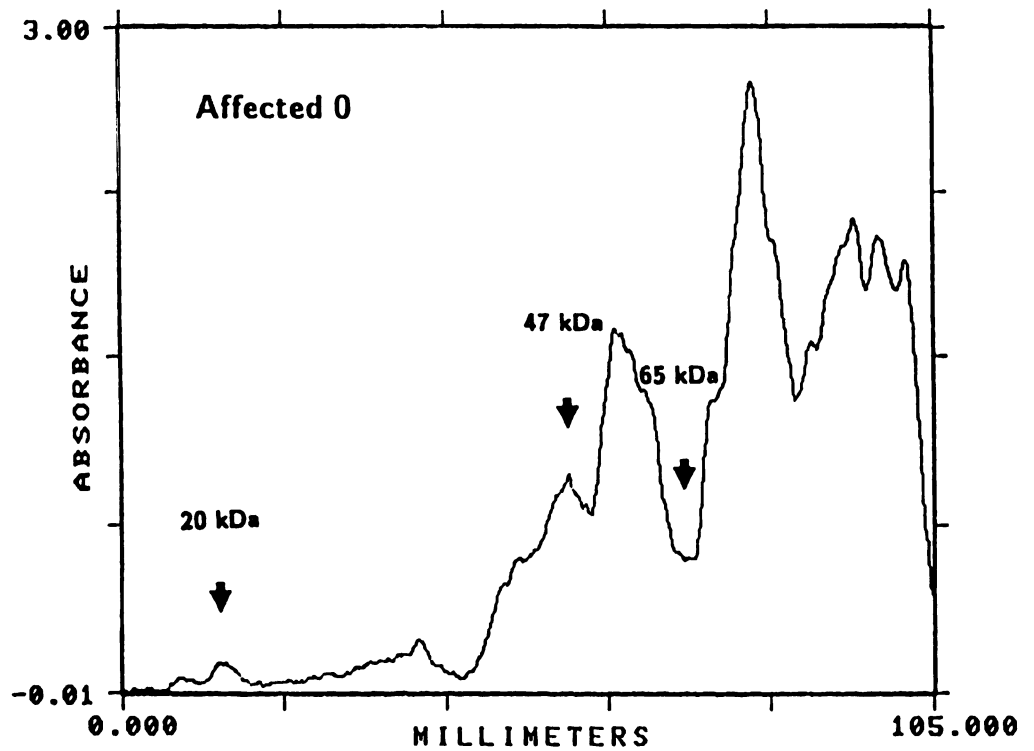
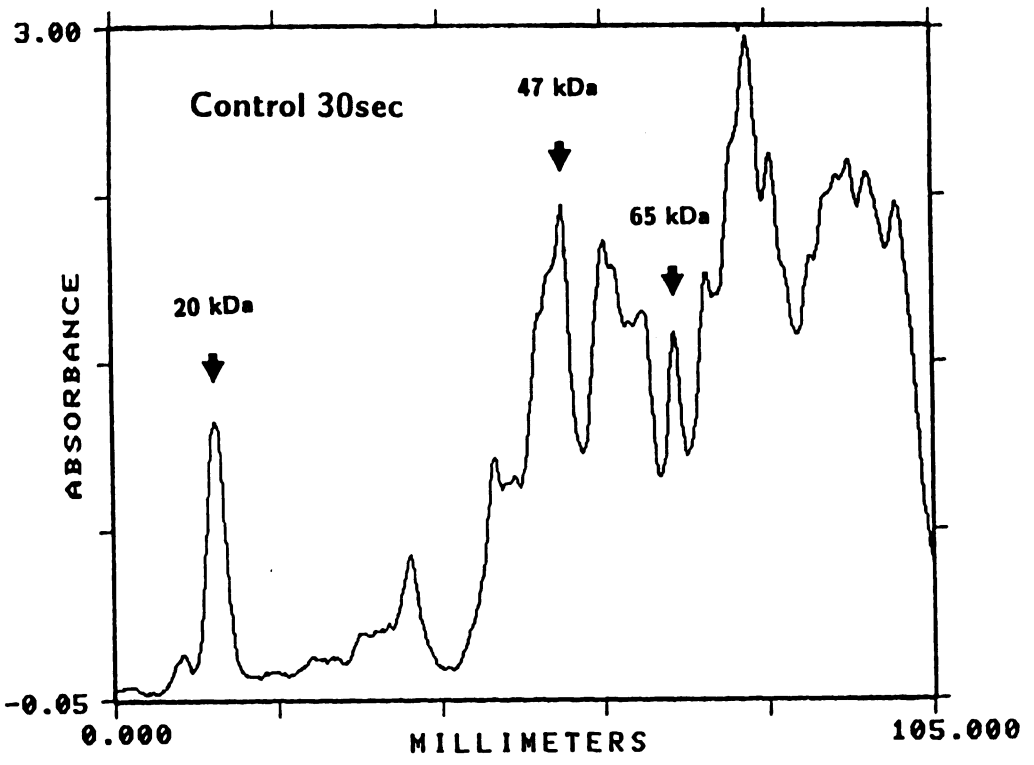

3.00
Affected 0
ABSORBANCE
47 kDa
65 kDa
20 kDa
-0.01
0.000
MILLIMETERS
105.000

Figure 64a. Densiometric scan of an autoradiograph of control ^{32}P-labelled platelets solubilized 30 seconds after the addition of 1.0 μM A23187 and separated by SDS-PAGE.

Figure 64b. Densiometric scan of an autoradiograph of affected ^{32}P-labelled platelets solubilized 30 seconds after the addition of 1.0 μM A23187 and separated by SDS-PAGE.


3.00
Control 30sec
47 kDa
65 kDa
20 kDa
ABSORBANCE
-0.05
0.000
MILLIMETERS
105.000

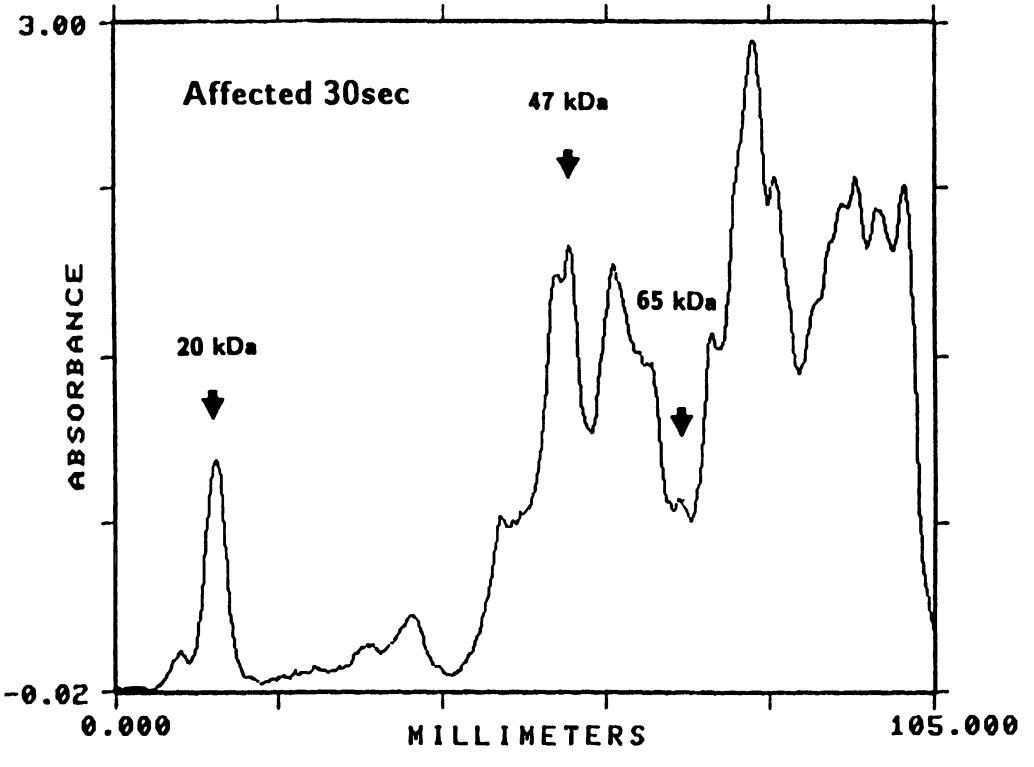
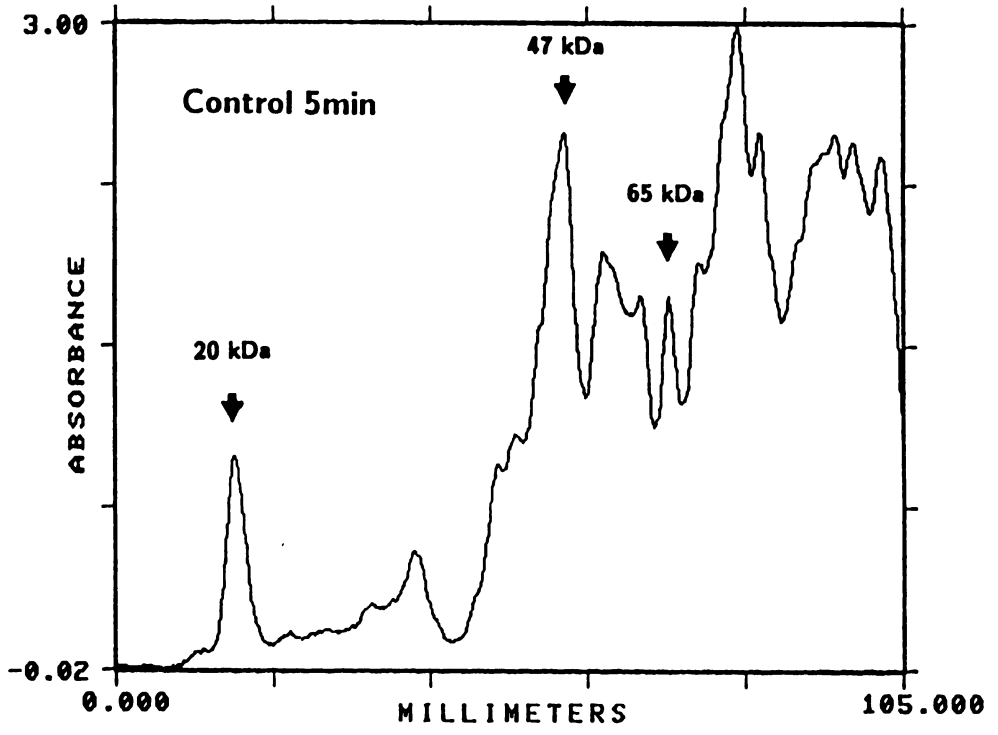

3.00
Affected 30sec
47 kDa
65 kDa
20 kDa
ABSORBANCE
-0.02
0.000
MILLIMETERS
105.000

Figure 65a. Densiometric scan of an autoradiograph of control ^{32}P-labelled platelets solubilized five minutes after the addition of 1.0 μM A23187 and separated by SDS-PAGE.

Figure 65b. Densiometric scan of an autoradiograph of affected ^{32}P-labelled platelets solubilized five minutes after the addition of 1.0 μM A23187 and separated by SDS-PAGE.


3.00
47 kDa
Control 5min
65 kDa
20 kDa
ABSORBANCE
-0.02
0.000
MILLIMETERS
105.000

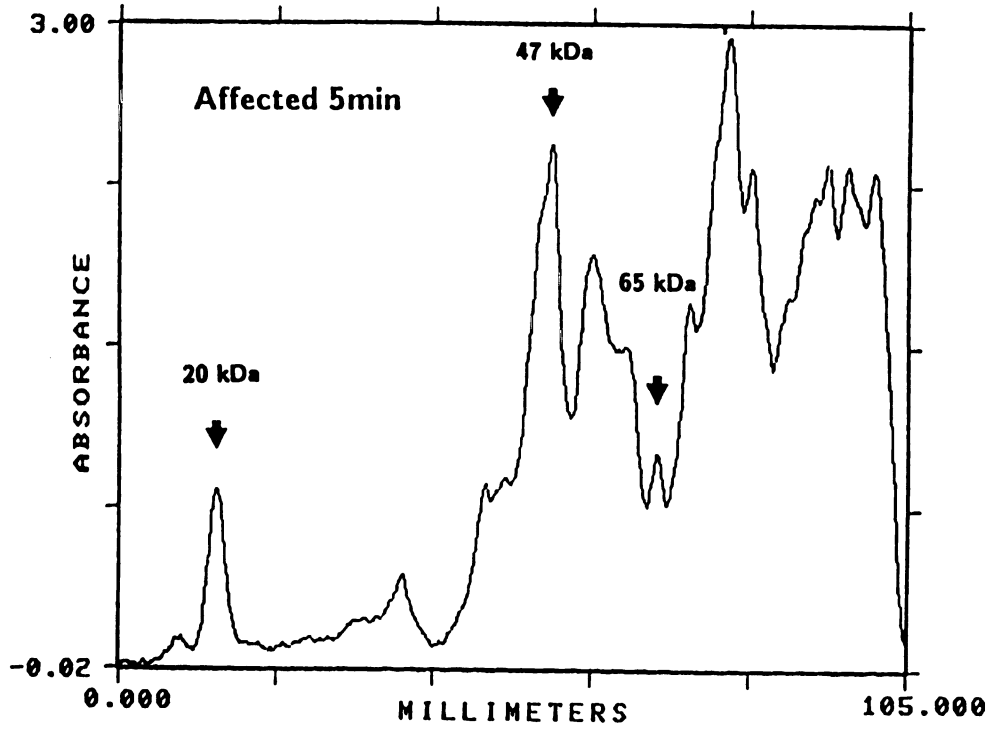
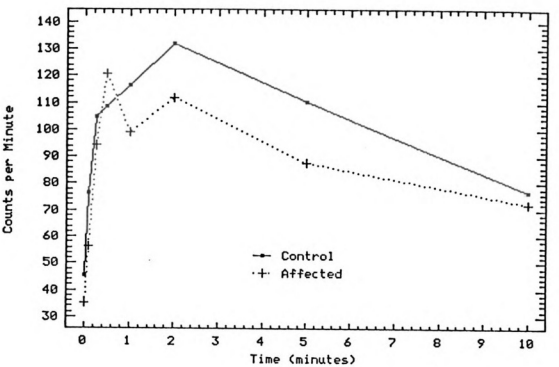
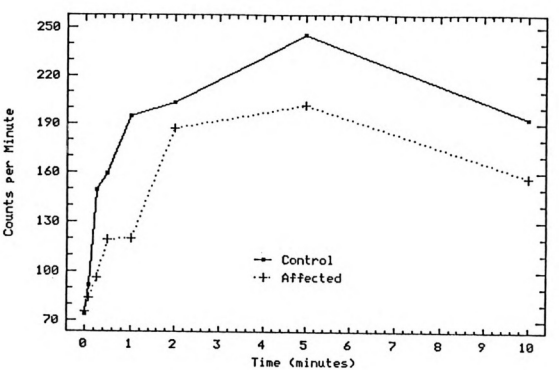

3.00
47 kDa
Affected 5min
65 kDa
20 kDa
ABSORBANCE
-0.02
0.000
MILLIMETERS
105.000

Figure 66. Phosphorylation (cpm) of the 20 kDa protein band of control and affected ^{32}P-labelled platelets solubilized after the addition of 1.0 μM A23187 and separated by SDS-PAGE.

Figure 67. Phosphorylation (cpm) of the 47 kDa protein band of control and affected ^{32}P-labelled platelets solubilized after the addition of 1.0 μM A23187 and separated by SDS-PAGE.


Counts per Minute
140
130
120
110
100
90
80
70
60
50
40
30
0
1
2
3
4
5
6
7
8
9
10
Time (minutes)
Control
Affected


Counts per Minute
250
220
190
160
130
100
70
0
1
2
3
4
5
6
7
8
9
10
Time (minutes)
Control
Affected

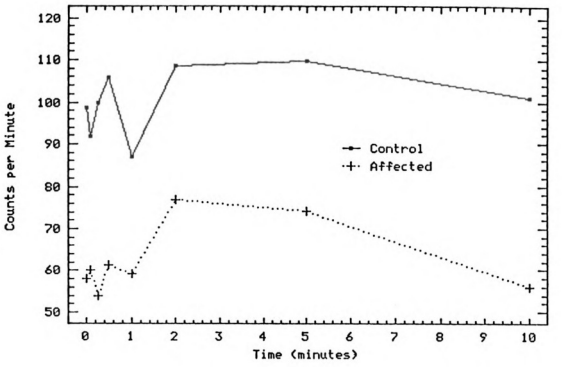


Figure 68. Phosphorylation (cpm) of the 65 kDa protein band of control and affected ^{32}P-labelled platelets solubilized after the addition of 1.0 μM A23187 and separated by SDS-PAGE.

DISCUSSION

Strong agonists and those of intermediate strength are able to mediate the release response from human platelets and stimulate the phosphorylation of the 20 kDa myosin light chain and 47 kDa protein in the absence of aggregation. These agonists include thrombin, PAF and calcium ionophore A23187.[143,183,252,277,281]

A weak agonist such as ADP does not induce phosphorylation of the 20 and 47 kDa proteins in normal human platelets under non-stirred, non-aggregating conditions in which release of granule contents and arachidonate metabolites does not occur.[143,183] Neither control or affected canine platelets phosphorylated the 20 kDa protein under non-aggregating conditions. This suggests, but does not prove, that the mechanism of activation of canine platelets in response to ADP is similar to that of human platelets. Although affected platelets in platelet rich plasma secrete dense granule contents in the absence of primary aggregation, their behavior in response to ADP is still apparently governed by the same factors that influence normal platelets. The baseline degree of ^{32}P incorporation into the 20 kDa protein of affected platelets in the 0 lane was less than that of control platelets while that of the 47 kDa protein was similar.

Platelet activating factor causes rapid phosphorylation of the 20 and 47 kDa proteins in human platelets, even in the absence of released ADP or arachidonate metabolites.[252,277] Phosphorylation of the 20 kDa protein in both control and affected canine platelets was very rapid,

peaking within 30 seconds of the addition of 1.0 μM or 0.1 μM PAF. Baseline incorporation of ^{32}P into the 20 kDa band was slightly less in affected compared to control platelets, but the percentage increase above baseline and rates of dephosphorylation were similar in most gels. Phosphorylation of the 47 kDa protein was also very rapid in both control and affected platelets, peaking in one minute or less. Baseline incorporation of ^{32}P was similar in both groups. Phosphorylation of the 20 kDa protein correlated well to the shape change seen in platelet rich plasma in both groups. However, it is difficult to relate it to the failure of dense granule secretion from affected platelets in response to 0.1 μM PAF as the effective concentration of PAF in an albumin-free buffer is higher than in plasma. In a later study, it was found that 0.1 μM PAF in a protein-free buffer has approximately the same effect as 1.0 μM PAF in platelet rich plasma. The lack of significant aggregation of affected platelets in response to PAF does not appear to be related to an inability to phosphorylate the 20 or 47 kDa proteins.

Thrombin has been reported to cause phosphorylation of the 20 and 47 kDa proteins in human platelets within 30 to 60 seconds under the non-aggregating conditions used in this study.[143] This is very similar to the response of control canine platelets which reached peak phosphorylation of the 20 kDa band within two minutes of the addition of 0.3 U/ml thrombin. Affected platelets also phosphorylated the 20 kDa band at the same initial rate as control platelets. The baseline degree of incorporation of ^{32}P into the 20 kDa protein, as well as peak phosphorylation, was less than that of control platelets, although the

percentage increase in phosphorylation above the baseline level was similar in both groups. The initial rate of phosphorylation of the 47 kDa protein in affected platelets was similar to controls, but did not reach as high a peak. This was a trend seen in most, but not all pairs of gels. The similar initial rate of phosphorylation of the 20 kDa protein in both groups correlates well with the occurrence of shape change and similar rate of dense granule secretion from both groups of platelets. The markedly slower initial rate of aggregation of affected platelets does not appear to be the directly related to an abnormal rate of phosphorylation of the 20 or 47 kDa proteins.

Activation of human platelets induced by PMA is associated with slow phosphorylation of the 20 kDa myosin light chain that is mediated mainly through protein kinase C.[245,346] The site phosphorylated in the myosin light chain is distinct from that phosphorylated by the myosin light chain kinase.[346] There is also some early reversible phosphorylation of the 20 kDa protein by myosin light chain kinase in PMA-stimulated platelets.[346] Endogenous 1,2-diacylglycerol produced by thrombin-stimulated platelets does not phosphorylate the 20 kDa band at the site phosphorylated by PMA-stimulated protein kinase C.[346] Therefore the phosphorylation of the 20 kDa protein by protein kinase C in PMA-stimulated platelets may not have any physiological significance. Both control and affected platelets stimulated by 3.0 μM PMA slowly phosphorylated the 20 kDa band with peak phosphorylation occurring at five to 10 minutes, although affected platelets incorporated less ^{32}P into the 20 kDa band than controls. Baseline ^{32}P incorporation into the 20 kDa band was similar in both groups, but the

radioactivity counted for the 0 lane of affected platelets was considerably higher than at 5, 15, 30 and 60 seconds, suggesting that the validity of the 0 value was questionable. There may have been some radioactive contamination of the scintillation vial.

Phorbol esters are able to substitute for 1,2-diacylglycerol causing irreversible activation of protein kinase C and phosphorylation of the 47 kDa protein.[73] Maximal phosphorylation of the 47 kDa protein by exogenous activators of protein kinase C in human platelets is not related to the onset of aggregation or dense granule release.[279,449] Control and affected canine platelets rapidly and extensively phosphorylated the 47 kDa protein. The rate of phosphorylation of the 47 kDa band did not correlate with the speed of onset of aggregation or dense granule secretion in either group, having peaked many minutes before aggregation or secretion was seen in platelet rich plasma. In some gels, the 47 kDa protein was phosphorylated to a lesser degree in affected platelets, but this was not a consistent finding.

The calcium ionophore A23187 has been reported to stimulate phosphorylation of the 20 and 47 kDa proteins in the presence and absence of 1.0 mM EGTA.[183,281] Both control and affected platelets rapidly phosphorylated the 20 kDa protein, reaching peak phosphorylation within two minutes of the addition of 1.0 μM A23187. Although baseline incorporation of ^{32}P into the 20 kDa band of affected platelets was slightly less than that of controls, the percentage increase above baseline phosphorylation was similar, or higher, than that of controls. The rate of dephosphorylation for the two groups was

also very similar. Phosphorylation of the 20 kDa protein correlated with the occurrence of shape change in both groups, but there did not appear to be a relationship between phosphorylation of the 20 kDa myosin light chain and dense granule secretion in affected platelets. In spite of similar phosphorylation of the 20 kDa protein in both groups, affected platelets secreted only 4 to 6% the amount of dense granule ATP secreted by control platelets.

Although activation of phospholipase C by A23187 is dependent upon formation of arachidonate metabolites, it has been reported that A23187 induces phosphorylation of the 47 kDa protein in the absence of phospholipase C activation.[281] The increase in intracellular Ca^{2+} is thought to activate protein kinase C without the formation of diacylglycerol. It has also been reported that the calcium ionophore ionomycin did not phosphorylate the 47 kDa protein under conditions in which phospholipase C was not activated.[412] Both control and affected platelets phosphorylated the 47 kDa protein with an initial rapid rate of phosphorylation which peaked within five minutes of the addition of A23187. However, this alone does not indicate that phospholipase C was activated and 1,2-diacylglycerol produced, the phosphorylation may have been the direct result of the increase in intracytoplasmic Ca^{2+} concentration. The failure of affected platelets to aggregate or secrete dense granule contents does not appear to be related to an inability to phosphorylate the 47 kDa protein.

Affected platelets phosphorylated the 20 kDa protein in response to all agonists that stimulated phosphorylation in control platelets.

This indicates that the enzyme and substrate are present and functional and that the intracytoplasmic Ca^{2+} concentration has been elevated sufficiently in response to the agonist for activation of the enzyme. A difference between the two groups was a slight decrease in baseline incorporation of ^{32}P into the 20 kDa band of affected platelets when compared to controls, although individual variation obliterated the difference in some pairs. The incorporation of ^{32}P into most other phosphoproteins, as measured by the baseline radioactivity of the 47 kDa band and the absorbance of prominent bands on densiometric scans of autoradiographs, was similar in both groups. This suggests that either affected platelets do not contain as much myosin light chain as control platelets, or that ^{32}P is not incorporated into it as well as in controls. It might also be related to the lack of Ca^{2+} in the external medium if there is any difference between the two groups in the way in which they handle Ca^{2+}.

Affected platelets also phosphorylated the 47 kDa protein in response to all agonists that stimulated phosphorylation in control platelets. This did not appear to be correlated to the occurrence of aggregation (thrombin, PMA) or the failure of aggregation (PAF, A23187). Similarly, phosphorylation of the 47 kDa protein did not appear to be correlated to the occurrence of dense granule secretion (PAF, thrombin, PMA) or the failure of secretion (A23187).

The affected platelets phosphorylated the 20 and 47 kDa proteins under the same circumstances as control platelets. There was, however, a tendency for the affected platelet proteins to phosphorylate less

extensively and/or dephosphorylate more rapidly. The significance of the difference is not clear, but could be related to the lack of external Ca^{2+} in the medium if affected platelets are in some way more dependent than control platelets upon the influx of external Ca^{2+} to maintain Ca^{2+}-dependent processes. Preliminary work using the calcium indicator Fura 2 indicated that the intracytoplasmic Ca^{2+} fluxes in response to 1.0 μM and 0.01 μM PAF, 0.25 U/ml and 0.01 U/ml thrombin, 10 μM and 2.0 μM ADP and 2.0 μM and 5.0 μM ionomycin were significantly less than that of controls when the external medium contained 1.0 mM EGTA. There was no difference between the two groups when PMA was the agonist. When the external medium contained 1.0 mM Ca^{2+}, the Ca^{2+} fluxes were either not significantly different between the two groups, or significantly higher in the affected platelets.

Although there were always some minor differences between pairs of autoradiographs and densiometric scans, a difference that was consistent and readily apparent was a marked decrease in the intensity of phosphorylation of a band of approximately 65 kDa in affected platelets relative to the controls both before and after agonist stimulation. This was sufficiently obvious to make all autoradiographs of affected platelets identifiable. Incubation with ADP, PAF or A23187 did not have any identifiable or consistent effect on phosphorylation of the 65 kDa band. Incubation with thrombin or PMA caused a time-dependent phosphorylation of the 65 kDa band that, while not dramatic, was consistent. It is interesting to note that the 65 kDa band was only phosphorylated in response to the two agonists that are also capable of aggregating affected platelets.

It is not possible to determine on 11 %T gels and autoradiographs whether the 65 kDa band on the autoradiographs is a single band or a cluster of bands that migrate closely together. A decrease in intensity on autoradiographs may be the result of a missing protein band in the gel, or failure to phosphorylate a band that is there. There was no identifiable difference between Coomassie blue-stained 11% T gels of affected and control platelets on visual examination and densiometric scans of the 65 kDa area. Future work will utilize two-dimensional and lower %T gels and autoradiographs to more closely examine the 65 kDa area.

The identity of this band and the functional significance of the difference between the two groups is open to speculation. A group of slowly phosphorylating proteins of 65, 41, 35-36 and 21 kDa in PMA-stimulated platelets has been described.[134] The time course for the phosphorylation of these proteins and the dose-response for PMA corresponded closely to the time course and dose-response for enhancement of arachidonic acid release by PMA. These slowly phosphorylating proteins have not been clearly identified, although the 41 kDa and 35-36 kDa proteins have probably been identified.[59,134,173,282,449]

The 41 kDa protein co-migrates with a 41 kDa protein that is ADP-ribosylated by pertussis toxin. It is probably the α-subunit of the inhibitory guanine nucleotide binding protein G_i that is linked to adenylate cyclase, and possibly phospholipase C.[59,134,173,282] It has been proposed that the α-subunit of the inhibitory guanine nucleotide

binding protein of adenylate cyclase may be a physiological substrate for protein kinase C which controls its functional status.[244] Phosphorylation of an approximately 40-41 kDa band that migrated immediately below the 47 kDa band was observed on some autoradiographs of gels of control and affected platelets exposed to PMA, PAF or thrombin. The band was more apparent on autoradiographs that were under-exposed for the 47 kDa band.

The 35-36 kDa protein has a pI similar to that of lipocortin which is phosphorylated in platelets by an unidentified kinase.[134,449] Lipocortin purified from bovine lung has been shown to be phosphorylated <u>in vitro</u> by protein kinase C.[248] Phosphorylation of this band was not identified in this study.

The α_2-adrenergic receptor of human platelets is a 64 kDa glycoprotein that has been characterized and purified to apparent homogeneity.[404] Although regulation of α_1- and $ß_2$-adrenergic receptors in smooth muscle cells by phosphorylation has been reported, no information is available regarding phosphorylation of α_2-adrenergic receptors.[49,285] The α_2-adrenergic receptor may also be very closely linked to the Na^+/H^+ exchange system, if it is not itself part of the ion transport system.[476] If the receptor does play a dual role as both receptor and ion-transporter, any defect in the receptor itself or the control of its function would have a major effect on platelet activation, particularly in response to weak agonists. Operation of Na^+/H^+ exchange is essential for the activation of the platelet release response by weak agonists.

A less likely candidate for the 65 kDa band is a 61 kDa membrane glycoprotein which has been identified in human platelets.[260] This protein specifically interacts through its extracellular domain with insoluble collagen and is possibly an important component of the initial platelet-matrix adhesion process which leads to platelet activation in vivo.

This study has determined that affected platelets have similar phosphorylation patterns of the two major proteins phosphorylated in control platelets in response to various agonists. Although phosphorylation of the 20 kDa myosin light chain correlated well with platelet shape change in both groups, there did not appear to be any consistent relationship between phosphorylation of the 20 and 47 kDa proteins and secretion of dense granule contents or aggregation. A major finding, decreased phosphorylation of an unidentified 65 kDa band in affected platelets, is of unknown functional significance.

CHAPTER V: MEASUREMENT OF THROMBOXANE B_2 PRODUCTION

INTRODUCTION

Platelets respond to Ca^{2+}-mobilizing stimulatory agonists by rapidly hydrolysing membrane phospholipids to generate a variety of intracellular and extracellular messengers. The activation of phospholipase C leads to the transient accumulation of intracellular inositol 1,4,5-trisphosphate and 1,2-diacylglycerol which increase the intracytoplasmic Ca^{2+} concentration and activate protein kinase C respectively. Both branches of this Ca^{2+}-messenger system may act synergistically to initiate the platelet responses of shape change, aggregation and secretion. Activated platelets also produce free arachidonic acid, the precursor for thromboxane A_2 (TxA_2) and other eicosanoids. These are able to leave the cell and function as intercellular messengers.

The eicosanoids are a group of chemical compounds which includes the 20-carbon fatty acids and their numerous oxygenated metabolites.[268,337,462] Arachidonic acid is the most common fatty acid present in the phospholipids of mammalian cells and, with its derivatives, makes up the most important eicosanoid group. The eicosanoids are potent local hormones or autocoids. They are not stored, but are formed rapidly in response to certain stimuli, exert

their biologic effect and are then rapidly degraded. The two main routes of metabolism of arachidonic acid are: (1) the cyclooxygenase enzyme system which produces prostaglandins and thromboxane; and (2) the lipoxygenase enzyme system which produces hydroxy and hydroperoxy fatty acids and leukotrienes.

Although platelets do metabolize arachidonate via the lipoxygenase pathway, they do not generate leukotrienes and the biological significance of the 12-hydroxy-eicosatetraenoic acid (12-HETE) produced by platelets is not clear.[268,337] In contrast, arachidonic acid and its oxygenated metabolites are of major importance in the normal function of platelets. Weak physiologic agonists such as adenosine diphosphate (ADP), epinephrine and low concentrations of platelet activating factor (PAF) and thrombin all share the requirement that arachidonic acid must be liberated and metabolized to TxA_2 for irreversible aggregation and dense granule secretion to occur. When the calcium ionophore A23187 or exogenous 1,4,5-inositol trisphosphate is used to stimulate platelets, the activation of phospholipase C is totally dependent on the formation of TxA_2.[58,413]

Since the level of free arachidonic acid in platelets is maintained at a very low level relative to its esterified level, the synthesis of prostaglandins and thromboxane requires the liberation of arachidonate from membrane phospholipids.[220,469] The bulk of arachidonate is esterified in the sn-2 position of glycerophospholipids and the level of free arachidonic acid represents a balance between the liberation of the acid by hydrolysis and its reesterification by acyltransferases.

The balance is weighted toward esterification because of the high affinity of the acyltransferase for the intermediate arachidonoyl-CoA, particularly if the acceptor is lysophosphatidylinositide. This preference probably explains the very high arachidonate content of the phosphoinositides.

Three major routes for arachidonate liberation from platelet membranes have been proposed: (1) direct deacylation of phospholipids, particularly phosphatidylcholine and phosphatidylethanolamine, by a Ca^{2+}-dependent phospholipase A_2; (2) phospholipase C-mediated release of 1,2-diacylglycerol from phosphoinositides followed by the sequential activation of diacyl- and monoacylglycerol lipases; and (3) phosphorylation of 1,2-diacylglycerol to phosphatidic acid and the activation of a phosphatidic acid-specific, Ca^{2+}-dependent phospholipase A_2.[81,218,302,392,397] The proportion of arachidonate derived from each of these is unclear, but the majority appears to be derived from the action of phospholipase A_2 on membrane phospholipids.

Arachidonic acid may be converted to prostaglandin H_2 in two separate steps catalyzed by prostaglandin endoperoxide synthase.[351,462,469] The cyclooxygenase and hydroperoxidase activities both reside in a single protein with a purified molecular weight of 70-72 kDa. The cyclooxygenase activity of prostaglandin endoperoxide synthase inserts two molecules of oxygen into arachidonate to yield the endoperoxide prostaglandin G_2, which is then reduced by the hydroperoxidase to the endoperoxide prostaglandin H_2. In platelets, the principle metabolite of prostaglandin H_2 is TxA_2 which is formed by the action of

thromboxane A synthase associated with the dense tubule membranes. Thromboxane A_2 is very unstable ($t_{\frac{1}{2}}$ = 30 seconds in aqueous solution) and spontaneously hydrolyses to its stable, but inactive metabolite, thromboxane B_2 (TxB_2). Alternative major metabolites are the 17-carbon 12L-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and the 3-carbon fragment malondialdehyde. In platelets, minute amounts of the endoperoxides are converted non-enzymatically to prostaglandin E_2, prostaglandin D_2 and prostaglandin $F_{2\alpha}$.

When arachidonic acid is added exogenously or liberated endogenously from platelets in response to a stimulus, it is converted by prostaglandin endoperoxide synthase to the endoperoxides prostaglandin G_2 and prostaglandin H_2 and by thromboxane A synthase to TxA_2. Platelets apparently possess one type of receptor that mediates the effects of both the endoperoxides and TxA_2.[65,318,429] Because the endoperoxides and TxA_2 are extremely labile, various stable analogues have been synthesized and used to investigate the effects of these compounds on platelets. Like endoperoxides and TxA_2, stable prostaglandin endoperoxide analogues bind to specific receptors on the platelet surface. This induces activation of phospholipase C and phosphoinositide hydrolysis, which initiates the platelet responses of shape change, aggregation and secretion.[445]

Irreversible inhibition of platelet cyclooxygenase by acetylsalicylic acid (aspirin) prevents secondary aggregation and granule secretion in response to weak agonists such as ADP and low concentrations of PAF, A23187 and thrombin. The secretion of dense granule contents from

control and affected canine platelets has been shown to be inhibited by incubation with aspirin. This suggests that both groups are able to synthesize TxA_2, but does not give any information on the quantity or rate of TxA_2 production. Inhibition of dense granule release from affected platelets appeared to be more sensitive to the effects of aspirin than control platelets. The abnormally rapid release of dense granule adenosine triphosphate (ATP) from affected platelets stimulated by ADP was markedly inhibited by aspirin, which suggests a relationship between TxA_2 production and this abnormal rate of production. Measurement of TxA_2 production by control and affected platelets was indicated to examine the relationship, if any, between the responses of both groups and TxA_2 production.

Because of the instability of TxA_2, bioassay is the only way that TxA_2 itself can be assayed.[162] Thromboxane A_2 is potent vasoconstrictor and this property can be used in a bioassay. Although lack of specificity limits the use the bioassay, it does provide information about the kinetics or formation and disappearance of the compound. The alternative is to measure TxB_2, the stable metabolite of TxA_2. This provides more information on the total thromboxane production, but less information on the kinetics of production. The vast majority of quantitative studies in this area measure TxB_2 by radioimmunoassay.

MATERIALS AND METHODS

Experimental Design

The control group consisted of the two resident Basset hounds (female) and one male Basset hound. The male was on loan from a breeder and was available for most, but not all, of this project. In the last part of this project, a normal Labrador x Husky female with normal aggregation responses was used and found to have very similar responses to the control Bassets. The affected group consisted of four (3 female and 1 male) Basset hounds that have been used throughout the project.

Baseline samples were obtained by stirring platelet rich plasma or gel-filtered platelets at 37.5°C in the aggregometer for approximately 30 seconds prior to the addition of 20 μl of 0.9% w/v NaCl. This measured the approximate concentration of TxB_2 present in the cuvette at the time of addition of an agonist. Two sample times were selected based on the aggregation responses of control platelets, one representing the initial stages of aggregation and the other representing complete or near-complete aggregation. A third sample time was added for 0.1 and 0.3 U/ml thrombin because of the prolonged aggregation response of affected platelets to this agonist. The agonists and concentrations selected as well as the time at which TxB_2 levels were measured are summarized in Table 17.

Table 17. Agonist concentrations and times at which TxB_2 was measured.

Medium	Agonist	Concentration	Time (minutes)
PRP	ADP	10 μM	0, 1 & 5
PRP	PAF	0.1 μM	0, 1 & 5
		1.0 μM	0, 1 & 5
PRP	A23187	15 μM	0, 1 & 5
		20 μM	0, 1 & 5
PRP	PMA	3.0 μM	0, 5 & 12
PRP	Arachidonic acid	0.5 mM	0, 1 & 5
		1.0 mM	0, 1 & 5
GFP	Thrombin	0.05 U/ml	0, 1 & 5
		0.1 U/ml	0, 1, 5 & 10
		0.3 U/ml	0, 1, 5 & 10

PRP = platelet rich plasma, GFP = gel-filtered platelets

The initial results of this study indicated that it would be useful to measure true "resting" TxB_2 levels in non-stirred samples as well as examine the effect of stirring in the absence of an agonist. To obtain resting TxB_2 levels, 20 μl of 0.9% w/v NaCl was added to non-stirred platelet rich plasma samples at room temperature or 37°C. To examine the effect of stirring on TxB_2 production, platelet rich plasma was allowed to stir in the aggregometer cuvette for one, five or twelve minutes before the addition of 20 μl of NaCl.

All agonists, except arachidonic acid, were used on two or more separate occasions in each dog. Arachidonic acid was used once with

each dog. The other exception was the male control dog which was not available for a second sample collection for all agonists. Each sample was assayed at least twice and the average used as the response of that individual for that collection. For example, n = 8 for most of the affected results represents eight average values from four dogs which had each been sampled on two separate occasions. For each collection from each dog, either one or two baseline samples, as defined above, were measured. The average baseline value for each individual was used to calculate the increase above baseline in response to an agonist for that individual for that collection. There was considerable variation in the same dog from one collection to another, as well as individual variation in the response to different agonists.

Reagents

Thrombin, ADP, PAF, phorbol myristate acetate (PMA) and calcium ionophore A23187 were purchased, stored and prepared as described previously (Page 131). Arachidonic acid was purchased from the Sigma Chemical Co. (St. Louis, MO), reconstituted in hexane (50 mg/ml) and stored at -20°C in a 1.0 ml volumetric flask. At the time of use, the required amount was removed, dried under nitrogen, then reconstituted in 0.1 M Tris, pH 8.0 and kept on ice. Anti-thromboxane B_2 was purchased from Advanced Magnetics (Cambridge, MA) and stored at -20°C until reconstituted in 0.1 M phosphate buffered saline, pH 7.4, containing 0.5% gelatin (PBSG). Standard TxB_2 was purchased from Advanced Magnetics, dissolved in ethanol (1.0 mg/ml) and stored at -20°C. This stock was then further diluted to 1000 ng/ml in PBSG and

aliquots frozen at -70°C. ^{3}H-labelled TxB_2 trace was purchased from New England Nuclear and diluted to a total volume of 1.0 ml in distilled water:acetonitrile at a ratio of 9:1 and stored at -20°C. RIA-Solve II scintillation fluid was purchased from Research Products Inc. (Mt. Prospect, IL). Dextran T-70 was purchased from Pharmacia (Piscataway, NJ) and charcoal (Norit A) from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade.

Preparation of Platelets and Thromboxane Samples

Platelets were collected and prepared for aggregation as described previously (Pages 132-134). Briefly, blood for the preparation of platelet rich plasma was collected from fasted dogs via an 18 gauge needle into 3.2% Na_3 citrate at a ratio of 9:1. Platelet rich plasma was prepared by centrifugation and the platelet count standardized to 3.0 x 10^8/ml with autologous platelet poor plasma. All platelet counts were done manually using a Unopette and hemocytometer. Platelets were allowed to rest for at least 30 minutes before starting aggregations.

Blood for the preparation of gel-filtered platelets was collected via an 18 gauge needle into 3.8% Na_3 citrate at a ratio of 9:1. Platelet rich plasma was prepared, 1.0 μM PGE_1 added, and the platelet rich plasma allowed to rest for 10 minutes before centrifugation at 800 x g for 15 minutes. The supernatant was discarded and the platelet pellet gently resuspended in 1.0 ml of Ca^{2+}-Mg^{2+}-free HEPES-Tyrodes containing 0.3% bovine serum albumin (HTA). The platelets were then gel-filtered over a Sepharose 4B column equilibrated with HTA, counted and diluted

to 3.0 x 10^8/ml with HTA. 1.0 mM $CaCl_2$ and 0.8 mM $MgCl_2$ were added and the platelets allowed to rest for at least 30 minutes before starting aggregations.

The majority of aggregations for the collection of thromboxane samples were done in a Lumi-aggregometer (Chronolog Corporation, Havertown, PA). Aggregations for the first collection from each dog were done in a Platelet Ionized Calcium Aggregometer (PICA, Chronolog Corporation, Havertown, PA) because the Lumi-aggregometer was not available.

For the collection of samples for TxB_2 radioimmunoassay, platelet rich plasma or gel-filtered platelets were stirred at 37.5°C in the aggregometer for approximately 30 seconds prior to the addition of 20 µl of 0.9% w/v NaCl or agonist. At the predetermined time, the contents of the cuvette were immediately transferred to an Eppendorf tube and centrifuged at 11,600 x g for 1.5 minutes. The supernatant was immediately removed, divided into aliquots and stored at -70°C. Baseline and stirred samples without agonist were prepared in the same way. Non-stirred samples were prepared in the same fashion, without being stirred in the aggregometer.

Radioimmunoassay for Thromboxane B_2

Radioimmunoassay (RIA) is a useful method for determining relatively small quantities (pg-ng) of substances in biologic fluids. The method used here to measure TxB_2 was adapted from the procedure described by Fitzpatrick.[140] It is based on the principle of competitive binding: the unlabelled TxB_2 in the sample or standard competes with ^3H-labelled TxB_2 ("trace") for binding sites on a specific antibody (Ab).

The trace was diluted in PBSG to a concentration that gave a total count of 5000-5500 cpm in 100 μl. The antibody was reconstituted in the volume of PBSG that gave an antibody concentration in 100 μl that bound 50% (45-52%) of the trace in the absence of any unlabelled TxB_2. This was established by using serial dilutions of antibody with a fixed concentration of trace. Fresh PBSG was made each day from gelatin and stock 0.1 M PBS containing 1.5 mM sodium azide, pH 7.4.

Because all tubes in the assay must contain the same proportion of plasma and/or buffer, charcoal-stripped plasma that is free of TxB_2 was prepared for diluting standard and samples when plasma samples were being assayed. Charcoal-stripped plasma was prepared by treating a dog with aspirin daily for 10 days before collecting approximately 150 mls of blood. The plasma was separated and incubated in an ice-bath for 30 minutes before the addition of 0.1 volume of ice-cold concentrated dextran-coated charcoal containing 1.5 gm Norit A charcoal and 0.15 gm Dextran T-70 in 75 mls of 0.1 M PBS. After 11 $\pm$ 1 minutes, the tubes were centrifuged at 1300 x g for 20 minutes at 0°C to separate the

charcoal and any TxB_2 from the plasma. The supernatant charcoal-stripped plasma was aliquoted and stored at -70°C.

For plasma samples, the proportion of plasma to buffer in all tubes was kept constant by initially diluting all samples in HTA to the lowest dilution used in the assay, for example 1/5. Medium containing 1/5 charcoal-stripped plasma to HTA was then used in all further dilutions of samples. The standard curve was set up by further diluting the 1000 ng/ml TxB_2 stock in either medium (for plasma samples) or HTA (for gel-filtered samples) to give a standard curve containing 9 duplicate points from 3.05 pg/100 µl to 780.0 pg/100 µl. Total count (TC), maximum binding (B_o) and non-specific binding (NSB) tubes also contained the same proportion of medium or HTA.

Duplicate tubes for each standard (100 µl each of standard, trace and Ab), sample (100 µl each of diluted sample, trace and Ab), TC and NSB (100 µl each of medium/HTA, trace and PBSG) and four tubes for B_o (100 µl each of medium/HTA, trace and Ab) were incubated at room temperature for one hour then at 4°C for 20-24 hours. The tubes were then incubated in an ice-bath for one hour before the addition to each tube of 1.0 ml of ice-cold dextran-coated charcoal containing 0.5% w/v charcoal and 0.05% w/v Dextran T-70 in 0.1 M PBS. After an incubation of precisely 11 ± 1 minutes, the tubes were centrifuged at 1300 x g for 20 minutes at 0°C to separate the charcoal and any unbound TxB_2 from the bound TxB_2 (labelled and unlabelled) which remained in the supernatant. Aliquots (1.0 ml) of the supernatant were added to scintillation vials containing 10 mls of RIA-Solve 11 and counted in a

Beckman LS 9000 counter for five minutes using the logit transformation in the RIA data reduction program.

The samples were diluted such that they fell on the straight part of a % bound versus ln standard concentration curve. The correct dilutions were found by trial and error. Values which did not fall on the straight part of the standard curve were discarded because of poor precision. The following parameters provided by the RIA data reduction program were recorded for each run: total count TC (cpm), non-specific binding NSB (cpm), maximum binding B_o (cpm), % bound for each standard point, slope, intercept, correlation (r), % NSB/TC, % Bo/TC, pipetting precision %, concentration at 95% B_o, and concentration at 50% B_o. Any run in which these deviated from the expected range was discarded.

Statistical Analysis

Results were entered into a statistics and graphics program (Statgraphics, STSC, Rockville, MD.) When normal distribution plots were run on sample groups many, but not all, groups were normally distributed. For this reason, as well as the relatively small sample sizes involved, the control and affected groups were compared using the Mann-Whitney non-parametric test for independent samples, $p < 0.05$. For comparison within control and affected groups of TxB_2 production at the selected time points, a one-way analysis of variance using Tukey's test ($p < 0.05$) for multiple comparisons was used. Although this is a parametric test, reasonable departures from normality do not affect the validity of the results.

RESULTS

Resting and Baseline Values

Baseline TxB_2 concentrations obtained by stirring platelet rich plasma in the aggregometer for 0.5 minute before the addition of 20 μl 0.9% w/v NaCl were significantly higher in the affected group than in the control group. The PICA aggregometer was used for the first group of platelet rich plasma samples collected from both groups and the Lumi-aggregometer used for the remainder of the study. Theoretically, these two machines should give identical results, but a difference between the two machines was noted in the baseline TxB_2 concentrations in the affected group. Within the affected group, the baseline TxB_2 concentrations measured in platelet rich plasma stirred in the PICA aggregometer were higher than those stirred in the Lumi-aggregometer. There was no difference in TxB_2 concentrations measured in response to the agonists used. The baseline TxB_2 concentrations measured in the Lumi-aggregometer were still significantly higher in the affected group than in the control group and results from the two machines have been pooled together in the one group. The control group was not affected by the aggregometer used.

Later in the study, TxB_2 production in platelet rich plasma stirred for 0.5, 5.5 and 12.5 minutes and in non-stirred platelet rich plasma at room temperature and 37.5°C was measured. These results are summarized in Table 18 and Figure 69. It should be noted that the stirred 0.5 minute sample is the same as that referred to as the

baseline sample throughout the rest of the study. The affected group had significantly higher TxB_2 concentrations at all times measured except 5.5 minutes, but within each group there was no significant effect of stirring. Thromboxane B_2 production is expressed in ng/ml of platelet suspension in which the concentration was 3.0×10^8/ml.

In contrast, the baseline TxB_2 concentration in gel-filtered platelets was higher than the baseline level in platelet rich plasma in both groups and did not differ significantly between the two groups.

Table 18. TxB_2 concentration (ng/ml) in non-stirred and stirred non-stimulated control and affected platelet suspensions.

	Control TxB_2 (ng/ml)	Affected TxB_2 (ng/ml)
Platelet rich plasma		
Non-stirred (room temperature)	0.32 ± 0.18 (4)	4.49 ± 2.24 (4)*
Non-stirred (37°C)	1.26 ± 0.55 (4)	6.16 ± 1.38 (4)*
0.5 minute (baseline)	1.31 ± 0.98 (19)	9.20 ± 3.87 (28)*
5.5 minutes	2.21 ± 2.58 (4)	11.55 ± 7.97 (4)
12.5 minutes	1.33 ± 0.49 (4)	7.49 ± 1.51 (4)*
Gel-filtered platelets		
0.5 minute (baseline)	16.12 ± 2.73 (15)	15.30 ± 2.14 (20)

Values = mean ± SD, (n) = number of samples, * = significantly different from control value ($p < 0.05$).

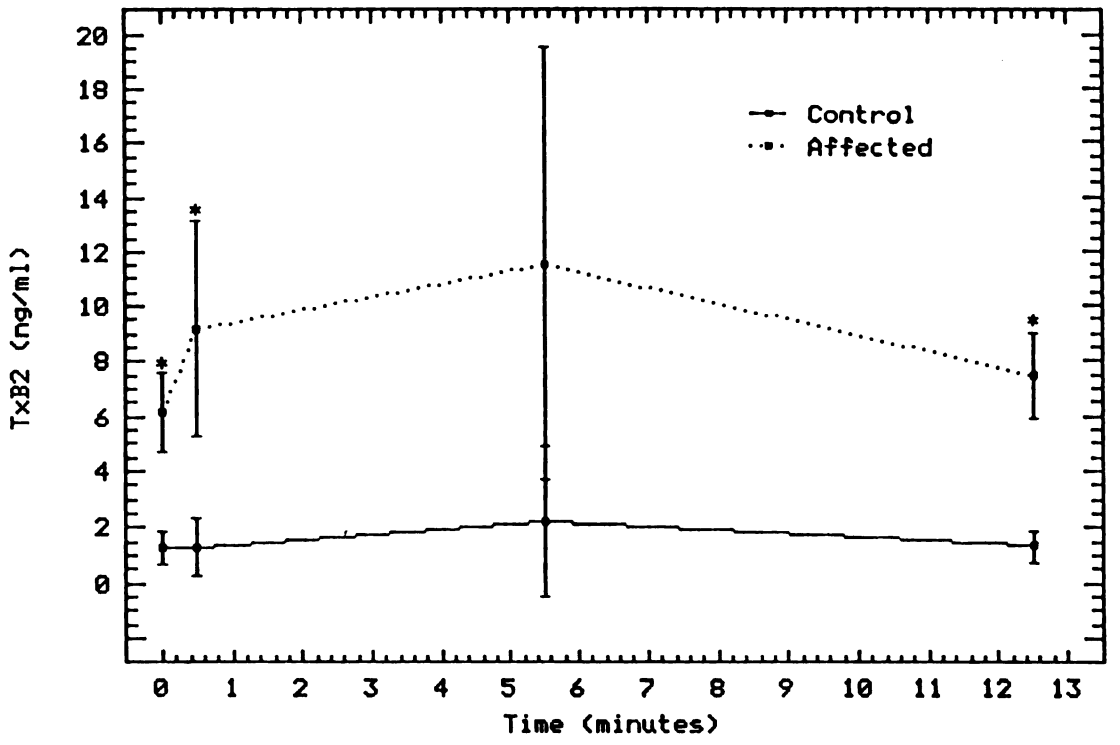


Figure 69. TxB_2 concentration in non-stirred (0 minute) and stirred (0.5, 5.5 and 12.5 minutes) control and affected platelet rich plasma at 37.5°C.

* = control and affected groups significantly different, ($p < 0.05$).

Adenosine Diphosphate

Although affected platelets failed to aggregate in response to 10 μM ADP, they produced significantly more TxB_2 than control platelets under the same conditions. Within the control group, the TxB_2 concentrations measured and the elevation above baseline in response to 10 μM ADP were significantly increased at five minutes, but not at one minute. In contrast, the TxB_2 concentrations measured and the elevation above

baseline of the affected group were significantly elevated at both one and five minutes. This suggests that the affected platelets were able to produce greater amounts of TxB_2 more rapidly than control platelets. These results are summarized in Table 19 and Figures 70 and 71.

Table 19. TxB_2 concentration (ng/ml) in control and affected platelet rich plasma stimulated by 10 μM ADP.

	Post-Agonist (minutes)	Control TxB_2 (ng/ml)	Affected TxB_2 (ng/ml)
Baseline	0	1.31 ± 0.98 (19)	9.20 ± 3.87 (28)*
TxB_2 measured	1	2.29 ± 0.54 (5)	37.97 ± 21.91 (8)*#
	5	4.58 ± 2.39 (5)#	38.61 ± 16.72 (8)*#
Increase above baseline	0	0	0
	1	0.92 ± 0.66 (5)	27.24 ± 20.41 (8)*#
	5	3.12 ± 2.51 (5)#	27.87 ± 16.06 (8)*#

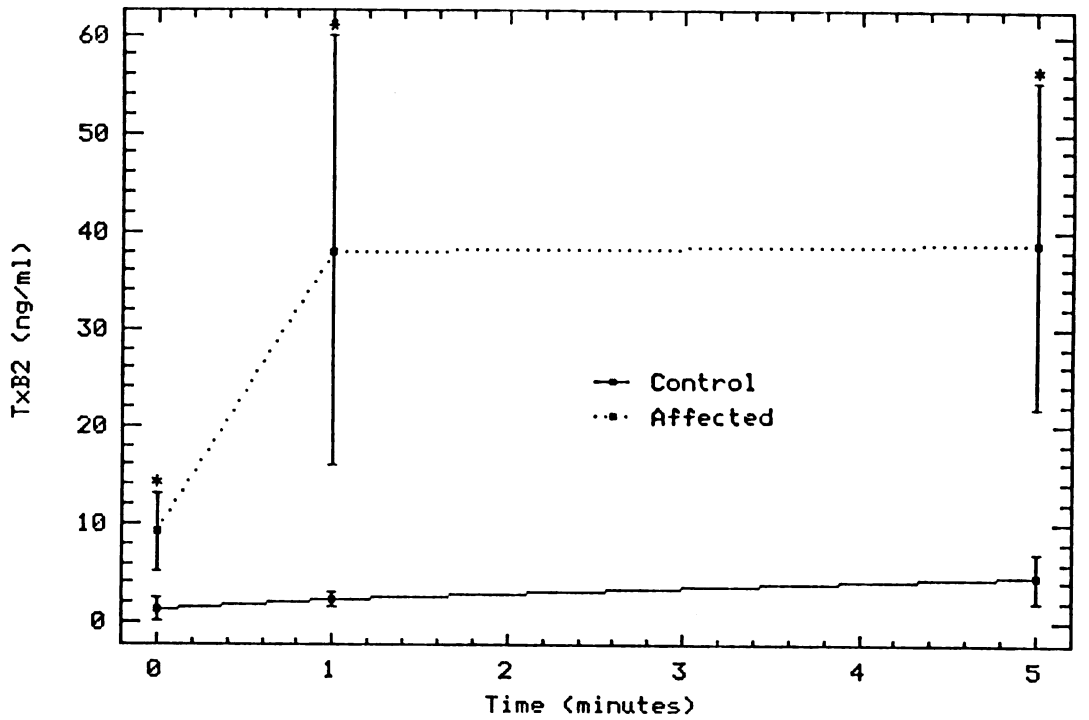
Values = mean ± SD, (n) = number of samples, * = significantly different from control value ($p < 0.05$), # = significantly different from baseline ($p < 0.05$).

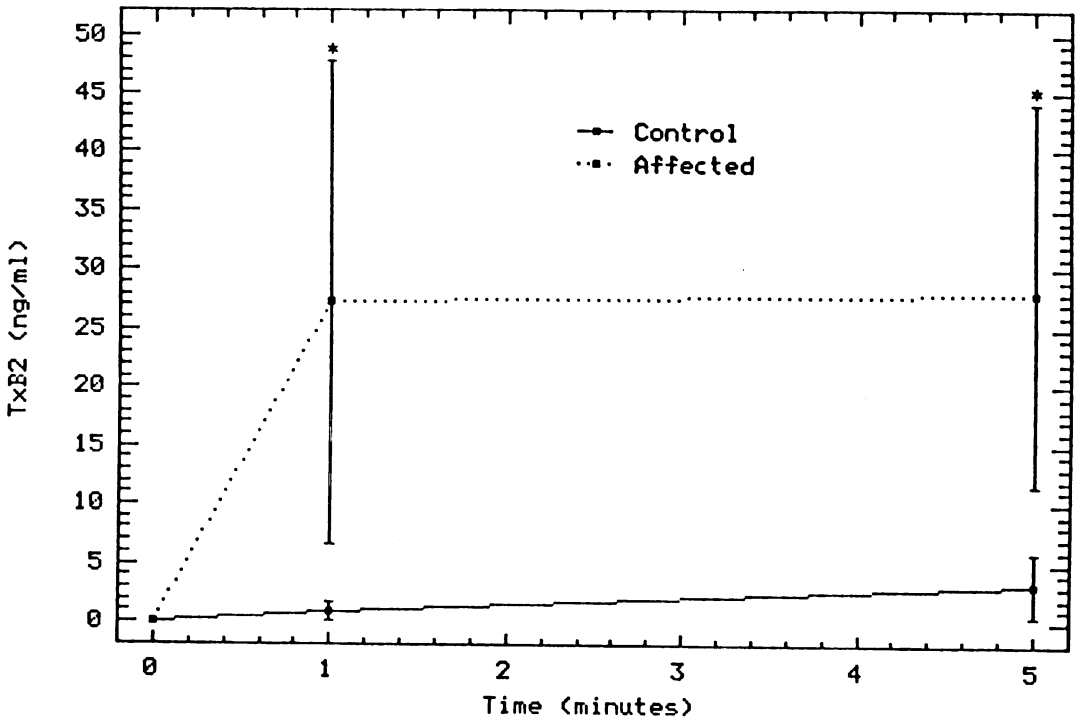
Figure 70. TxB_2 concentration (ng/ml) in control and affected platelet rich plasma stimulated by 10 μM ADP.

* = control and affected groups significantly different, ($p < 0.05$).

Figure 71. Increase in TxB_2 concentration (ng/ml) above the baseline in control and affected platelet rich plasma stimulated by 10 μM ADP.

* = control and affected groups significantly different, ($p < 0.05$).


TxB2 (ng/ml)
60
50
40
30
20
10
0
0
1
2
3
4
5
Time (minutes)
Control
Affected


TxB2 (ng/ml)
50
45
40
35
30
25
20
15
10
5
0
0
1
2
3
4
5
Time (minutes)
Control
Affected

Platelet Activating Factor

Production of TxB_2 was measured in response to 1.0 and 0.1 μM PAF because the aggregation and secretion responses of affected platelets to these two concentrations were quite different. There was rapid secretion of dense granule ATP in response to 1.0 μM PAF, but failure of secretion in response to 0.1 μM. In contrast, platelets from the controls used in this study secreted in response to both concentrations. Interestingly, the occurrence of dense granule secretion correlated with the production of TxB_2.

When control platelets were stimulated by 0.1 μM PAF, TxB_2 production measured was significantly increased above baseline concentration at one minute with a slight, further increase by five minutes. When the increase above baseline was examined, the control group TxB2 was significantly increased by five minutes, but not at one minute. TxB_2 production by the affected group was not significantly increased when either the concentration measured or the increase above baseline were analyzed. There was no significant difference in the concentrations measured between the two groups at one and five minutes, but the elevations above baseline were significantly higher in the control group. These results are summarized in Table 20 and Figures 72 and 73.

Table 20. TxB_2 concentration (ng/ml) in control and affected platelet rich plasma stimulated by 0.1 μM PAF.

	Post-Agonist (minutes)	Control TxB_2 (ng/ml)	Affected TxB_2 (ng/ml)
Baseline	0	1.31 ± 0.98 (19)	9.20 ± 3.87 (28)*
TxB_2 measured	1	13.74 ± 13.02 (5)#	12.47 ± 3.11 (8)
	5	18.15 ± 9.45 (5)#	9.62 ± 1.14 (8)
Increase above baseline	0	0	0
	1	12.34 ± 13.05 (5)	1.73 ± 1.93 (8)*
	5	16.84 ± 9.59 (5)#	1.04 ± 1.61 (8)*

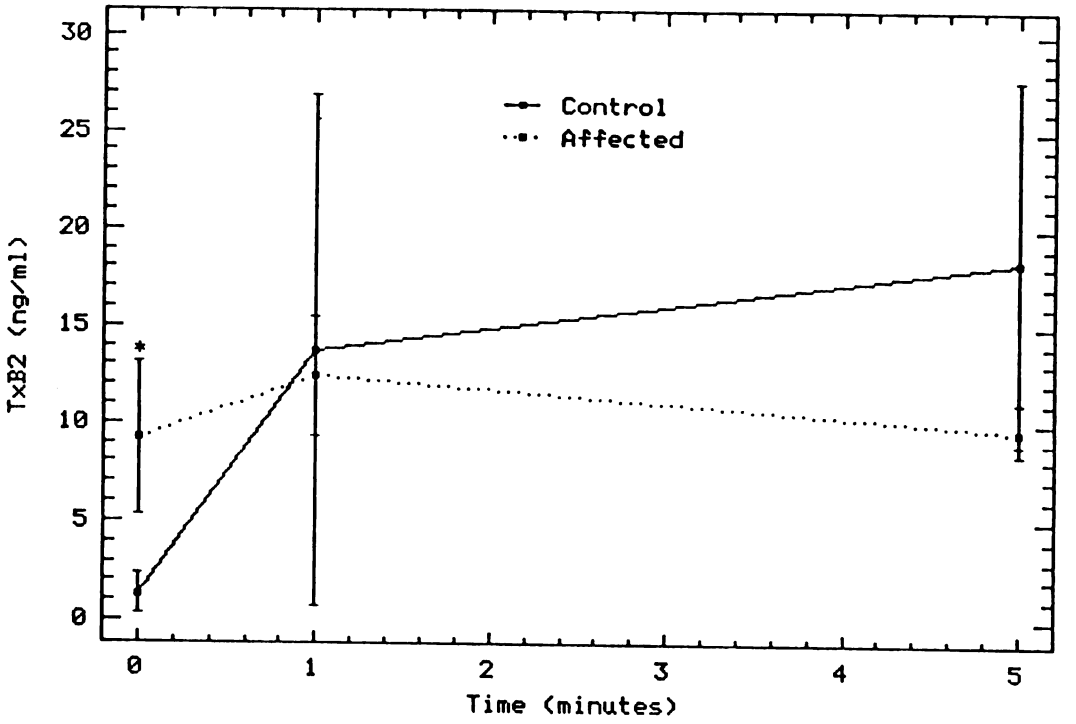
Values = mean ± SD, (n) = number of samples, * = significantly different from control value ($p < 0.05$), # = significantly different from baseline ($p < 0.05$).

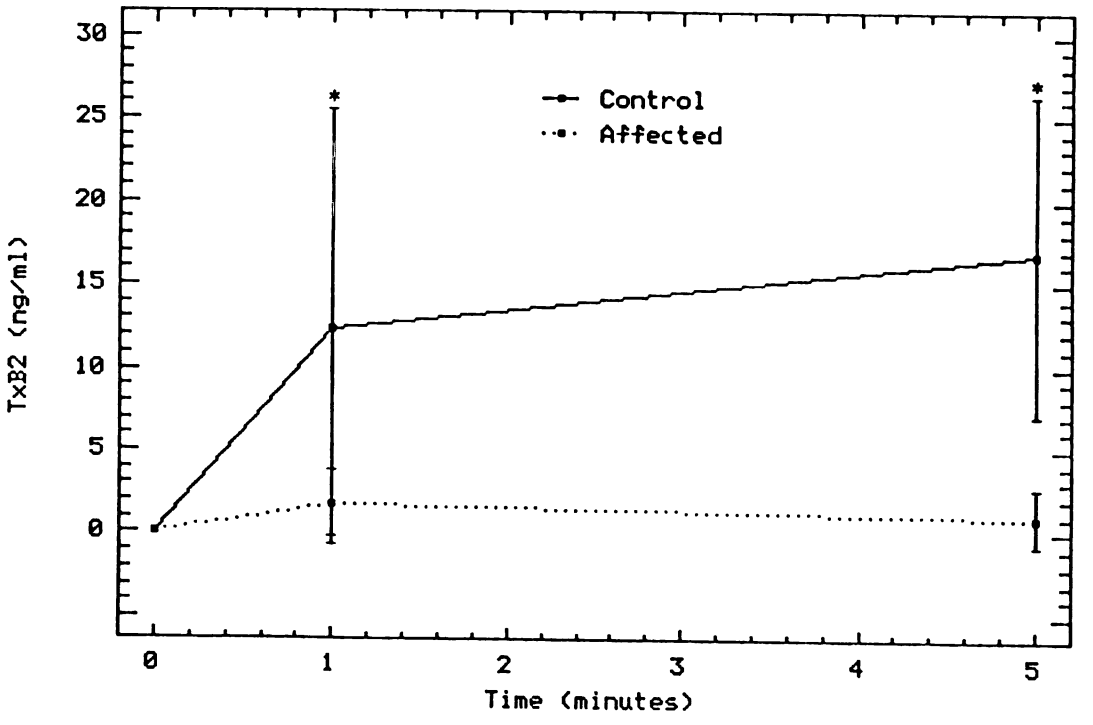
Figure 72. TxB_2 concentration (ng/ml) in control and affected platelet rich plasma stimulated by 0.1 μM PAF.

* = control and affected groups significantly different, ($p < 0.05$).

Figure 73. Increase in TxB_2 concentration (ng/ml) above the baseline in control and affected platelet rich plasma stimulated by 0.1 μM PAF.

* = control and affected groups significantly different, ($p < 0.05$).


30
25
20
15
10
5
0
TxB2 (ng/ml)
Control
Affected
*
0
1
2
3
4
5
Time (minutes)


30
25
20
15
10
5
0
TxB2 (ng/ml)
Control
Affected
*
*
0
1
2
3
4
5
Time (minutes)

Both control and affected platelets stimulated by 1.0 μM PAF had significantly increased TxB_2 production at one and five minutes when both the concentration measured and increase above baseline were analyzed. Within both groups, there was no significant difference between the one and five minute concentrations. However, the affected group produced significantly more TxB_2 than the control group at both one and five minutes. These results are summarized in Table 21 and Figures 74 and 75.

Table 21. TxB_2 concentration (ng/ml) in control and affected platelet rich plasma stimulated by 1.0 μM PAF.

	Post-Agonist (minutes)	Control TxB_2 (ng/ml)	Affected TxB_2 (ng/ml)
Baseline	0	1.31 ± 0.98 (19)	9.20 ± 3.87 (28)*
TxB_2 measured	1	29.72 ± 10.55 (5)#	93.83 ± 44.52 (8)*#
	5	35.59 ± 16.98 (5)#	83.06 ± 47.12 (8)*#
Increase above baseline	0	0	0
	1	28.31 ± 10.67 (5)#	83.10 ± 41.91 (8)*#
	5	34.18 ± 17.07 (5)#	72.32 ± 44.96 (8)*#

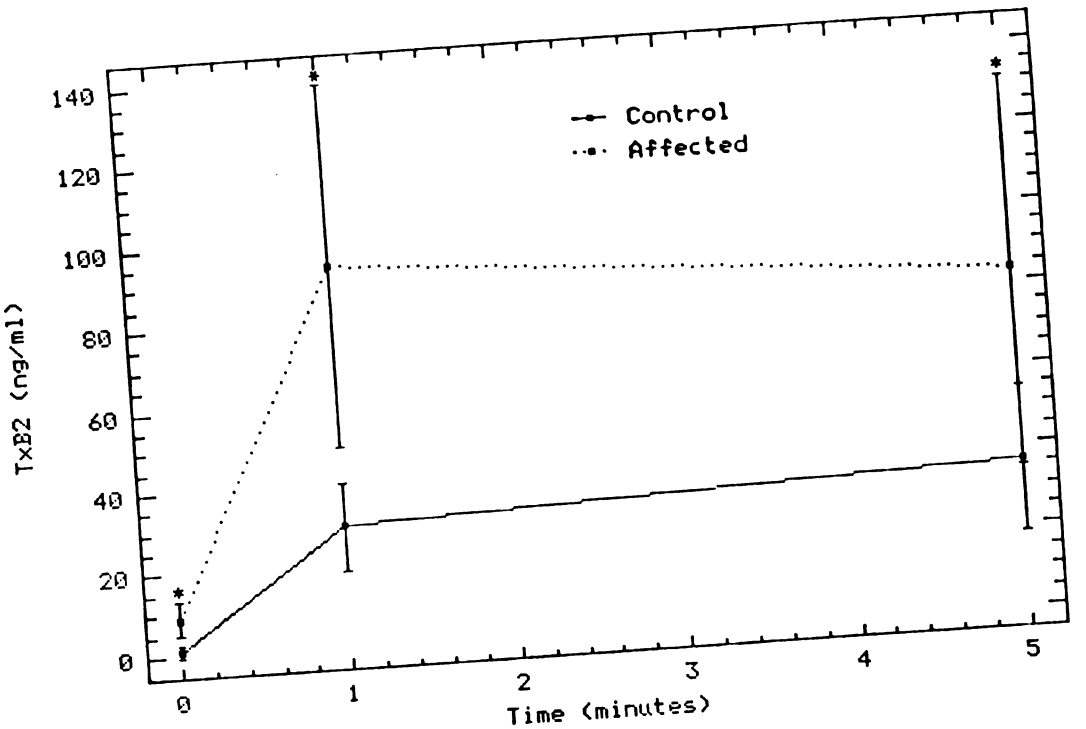
Values = mean ± SD, (n) = number of samples, * = significantly different from control value ($p < 0.05$), # = significantly different from baseline ($p < 0.05$).

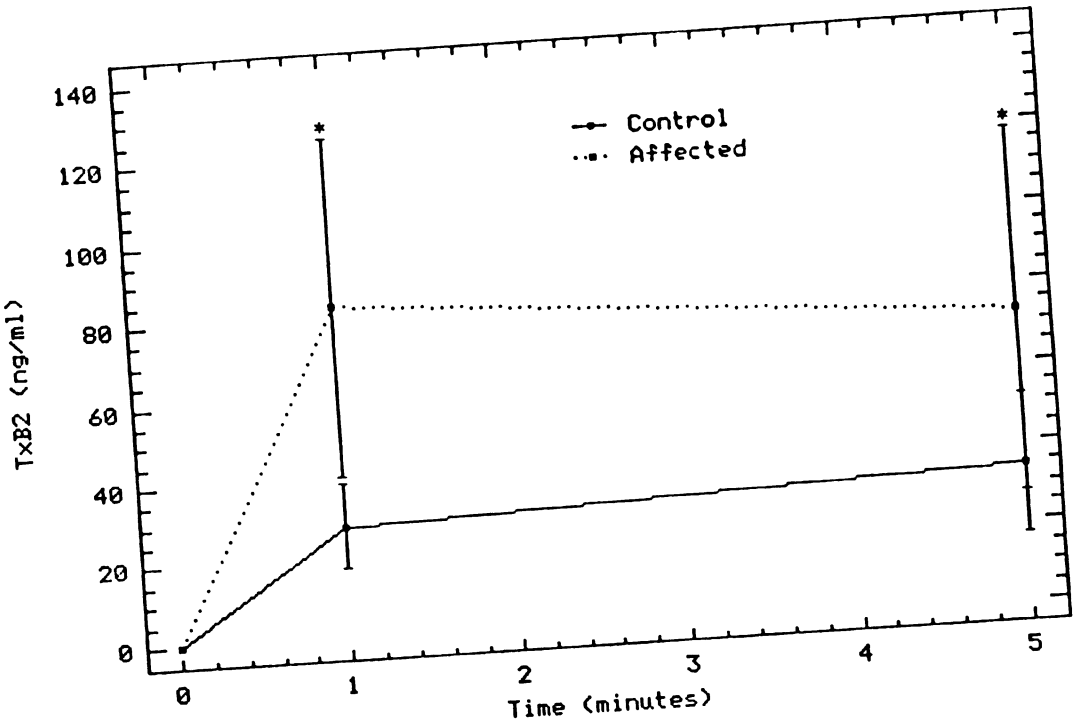
Figure 74. TxB_2 concentration (ng/ml) in control and affected platelet rich plasma stimulated by 1.0 μM PAF.

* = control and affected groups significantly different, ($p < 0.05$).

Figure 75. Increase in TxB_2 concentration (ng/ml) above the baseline in control and affected platelet rich plasma stimulated by 1.0 μM PAF.

* = control and affected groups significantly different, ($p < 0.05$).


Control
Affected
TxB2 (ng/ml)
Time (minutes)
0
20
40
60
80
100
120
140
0
1
2
3
4
5


Control
Affected
TxB2 (ng/ml)
Time (minutes)
0
20
40
60
80
100
120
140
0
1
2
3
4
5

Thrombin

In contrast to platelet rich plasma, the baseline TxB_2 production for gel-filtered platelets was not significantly different between the two groups. Thromboxane B_2 production in response to 0.05 U/ml thrombin was very variable from one individual to the other, but the occurrence of platelet aggregation was associated with TxB_2 production. The majority of times, aggregation did not occur with this concentration of thrombin and TxB_2 production was minimal. It was noted that on the one occasion that an affected dog had aggregated measurably by five minutes (10.7%), the increase above baseline TxB_2 production was 144.25 ng/ml. In contrast, stronger aggregation responses (69.8% and 19.3%) of control platelets were associated with much smaller increases above baseline in TxB_2 production of 16.46 and 11.63 ng/ml respectively. Both the levels measured and the increase above baseline were significantly greater in the affected group at one minute. Because of the great individual variation and large standard deviations, it is difficult to make any general interpretations from the data that is summarized in Table 22 and Figures 76 and 77. However, it does appear that aggregation by affected platelets in response to 0.05 U/ml thrombin is associated with greater TxB_2 formation than in control platelets.

Table 22. TxB_2 concentration (ng/ml) in control and affected gel-filtered platelets stimulated by 0.05 U/ml thrombin.

	Post-Agonist (minutes)	Control TxB_2 (ng/ml)	Affected TxB_2 (ng/ml)
Baseline	0	16.12 ± 2.73 (15)	15.30 ± 2.14 (20)
TxB_2 measured	1	17.77 ± 4.46 (6)	38.80 ± 33.57 (8)*
	5	22.17± 8.04 (6)#	37.38 ± 48.54 (8)
Increase above baseline	0	0	0
	1	1.76 ± 2.54 (6)	23.13 ± 34.45 (8)*
	5	5.85 ± 6.67 (6)	21.71 ± 49.59 (8)

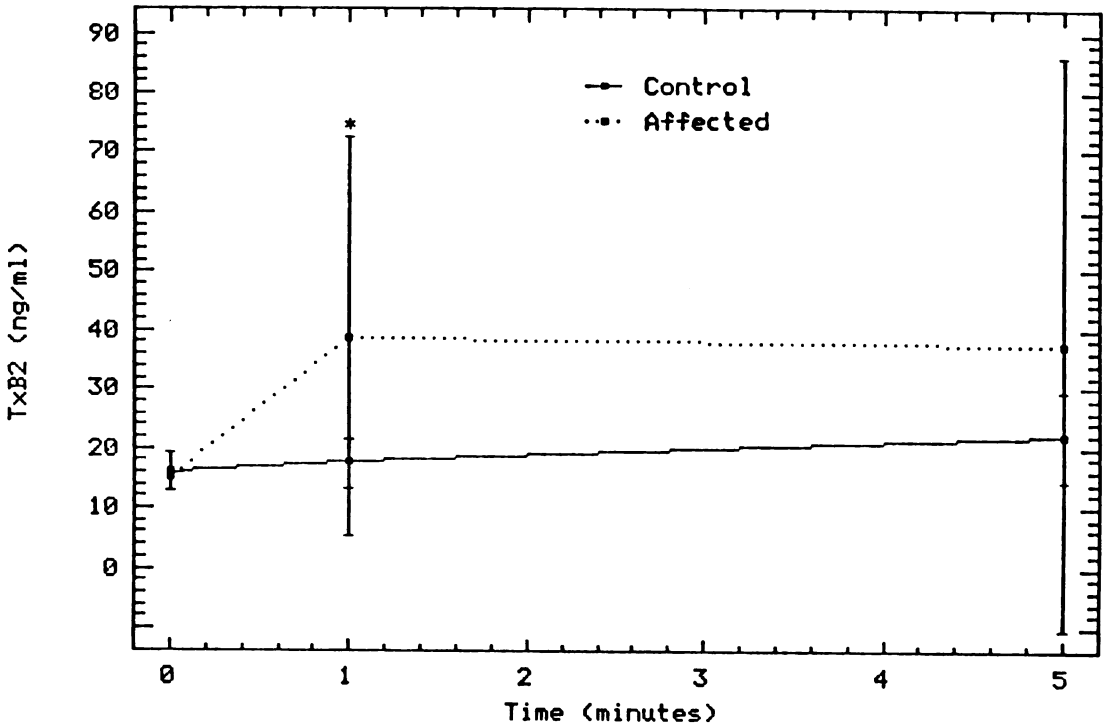
Values = mean ± SD, (n) = number of samples, * = significantly different from control value ($p < 0.05$), # = significantly different from baseline ($p < 0.05$).

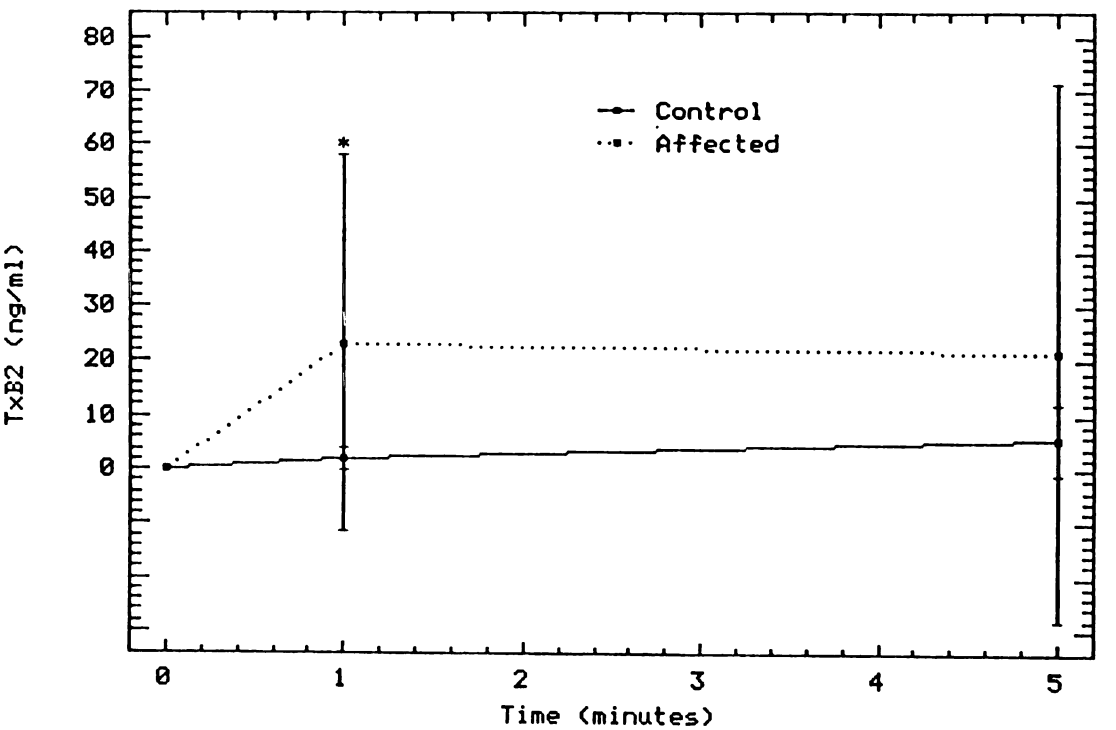
Figure 76. TxB_2 concentration (ng/ml) in control and affected gel-filtered platelets stimulated by 0.05 U/ml thrombin.

* - control and affected groups significantly different, ($p < 0.05$).

Figure 77. Increase in TxB_2 concentration (ng/ml) above the baseline in control and affected gel-filtered platelets stimulated by 0.05 U/ml thrombin.

* - control and affected groups significantly different, ($p < 0.05$).


90
80
70
60
50
40
30
20
10
0
TxB2 (ng/ml)
Control
Affected
*
0
1
2
3
4
5
Time (minutes)


80
70
60
50
40
30
20
10
0
TxB2 (ng/ml)
Control
Affected
*
0
1
2
3
4
5
Time (minutes)

When control platelets were stimulated by 0.1 U/ml thrombin, TxB_2 production was quite variable. All controls aggregated in response to 0.1 U/ml thrombin with aggregation being complete, or near completion, by five minutes. There was no significant relationship between the percent aggregation measured at five minutes and the increase in TxB_2 production above baseline ($r = 0.48$ by regression analysis).

Affected platelets were variable in their response to 0.1 U/ml thrombin and did not always aggregate. Even when they did aggregate, the response was far from complete at five minutes because of the initial very slow rate of aggregation of affected platelets. Thromboxane B_2 production by affected platelets was also very variable, but there was a significant positive correlation between the percent aggregation measured at five minutes and the amount of TxB_2 produced ($r = 0.90$ by regression analysis). In contrast to 0.05 U/ml thrombin, affected platelets which had not aggregated by five minutes (5/12) still produced quite large amounts of TxB_2 (21.89 - 89.88 ng/ml).

Because of the variable occurrence and slow onset of aggregation of affected platelets and apparent relationship between the quantity of TxB_2 produced and the extent of aggregation, TxB_2 production at 10 minutes was measured in both groups. The increase in TxB_2 production above baseline at 10 minutes in the control group was significantly higher than at five minutes. The response of the affected group was very variable with only 1/4 dogs responding with measurable aggregation. In this individual, the increase above baseline TxB_2 was 325.05 ng/ml at five minutes (35.0% aggregation) and 240.64 at 10

minutes (64.0% aggregation). At one minute, affected platelets produced significantly more TxB_2 than the controls, but there was no difference at the other times measured. However, in spite of the similar TxB_2 production, there was a marked difference in the aggregation responses of the two groups. These results are summarized in Table 23 and Figures 78 and 79.

Table 23. TxB_2 concentration (ng/ml) in control and affected gel-filtered platelets stimulated by 0.1 U/ml thrombin.

	Post-Agonist (minutes)	Control TxB_2 (ng/ml)	Affected TxB_2 (ng/ml)
Baseline	0	16.12 ± 2.73 (15)	15.30 ± 2.14 (20)
TxB_2 measured	1	43.80 ± 23.86 (6)#	115.73 ± 74.02 (8)*#
	5	69.63 ± 35.48 (9)#	145.62 ± 111.33 (12)#
	10	112.32 ± 12.25 (3)#@	90.01 ± 105.50 (4)
Increase above baseline	0	0	0
	1	27.38 ± 22.65 (6)	100.06 ± 74.33 (8)*
	5	53.43 ± 35.26 (9)#	130.56 ± 111.28 (12)#
	10	96.58 ± 12.42 (3)#	76.17 ± 105.52 (4)

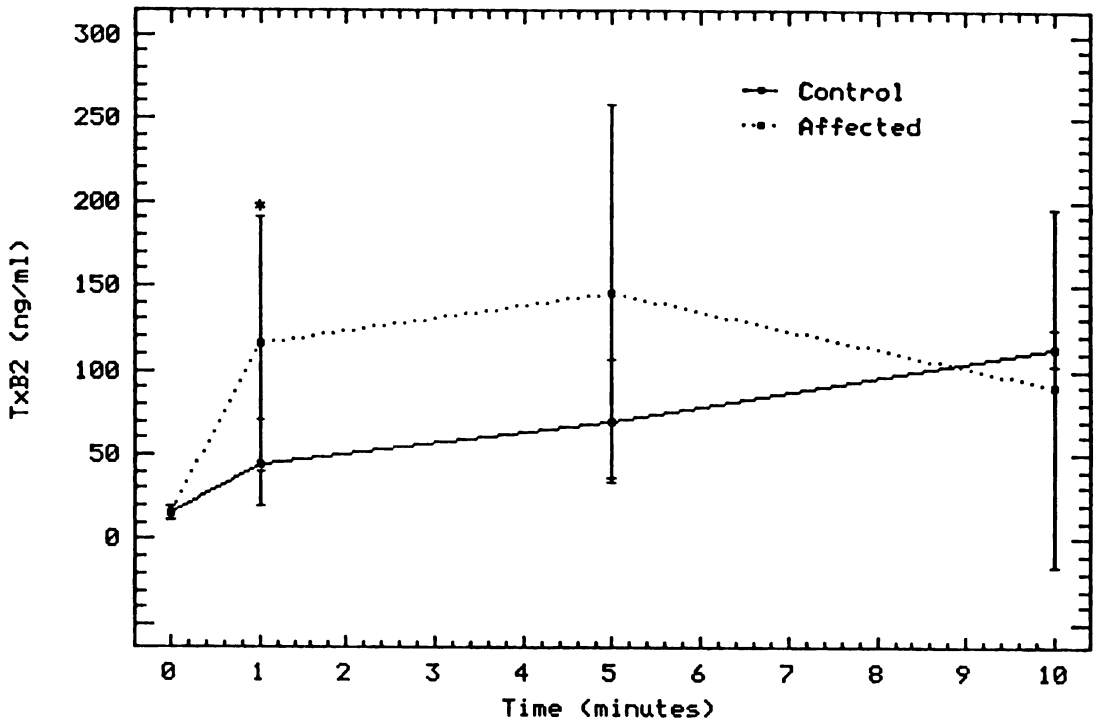
Values = mean ± SD, (n) = number of samples, * = significantly different from control value, # = significantly different from baseline ($p < 0.05$), @ = significantly different from "5 minutes" ($p < 0.05$).

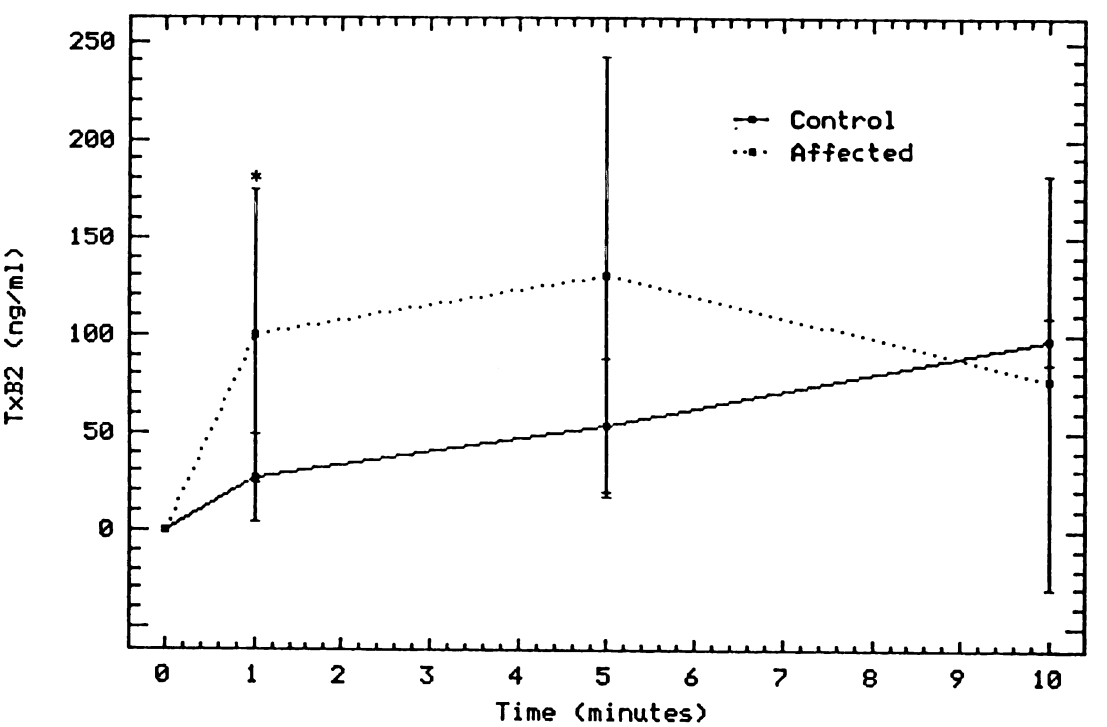
Figure 78. TxB_2 concentration (ng/ml) in control and affected gel-filtered platelets stimulated by 0.1 U/ml thrombin.

* = control and affected groups significantly different, ($p < 0.05$).

Figure 79. Increase in TxB_2 concentration (ng/ml) above the baseline in control and affected gel-filtered platelets stimulated by 0.1 U/ml thrombin.

* = control and affected groups significantly different, ($p < 0.05$).


300
250
200
150
100
50
0
TxB2 (ng/ml)
Control
Affected
*
0
1
2
3
4
5
6
7
8
9
10
Time (minutes)


250
200
150
100
50
0
TxB2 (ng/ml)
Control
Affected
*
0
1
2
3
4
5
6
7
8
9
10
Time (minutes)

Both control and affected platelets aggregated and produced TxB_2 in response to 0.3 U/ml thrombin. Affected platelets produced significantly more TxB_2 than controls both one and five minutes after the addition of agonist. In contrast to 0.1 U/ml thrombin, there was no significant relationship between percent aggregation at five minutes and TxB_2 production by affected platelets ($r = 0.51$ by regression analysis). Neither group produced significantly more TxB_2 at 10 minutes after the addition of 0.3 U/ml thrombin than they had at five minutes. While there was no significant difference between TxB_2 production by affected platelets at one, five or 10 minutes, control platelets produced more TxB_2 at five and 10 minutes than they had at one minute. These results are summarized in Table 24 and Figures 80 and 81.

Table 24. TxB_2 concentration (ng/ml) in control and affected gel-filtered platelets stimulated by 0.3 U/ml thrombin.

	Post-Agonist (minutes)	Control TxB_2 (ng/ml)	Affected TxB_2 (ng/ml)
Baseline	0	16.12 ± 2.73 (15)	15.30 ± 2.14 (20)
TxB_2 measured	1	130.85 ± 46.76 (6)#	331.69 ± 121.55 (8)*#
	5	238.37 ± 102.26(9)#@	441.44 ± 138.25 (12)*#
	10	280.45 ± 5.98 (3)#@	476.64 ± 194.52 (4)#
Increase above baseline	0	0	0
	1	114.43 ± 45.60 (6)#	316.02 ± 120.40 (8)*#
	5	222.14 ± 101.96 (9)#@	426.39 ± 138.01 (12)*#
	10	264.72 ± 6.47 (3)#@	462.74 ± 194.21 (4)#

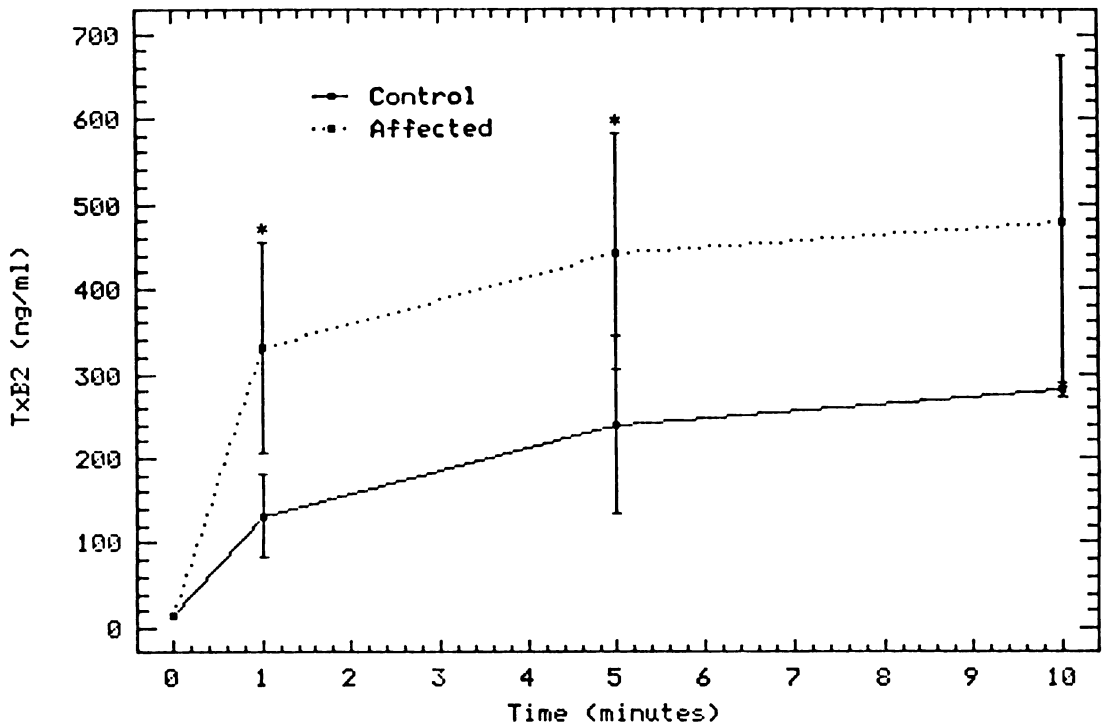
Values = mean ± SD, (n) = number of samples, * = significantly different from control value ($p < 0.05$), # = significantly different from baseline ($p < 0.05$), @ = significantly different from "1 minute" ($p < 0.05$).

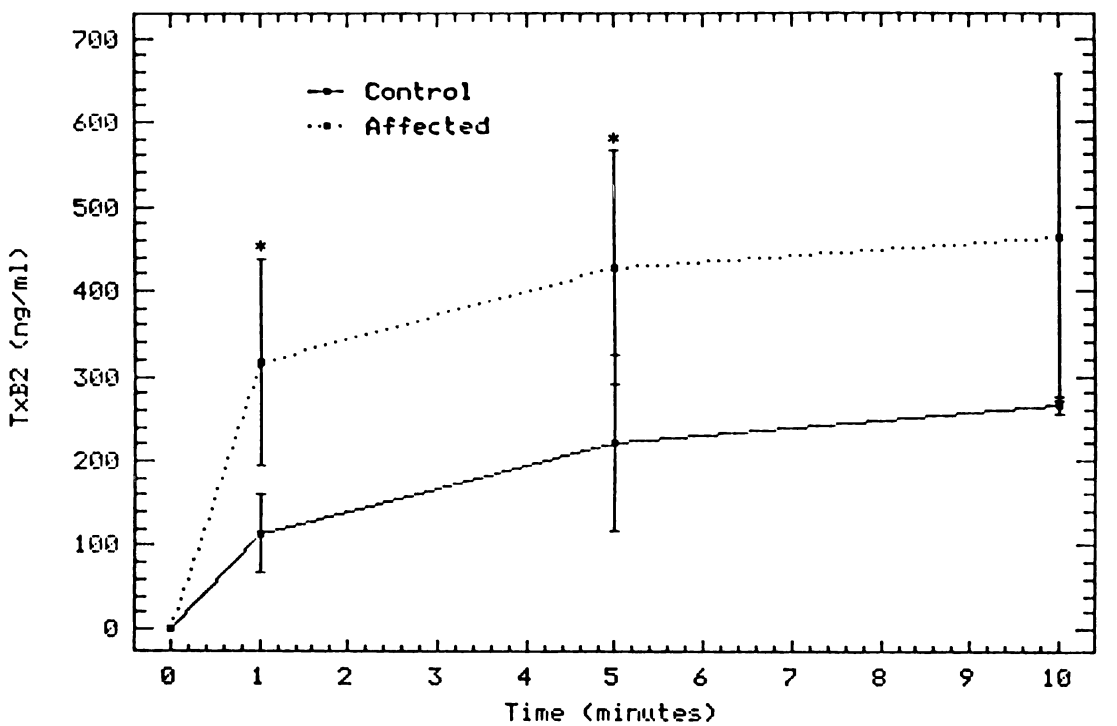
Figure 80. TxB_2 concentration (ng/ml) in control and affected gel-filtered platelets stimulated by 0.3 U/ml thrombin.

* = control and affected groups significantly different, ($p < 0.05$).

Figure 81. Increase in TxB_2 concentration (ng/ml) above the baseline in control and affected gel-filtered platelets stimulated by 0.3 U/ml thrombin.

* = control and affected groups significantly different, ($p < 0.05$).


Control
Affected
TxB2 (ng/ml)
Time (minutes)
0
100
200
300
400
500
600
700
0
1
2
3
4
5
6
7
8
9
10
*
*


Control
Affected
TxB2 (ng/ml)
Time (minutes)
0
100
200
300
400
500
600
700
0
1
2
3
4
5
6
7
8
9
10
*
*

Phorbol Myristate Acetate

Both control and affected platelets aggregated fully and irreversibly in response to 3.0 μM PMA, but there was no increase in TxB_2 production by either group five or 12 minutes after the addition of agonist. Interestingly, the TxB_2 concentrations measured for the affected group at both five and 12 minutes after the addition of PMA were significantly lower than baseline concentration and were not significantly different from the control concentrations for these times. These results are summarized in Table 25 and Figures 82 and 83.

Table 25. TxB_2 concentration (ng/ml) in control and affected platelet rich plasma stimulated by 3.0 μM PMA.

	Post-Agonist (minutes)	Control TxB_2 (ng/ml)	Affected TxB_2 (ng/ml)
Baseline	0	1.31 ± 0.98 (19)	9.20 ± 3.87 (28)*
TxB_2 measured	1	1.15 ± 0.61 (5)	1.25 ± 0.61 (8)#
	5	1.61 ± 0.45 (5)	1.54 ± 0.75 (8)#
Increase above baseline	0	0	0
	5	0.16 ± 0.35 (5)	0 (8)
	12	0.28 ± 0.44 (5)	0 (8)

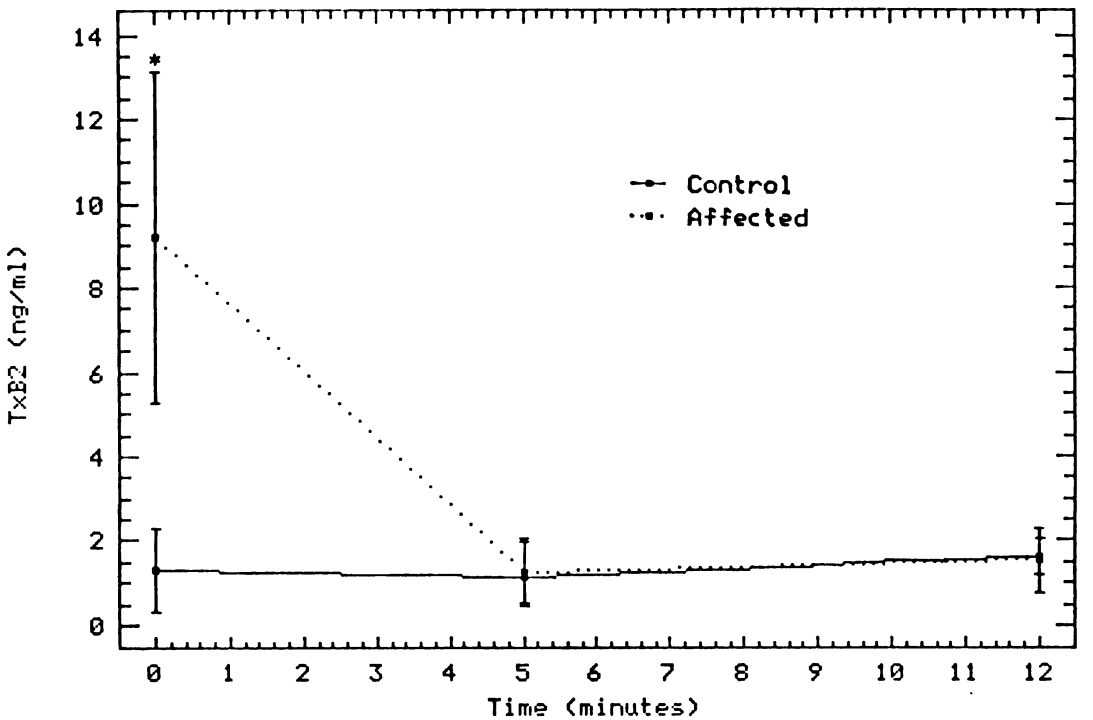
Values = mean ± SD, (n) = number of samples, * = significantly different from control value ($p < 0.05$), # = significantly different from baseline ($p < 0.05$).

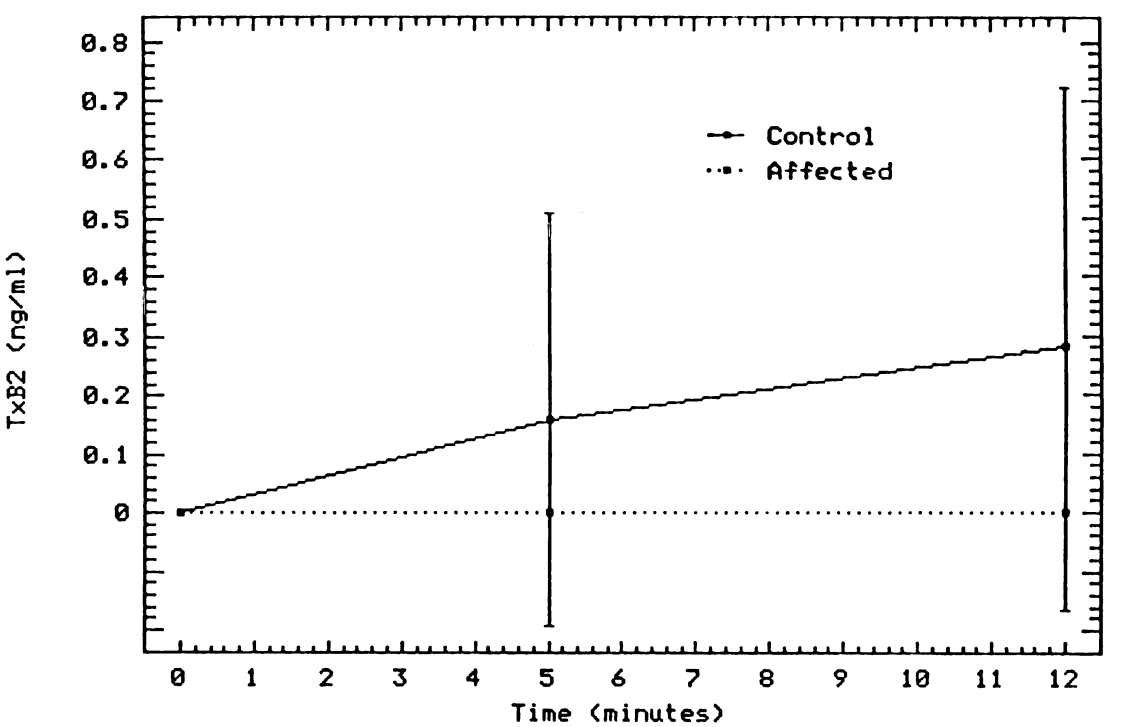
Figure 82. TxB_2 concentration (ng/ml) in control and affected platelet rich plasma stimulated by 3.0 μM PMA.

* - control and affected groups significantly different, ($p < 0.05$).

Figure 83. Increase in TxB_2 concentration (ng/ml) above the baseline in control and affected platelet rich plasma stimulated by 3.0 μM PMA.

* - control and affected groups significantly different, ($p < 0.05$).


14
12
10
8
6
4
2
0
*
Control
Affected
TxB2 (ng/ml)
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (minutes)


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
Affected
TxB2 (ng/ml)
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (minutes)

Calcium Ionophore A23187

Control platelets stimulated by 15 or 20 μM A23187 aggregated and produced large amounts of TxB_2. Although 20 μM A23187 stimulated greater TxB_2 production than 15 μM A23187, the responses were qualitatively similar. Both the concentrations measured and the increase above baseline were significantly increased at one minute and had further increased by five minutes. Affected platelets did not aggregate in response to 15 or 20 μM A23187. Although TxB_2 production by affected platelets was significantly increased by one minute, both the concentration measured and the increase above baseline were significantly less than that measured for the control group. At five minutes, the concentration measured and increase above baseline were less than that measured at one minute and were not significantly increased over the baseline level. Thromboxane B_2 production by affected platelets was slightly greater in response to 20 μM A23187 than 15 μM, but it was still significantly less than that of control platelets. These results are summarized in Tables 26 and 27 and Figures 84, 85, 86 and 87.

Table 26. TxB_2 concentration (ng/ml) in control and affected platelet rich plasma stimulated by 15 μM A23187.

	Post-Agonist (minutes)	Control TxB_2 (ng/ml)	Affected TxB_2 (ng/ml)
Baseline	0	1.31 ± 0.98 (19)	9.20 ± 3.87 (28)*
TxB_2 measured	1	179.90 ± 34.11 (5)#	46.79 ± 9.66 (8)*#
	5	306.55 ± 165.70 (5)#@	12.80 ± 3.78 (8)*
Increase above baseline	0	0	0
	1	178.19 ± 32.78 (5)#	40.61 ± 9.08 (8)*#
	5	304.90 ± 165.01 (5)#@	6.62 ± 3.33 (8)*

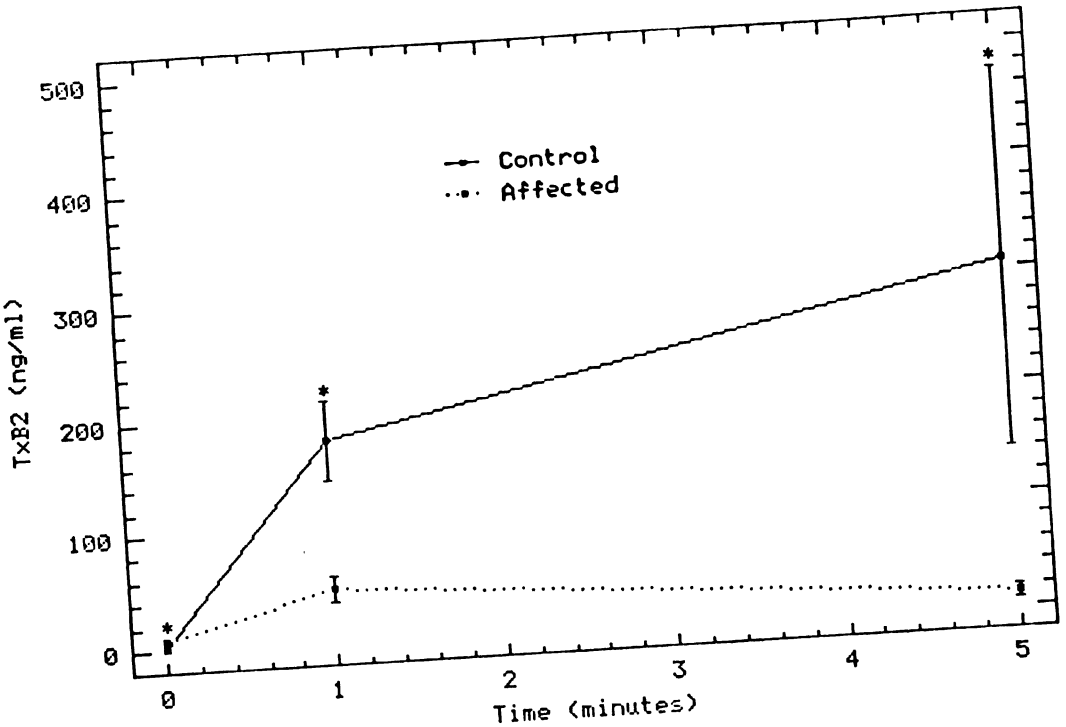
Values = mean ± SD, (n) = number of samples, * = significantly different from control value ($p < 0.05$), # = significantly different from baseline ($p < 0.05$), @ = significantly different from "1 minute" ($p < 0.05$).

Figure 84. TxB_2 concentration (ng/ml) in control and affected platelet rich plasma stimulated by 15 μM A23187.

* = control and affected groups significantly different, ($p < 0.05$).

Figure 85. Increase in TxB_2 concentration (ng/ml) above the baseline in control and affected platelet rich plasma stimulated by 15 μM A23187.

* = control and affected groups significantly different, ($p < 0.05$).


500
400
300
200
100
0
TxB2 (ng/ml)
Control
Affected
0
1
2
3
4
5
Time (minutes)

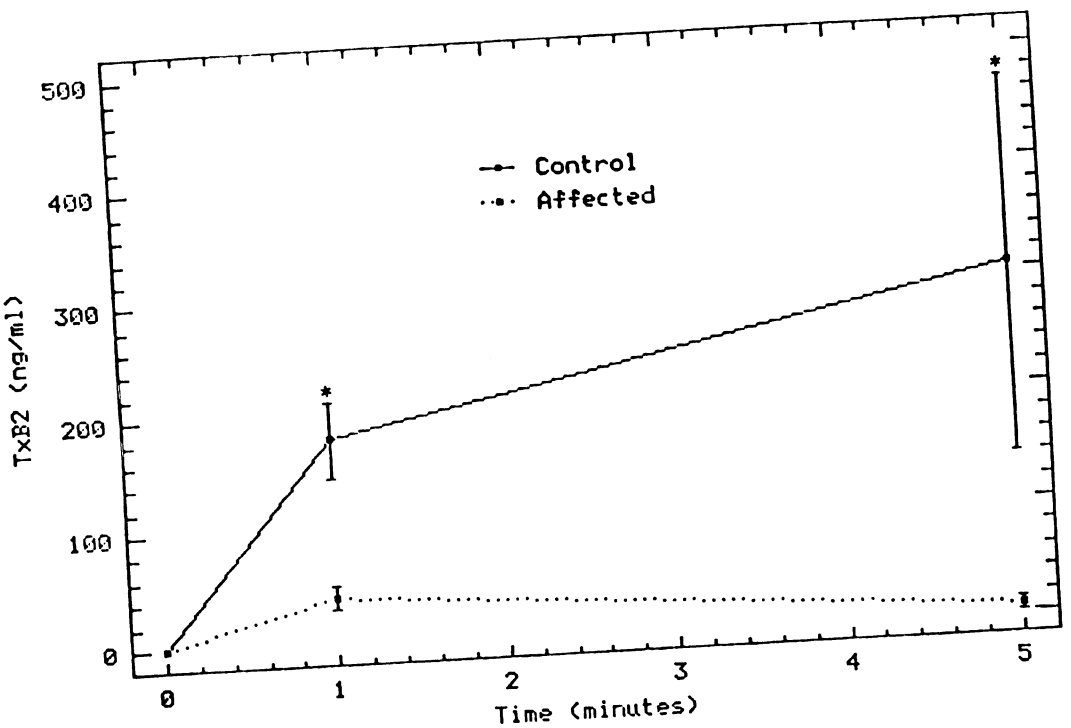

500
400
300
200
100
0
TxB2 (ng/ml)
Control
Affected
0
1
2
3
4
5
Time (minutes)

Table 27. TxB_2 concentration (ng/ml) in control and affected platelet rich plasma stimulated by 20 μM A23187.

	Post-Agonist (minutes)	Control TxB_2 (ng/ml)	Affected TxB_2 (ng/ml)
Baseline	0	1.31 ± 0.98 (19)	9.20 ± 3.87 (28)*
TxB_2 measured	1	251.00 ± 110.55 (7)#	68.59 ± 30.30 (12)*#
	5	437.70 ± 87.41 (8)#@	19.18 ± 8.96 (12)*
Increase above baseline	0	0	0
	1	249.40 ± 109.79 (7)#	59.64 ± 28.95 (12)*#
	5	436.29 ± 86.92 (7)#@	10.30 ± 7.48 (12)*

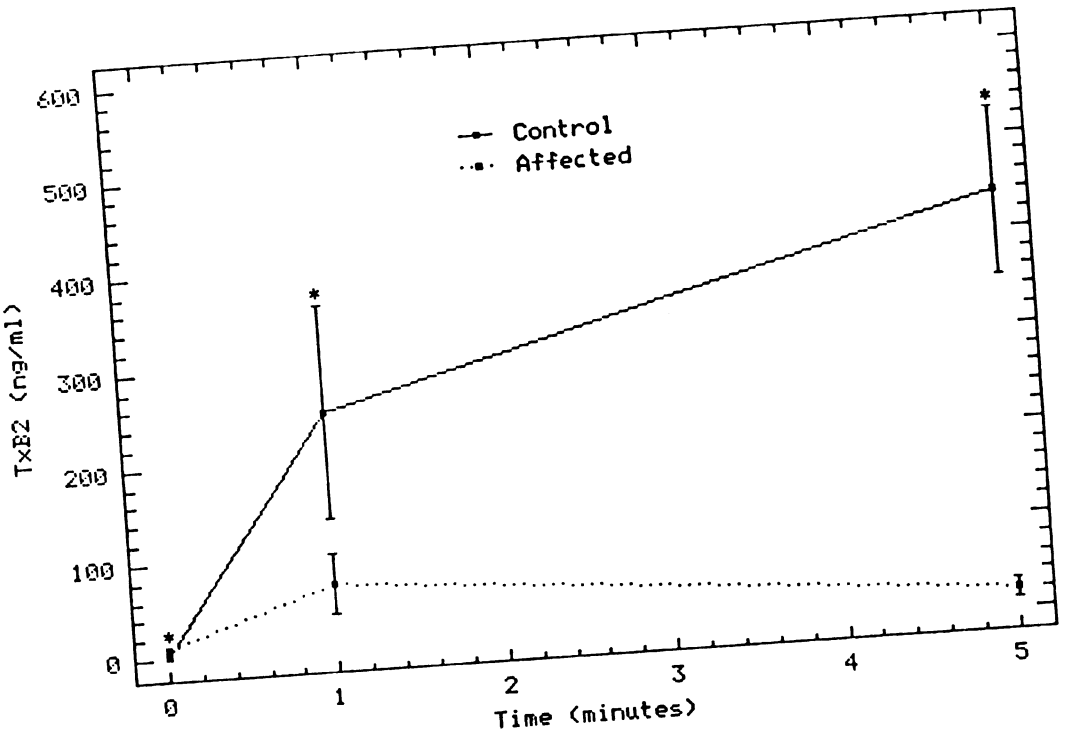
Values = mean ± SD, (n) = number of samples, * = significantly different from control value ($p < 0.05$), # = significantly different from baseline ($p < 0.05$), @ = significantly different from "1 minute" ($p < 0.05$).

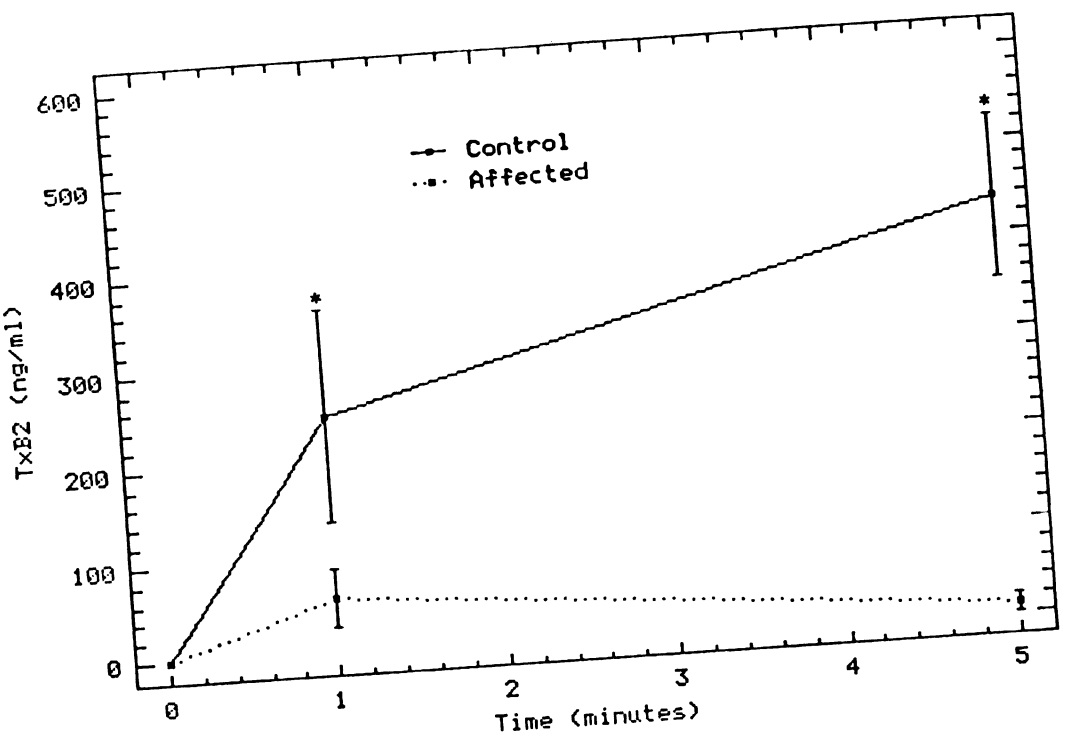
Figure 86. TxB_2 concentration (ng/ml) in control and affected platelet rich plasma stimulated by 20 μM A23187.

* - control and affected groups significantly different, ($p < 0.05$).

Figure 87. Increase in TxB_2 concentration (ng/ml) above the baseline in control and affected platelet rich plasma stimulated by 20 μM A23187.

* - control and affected groups significantly different, ($p < 0.05$).


Control
Affected
TxB2 (ng/ml)
600
500
400
300
200
100
0
0
1
2
3
4
5
Time (minutes)


Control
Affected
TxB2 (ng/ml)
600
500
400
300
200
100
0
0
1
2
3
4
5
Time (minutes)

Arachidonic Acid

In view of the significant differences in TxB_2 production by control and affected platelets stimulated by a range of physiologic and non-physiologic agonists, it was logical to examine TxB_2 production in response to exogenous arachidonic acid. Arachidonic acid does not always induce aggregation of normal canine platelets. For this study, concentrations of arachidonic acid which induced aggregation of normal human platelet rich plasma were selected and tested on the control group of three normal Basset hounds and one normal Labrador x Husky. One of the control Basset hounds aggregated strongly in response to 1.0 mM and 0.75 mM and weakly in response to 0.5 mM arachidonic acid. Platelets of the other controls and all affected dogs changed shape, but did not aggregate in response to these concentrations of arachidonic acid.

Both control and affected platelets produced very large quantities of TxB_2 in response to 0.5 and 1.0 mM arachidonic acid, with greater quantities produced in response to 1.0 mM arachidonic acid. There was no relationship between the occurrence of aggregation and the amount of TxB_2 produced. Neither the level measured or the increase above baseline differed significantly between the two groups at either one or five minutes. Within both groups, the TxB_2 was significantly increased by one minute and further increased by five minutes. These results are summarized in Tables 28 and 29 and Figures 88, 89, 90 and 91.

Table 28. TxB_2 concentration (ng/ml) in control and affected platelet rich plasma stimulated by 0.5 mM arachidonic acid.

	Post-Agonist (minutes)	Control TxB_2 (ng/ml)	Affected TxB_2 (ng/ml)
Baseline	0	1.31 ± 0.98 (19)	9.20 ± 3.87 (28)*
TxB_2 measured	1	546.80 ± 135.66 (4)#	514.15 ± 84.12 (4)#
	5	829.14 ± 207.70 (4)#@	859.07 ± 110.23 (4)#@
Increase above baseline	0	0	0
	1	546.13 ± 135.39 (4)#	505.12 ± 85.76 (4)#
	5	828.48 ± 207.40 (4)#@	850.04 ± 111.55 (4)#@

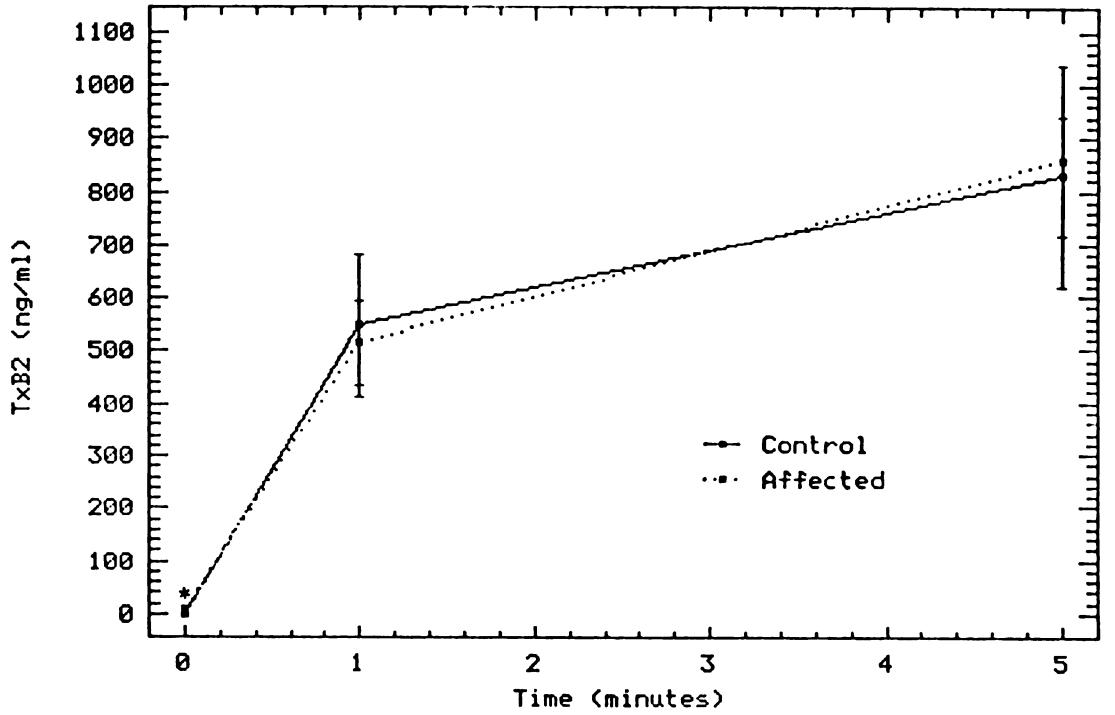
Values = mean ± SD, (n) = number of samples, * = significantly different from control value ($p < 0.05$), # = significantly different from baseline ($p < 0.05$), @ = significantly different from "1 minute" ($p < 0.05$).

Figure 88. TxB_2 concentration (ng/ml) in control and affected platelet rich plasma stimulated by 0.5 mM arachidonic acid.

* = control and affected groups significantly different, ($p < 0.05$).

Figure 89. Increase in TxB_2 concentration (ng/ml) above the baseline in control and affected platelet rich plasma stimulated by 0.5 mM arachidonic acid.

* = control and affected groups significantly different, ($p < 0.05$).


TxB2 (ng/ml)
1100
1000
900
800
700
600
500
400
300
200
100
0
0
1
2
3
4
5
Time (minutes)
Control
Affected

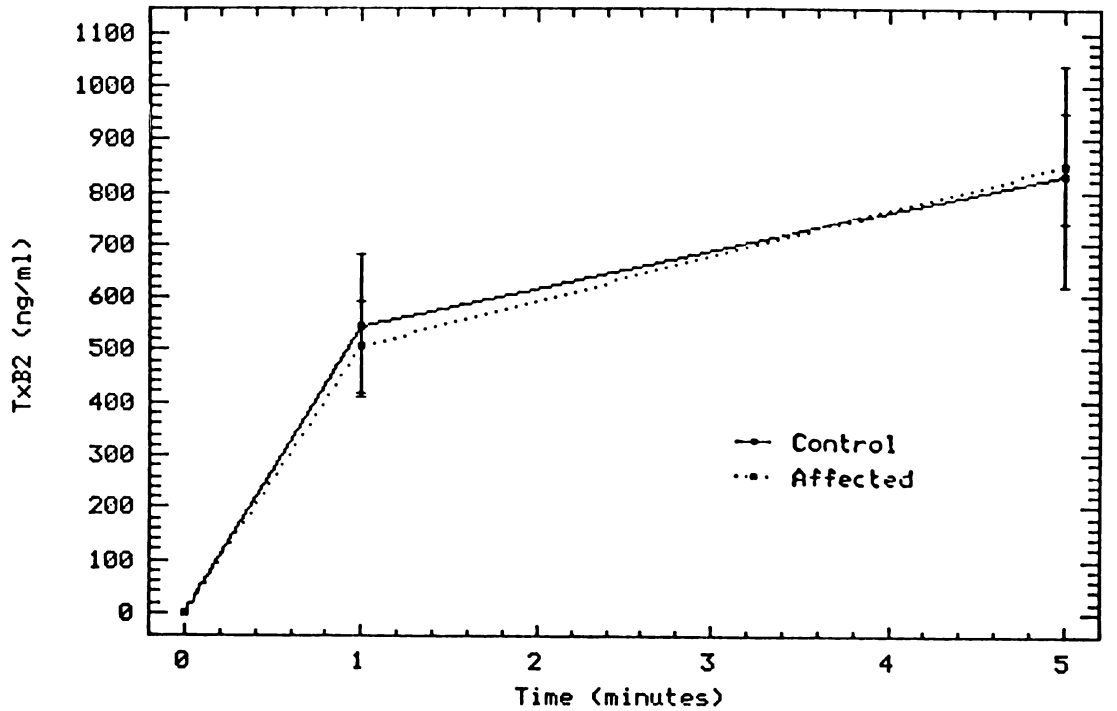

TxB2 (ng/ml)
1100
1000
900
800
700
600
500
400
300
200
100
0
0
1
2
3
4
5
Time (minutes)
Control
Affected

Table 29. TxB_2 concentration (ng/ml) in control and affected platelet rich plasma stimulated by 1.0 mM arachidonic acid.

	Post-Agonist (minutes)	Control TxB_2 (ng/ml)	Affected TxB_2 (ng/ml)
Baseline	0	1.31 ± 0.98 (19)	9.20 ± 3.87 (28)*
TxB_2 measured	1	815.25 ± 172.43 (4)#	779.13 ± 55.16 (4)#
	5	1060.55 ± 253.49 (4)#@	1022.61 ± 108.73 (4)#@
Increase above baseline	0	0	0
	1	814.59 ± 172.12 (4)#	770.10 ± 55.67 (4)#
	5	1059.89 ± 253.20 (4)#	1013.75 ± 109.73 (4)#@

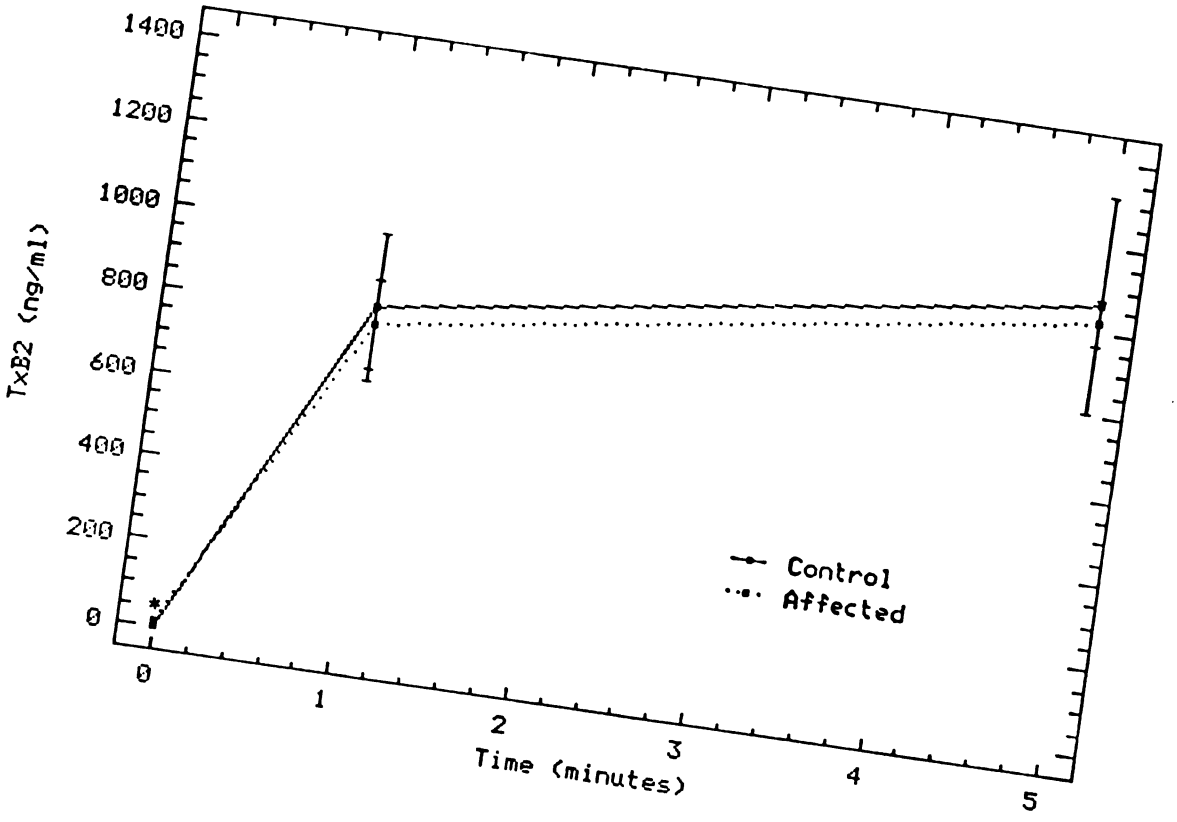
Values = mean ± SD, (n) = number of samples, * = significantly different from control value ($p < 0.05$), # = significantly different from baseline ($p < 0.05$), @ = significantly different from "1 minute" ($p < 0.05$).

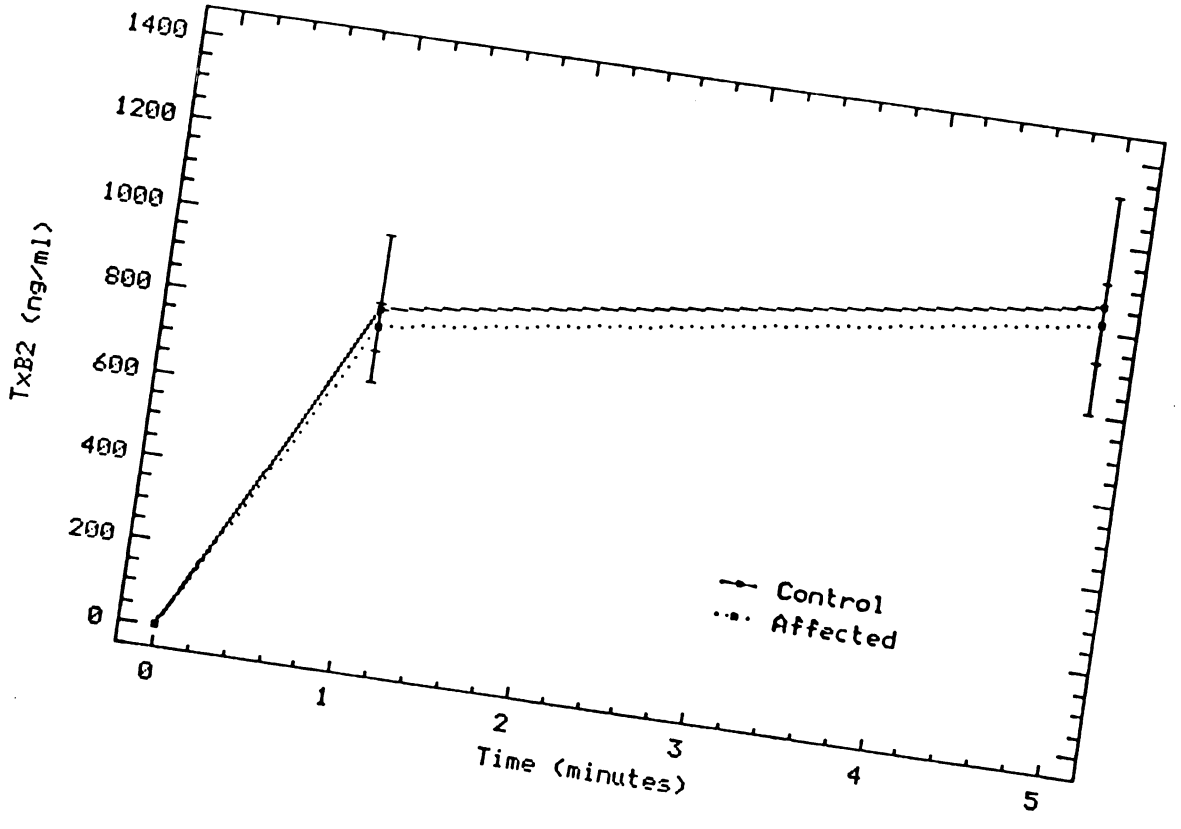
Figure 90. TxB_2 concentration (ng/ml) in control and affected platelet rich plasma stimulated by 1.0 mM arachidonic acid.

* = control and affected groups significantly different, ($p < 0.05$).

Figure 91. Increase in TxB_2 concentration (ng/ml) above the baseline in control and affected platelet rich plasma stimulated by 1.0 mM arachidonic acid.

* = control and affected groups significantly different, ($p < 0.05$).


1400
1200
1000
800
600
400
200
0
TxB2 (ng/ml)
0
1
2
3
4
5
Time (minutes)
Control
Affected


1400
1200
1000
800
600
400
200
0
TxB2 (ng/ml)
0
1
2
3
4
5
Time (minutes)
Control
Affected

DISCUSSION

There are dramatic differences between the control and affected groups in the amount of TxB_2 produced and the pattern of production. Although the measurement of TxB_2 does not give a lot of information on the kinetics of TxA_2 production, the results suggest that affected platelets are capable of more rapid, and greater, TxB_2 production than controls in response to certain stimuli.

The baseline TxB_2 concentration in affected platelet rich plasma was consistently and significantly higher than that of controls. Initially, for two reasons, this was thought to be a reflection of increased sensitivity to activation induced by stirring. Firstly, experience with affected platelets has indicated that, although they do not aggregate normally, they are very susceptible to spontaneous shape change when stirred in the aggregometer cuvette. Presumably, this reflects spontaneous activation induced by stirring. This occurs occasionally, but much less frequently, in control platelets. In both groups, gentle handling of platelets is essential to minimize this problem. Secondly, the baseline concentrations measured when the platelet rich plasma was stirred in the PICA aggregometer were higher than those stirred in the Lumi-aggregometer. Although these are similar instruments from the same manufacturer, set at the same stir bar speed (900 rpm) and using the same cuvettes and stir bars, previous experience with these instruments suggests that platelets stimulated with a threshold concentration of agonist are more likely to aggregate in the PICA than in the Lumi-aggregometer. This may indicate that the

actual stir speed differs slightly from the theoretical setting, being relatively slightly faster in the PICA.

When resting concentrations of TxB_2 in non-stirred platelet rich plasma were measured at room temperature and at 37°C, they were found to not differ significantly from those measured after stirring for 0.5, 5.5 or 12.5 minutes. Thromboxane B_2 production after stirring in the absence of an agonist was investigated to eliminate stirring alone as the source of the greater TxB_2 production by affected platelets in response to some agonists. It is possible that the handling of affected platelets during collection and preparation is sufficient to stimulate TxB_2 production, making it impossible to measure true resting TxB_2 concentrations. If this is so, there must be a reason for the increased susceptibility to activation induced by handling and stirring. It might reflect an increased sensitivity to the traces of the ADP released in hemolysis and agents such as PAF, eicosanoids and bioamines released from contaminating leukocytes.[332]

When platelets are gel-filtered, there is considerably more manipulation, including centrifugation and resuspension and incubation with prostaglandin E_1, than when platelets are prepared in plasma. The TxB_2 concentrations measured after 0.5 minute stirring did not differ significantly between control and affected gel-filtered platelets. This possibly indicates that both groups are susceptible to the partial activation that is known to occur during gel-filtration. This activation is usually reversible and platelets recover fully when rested for 30 minutes after gel-filtration.[165]

The higher baseline concentrations of TxB_2 measured in platelets in buffer compared to plasma may reflect increased TxB_2 production by platelets during preparation of gel-filtered platelets. However, it more likely is a reflection of the different protein contents of the two media. The gel-filtered platelets were in a buffer which contained only 0.3 gm/100 ml bovine serum albumin, whereas normal canine plasma contains 2.3 - 3.2 gm/100 ml albumin.[118] Albumin binds strongly to TxA_2 and the higher levels measured in buffer are most likely a reflection of decreased binding to albumin.[139,143,460]

In spite of the failure of affected platelets to aggregate in response to 10 μM ADP, they very rapidly produced larger amounts of TxB_2 than control platelets. This contradicts current knowledge which maintains that TxA_2 production in response to ADP is secondary to primary aggregation. Secretion of dense granule contents from normal platelets stimulated by ADP is dependent upon aggregation and the formation of TxA_2, and is almost completely inhibited by incubation with aspirin.[80,263] In affected platelets, ATP secretion was also assumed to be dependent upon TxA_2 formation because it was almost completely inhibited by aspirin. It is probable that the rapid ATP release from affected platelets stimulated by ADP is not a primary defect, but is secondary to abnormally rapid TxA_2 production. The ability of affected platelets to produce TxA_2 in the absence of aggregation suggests that the defect is related to abnormal control of TxA_2 production, with the rapid release of dense granule ATP being a secondary event.

When control platelets were stimulated by 0.1 or 1.0 μM PAF, they aggregated and secreted dense granule ATP. Aggregation was not significantly inhibited and ATP secretion only partially inhibited by incubation with aspirin. This is compatible with PAF being an agonist of intermediate strength in which TxA_2 amplifies the response of platelets, but is not essential for secondary aggregation and secretion.[77,174,310,470] Affected platelets aggregated minimally in response to 1.0 μM PAF, but did release dense granule ATP. When incubated with aspirin, dense granule ATP release was inhibited to a greater extent than in controls. Affected platelets stimulated by 0.1 μM PAF did not aggregate or release dense granule ATP.

Control platelets rapidly produced TxB_2 when stimulated by 0.1 or 1.0 μM PAF, with more TxB_2 being produced in response to the higher concentration of PAF. Affected platelets stimulated by 1.0 μM PAF rapidly produced approximately three times the amount of TxB_2 generated by control platelets, but when stimulated by 0.1 μM PAF, they did not produce any TxB_2. The production of TxB_2 by affected platelets correlates with the occurrence of dense granule ATP secretion. It is possible that released TxA_2 plays a more important role in induction of dense granule ATP secretion in affected platelets than in controls. Affected platelets stimulated by 1.0 μM PAF produced more TxB_2 than controls, but ATP release was inhibited to a greater degree by incubation with aspirin than in control platelets.

However, failure to produce TxB_2 when stimulated by 0.1 μM PAF does not explain the complete failure of affected platelets to secrete dense

granule ATP. Control platelets incubated with aspirin still secreted some dense granule ATP in the absence of TxA_2 production. This suggests two possible abnormalities in affected platelets. Firstly, there may be defective control of TxA_2 production which results in increased production, or failure of production of TxA_2, depending on the stimulus. Secondly, there may be failure of, or a defect in, the pathway that mediates TxA_2-independent dense granule secretion.

Although not all control or affected dogs aggregated in response to 0.05 U/ml thrombin, aggregation was associated with the production of TxB_2 and failure of aggregation with lack of production of TxB_2. This was to be expected, as low concentrations of thrombin such as 0.05 U/ml are weak agonists and dependent on TxA_2 production and ADP release for aggregation and secretion to occur.[441]

At the slightly higher concentration of 0.1 U/ml, thrombin is considered to be an agonist of intermediate strength. It was found that incubation with aspirin reduced, but did not prevent, the responses of control platelets to 0.1 U/ml thrombin. In control platelets, TxB_2 production steadily increased for 10 minutes after the addition of 0.1 U/ml thrombin. Affected platelets varied in their ability to aggregate in response to 0.1 U/ml thrombin, but there was a significant correlation between the quantity of TxB_2 produced and the percent aggregation five minutes after the addition of thrombin. This is compatible with the complete inhibition of aggregation of affected platelets in response to 0.1 U/ml thrombin and suggests that TxA_2 plays

a much more important role in the activation of affected platelets by thrombin than in controls.

Both control and affected platelets aggregated and secreted dense granule ATP in response to 0.3 U/ml thrombin, although the initial rate of aggregation of affected platelets was very much slower than that of controls. In control platelets, TxB_2 production steadily increased for 10 minutes after the addition of 0.3 U/ml thrombin. This was similar to their response to 0.1 U/ml, although quantitatively greater. Affected platelets produced significantly more TxB_2 than controls one and five minutes after the addition of 0.3 U/ml thrombin. When affected platelets were incubated with aspirin, aggregation was inhibited much more strongly than in controls and the rate of aggregation greatly decreased. This also suggests that TxA_2 plays a much more important role in the activation of affected platelets by thrombin than controls. It seems probable that much of the response seen in affected platelets stimulated by thrombin is due to TxA_2, even at higher concentrations of thrombin that are not dependent on TxA_2 production in normal platelets.

It has been reported previously that aggregation of human and rabbit platelets by the tumor-promoting phorbol esters is not inhibited by cyclooxygenase inhibitors.[127,233,341] Thromboxane B_2 production by phorbol-ester-stimulated platelets has been reported to be not significant or very slight and TxA_2 activity by bioassay not detectable.[127,172,264,341] Both control and affected platelets aggregated irreversibly in response to 3.0 μM PMA without producing significant

amounts of TxB_2. While the lack of production by the control group is compatible with published findings, the reason for the significant decrease in TxB_2 production by the affected group is not clear. It is possible that binding to plasma albumin may decrease the levels measured five and 10 minutes after the addition of PMA, but the TxB_2 concentration in affected platelet rich plasma stirred for 12.5 minutes did not decrease.

It has been reported that the conversion of exogenously added arachidonate to TxB_2 is inhibited by a 10 second to five minutes pre-incubation with PMA.[264] In the same study, both arachidonate release and TxB_2 production by thrombin were inhibited by preincubation with PMA. Similarly, preincubation with PMA inhibited TxB_2 formation by maximally effective doses of ADP, PAF and epinephrine.[265] The presence of PMA may interfere with the TxB_2 production induced by stirring in affected platelets. Alternatively, it is possible that PMA interferes with the TxB_2 radioimmunoassay, but the lack of inhibition of TxB_2 production by control group makes this seem less likely. At this time is only possible to speculate that PMA has a not yet clearly identified effect on TxB_2 production, and possibly arachidonate release, in affected platelets and normal platelets stimulated by a range of other agonists.

Calcium ionophores such as A23187 are able to cross the plasma membrane and directly mobilize Ca^{2+} from intracellular stores to increase the intracytoplasmic Ca^{2+} concentration and activate phospholipase A_2. The released arachidonic acid is metabolized to

endoperoxides and TxA_2 which activate phospholipase C and stimulate phosphoinositide hydrolysis.[275,281,413] Control platelets stimulated by A23187 generated large amounts of TxB_2, as has been reported for human platelets.[275] Although the amount of TxB_2 generated by affected platelets at one minute was quite large relative to that generated by some agonists, it was significantly less than that of the control platelets. By five minutes, the TxB_2 concentration measured was not significantly different to the baseline concentration.

Albumin has been shown to bind to TxA_2 in plasma and prolong its $T_{\frac{1}{2}}$ to up to three minutes, but as TxB_2 has been shown to be stable in plasma, it is difficult to understand why less TxB_2 was detected at five than at one minute.[139,143,460] It is possible that A23187 stimulates an extremely short-lived burst of TxA_2 generation in affected platelets compared to controls. In the absence of continued TxA_2 production, the binding of TxA_2 to albumin between one and five minutes may have exceeded production, causing a decrease in the amount measured at five minutes relative to one minute. Also, the contribution of aggregation-dependent positive feedback on TxB_2 production in control platelets stimulated by A23187 is not known and may explain at least some of the difference between the two groups. A decrease in the TxB_2 concentration measured has been reported under conditions in which the reaction was quenched with citric acid.[139] It was proposed that an early "burst" (< 60 seconds) of TxB_2 formation after the addition of prostaglandin H_2 to platelet rich plasma could be accounted for by the rapid hydrolysis of the TxA_2 present to TxB_2 by the addition of acid before the TxA_2 could bind to the protein present. Samples collected

after 60 seconds contained less TxB_2 because much of the TxA_2 had bound to albumin and the TxB_2 present could be accounted for by the hydrolysis of the unbound TxA_2 in the plasma.

Both control and affected platelets stimulated by arachidonic acid generated very large amounts of TxB_2 in a concentration-dependent manner, similar to that described for human platelets.[139,460] The ability of platelets to convert exogenous arachidonic acid to TxB_2 indicates that the enzymes involved are present and functional. The generation by affected platelets of a similar quantity of TxB_2 from arachidonic acid at a similar rate to controls, indicates that they have functional prostaglandin endoperoxide synthase and thromboxane A synthase enzyme systems.

The only agonist used in this study that was able to stimulate similar TxB_2 generation in both control and affected groups was exogenous arachidonic acid. Thromboxane B_2 generation in response to all other agonists used was significantly different between the two groups. Thus, it is possible that the defect occurs before the conversion of arachidonic acid to thromboxane. Possible sites for a defect include arachidonic acid mobilization from membrane phospholipids, phospholipase A_2, and Ca^{2+} mobilization.

The range of abnormal TxB_2 responses of affected platelets is unique and cannot be explained by current knowledge of the area. Although the major routes of arachidonic acid release from membrane phospholipids are known, relatively little is known about the control of these and

the preceding events. There is growing evidence for a role for protein kinase C in the regulation of arachidonic acid metabolites. It has been shown that activators of protein kinase C inhibit the lysoacyltransferase which increases the availability of arachidonic acid for eicosanoid synthesis.[140,382] However, it has also been demonstrated that synthetic diacylglycerols are able to inhibit lysophosphatidate acyltransferase activity in macrophages by a direct interaction independent of protein kinase C.[161] If the lysoacyltransferase in macrophages is inhibited by pre-incubation with merthiolate, incorporation of arachidonic acid into phospholipids is inhibited and the production of prostanoids greatly increased in response to phagocytic stimuli.[160] In platelets, arachidonic acid liberation by A23187 is potentiated by preincubation with stimulators of protein kinase C, an effect coincident with the slow phosphorylation of five proteins with molecular weights of 65, 41, 35-36 and 21 kDa.[134,172] This potentiation of arachidonic acid release by PMA appeared to be due to phospholipase A_2 activation. If the 65 kDa protein that is not phosphorylated normally in affected platelets is the same 65 kDa protein as that described above, it may be linked to abnormal regulation of arachidonic acid liberation.

It is generally assumed that phospholipase A_2 can be activated by an increase in Ca^{2+} alone, if the increase is sufficiently large.[457] Evidence that an increase in Ca^{2+} alone may be sufficient to activate phospholipase A_2 is provided by calcium ionophores such as A23187 which can release arachidonic acid from membrane phospholipids even when phospholipase C is not activated.[413] Although phospholipase A_2 requires

Ca^{2+} for activity, its role in the regulation of phospholipase A_2 is not clear. Protein kinase C may play an inhibitory role in Ca^{2+} mobilization by phosphorylating and activating the 5'-phosphomonoesterase which inactivates inositol 1,4,5-trisphosphate.[97]

There is also evidence that phospholipase A_2 in human platelets is under the control of GTP-binding proteins that are distinct from those involved in the regulation of phospholipase C.[152,348] As protein kinase C has been shown to affect the state of association of G-protein oligomers and the function of α-subunits, this is another possible way in which protein kinase C may influence the release of arachidonic acid.[173,244]

The Na^+/H^+ exchanger may also play a role in arachidonic acid release, particularly in response to weak agonists.[472,473,476] Phospholipase A_2 has an alkaline pH optimum and the small alkalinization of the cytoplasm that occurs when the Na^+/H^+ exchanger is activated may play an important role in activation by weak agonists, possibly by enabling phospholipase A_2 to function at low concentrations of intraplatelet Ca^{2+}.[26] However, it does not appear to play a role in activation by higher concentrations of thrombin. Again, protein kinase C is thought to have a regulatory role. Activators of protein kinase C induce acceleration of Na^+/H^+ exchange and an increase in intracytoplasmic pH.[455]

The range of responses of affected platelets to different stimuli suggests that the mechanism for the production of TxB_2 is intact, but

not under the normal control mechanisms. Whether the defect is at the level of liberation of arachidonic acid or whether it is secondary to another defect higher up in the activation process cannot be answered by this study. Further investigation of this aspect of the defect in Basset hound hereditary thrombopathy platelets may provide new insights into the control of eicosanoid production in normal platelets.

CHAPTER VI: SUMMARY AND CONCLUSIONS

Affected platelets aggregated when phosphoinositide hydrolysis was able to be bypassed (phorbol ester, higher concentrations of thrombin) or when membrane properties were altered by a reducing agent, enabling GPIIb-IIIa to bind fibrinogen (dithiothreitol). Primary aggregation of affected platelets did not occur in response to agonists of weak (ADP) or intermediate strength (PAF) which are fully or partially dependent upon activation of Na^+/H^+ exchange and generation of arachidonate metabolites. Primary aggregation and dense granule ATP release did not occur in response to the calcium ionophore A23187 which is dependent upon the formation of arachidonate metabolites for activation of phospholipase C and complete aggregation.

The failure of primary aggregation in response to ADP and PAF was not associated with an inability of affected platelets to secrete ATP or generate TxA_2. Secretion of ATP by affected platelets was inhibited by pre-treatment of platelets with the cyclooxygenase inhibitor aspirin. Secretion of ATP from affected platelets was more susceptible to inhibition by aspirin for all agonists which induced secretion (PAF, ADP, thrombin). Thrombin-induced aggregation of affected platelets was much more sensitive to inhibition by aspirin than control platelets. The responses of affected platelets to thrombin were more dependent on the formation of arachidonate metabolites than control platelets.

Affected platelets had similar phosphorylation patterns of the two major proteins (20 and 47 kDa) phosphorylated in control platelets in response to PAF, thrombin, phorbol ester and A23187 under non-aggregating conditions. Phosphorylation of the 20 kDa myosin light chain correlated well with platelet shape change in both groups, but there did not appear to be any consistent relationship between phosphorylation of the 20 and 47 kDa proteins and aggregation or secretion of dense granule contents. Decreased phosphorylation of a 65 kDa band in affected platelets may represent either the absence of a protein band or an inability or decreased ability to phosphorylate a protein that is present. The identity and functional significance of this 65 kDa protein are not known, but it may be of major importance in the pathogenesis of this defect.

Measurement of TxB_2 levels confirmed that affected platelets produce TxA_2 in response to most agonists. In non-stirred and stirred platelet rich plasma, affected platelets had significantly higher TxB_2 levels than control platelets. Agonists which induced dense granule ATP release from affected platelets also induced significantly greater TxB_2 production from affected platelets than controls (ADP, 1.0 μM PAF, thrombin). When stimulated by agonists which did not induce either aggregation or ATP secretion, affected platelets produced no TxB_2 (0.1 μM PAF) or significantly less TxB_2 (A23187) than control platelets. When affected platelets were stimulated by thrombin, aggregation was associated with TxB_2 production and the extent of aggregation was proportional to the amount of TxB_2 produced. The amount of TxB_2 produced by control platelets stimulated by thrombin was not related to

the extent of aggregation. Arachidonic acid was the only agonist to induce the production of similar quantities of TxB_2 by both groups of platelets. Thromboxane production in response to arachidonic acid was not related to failure of aggregation (affected and most control platelets) or occurrence of aggregation (one control). Neither control or affected platelets produced TxB_2 in response to stimulation by phorbol ester. These results are summarized in Table 30.

Table 30. Summary of control and affected platelet aggregation, dense granule ATP secretion, TxB_2 generation and 20 and 47 kDa protein phosphorylation (control/affected).

Response	ADP 10μM	PAF 0.1μM	PAF 1.0μM	Thrombin 0.1U/ml	Thrombin 0.3U/ml	A23187 20μM	PMA 3.0μM
Aggregation	+/-	+/-	+/±	+/±	+/+	+/-	+/+
Secretion	+/+	+/-	+/+	+/+	+/+	+/-	+/+
TxB_2	+/++	+/-	+/++	+/++	+/++	++/+	-/-
20 kDa P^n	-/-	+/+*	ND	ND	+/+	+/+#	+/+
47 kDa P^n	-/-	+/+*	ND	ND	+/+	+/+#	+/+

+ = event occurs, - = event does not occur, ± = event may or may not occur, ND = not done. * = under protein-free conditions, the effective concentration of PAF was approximately 1.0 μM (see Page 290).
# = under protein-free conditions, 1.0 μM A23187 was used.

From these results, the following conclusions have been made:

1. At least one pathway of platelet activation is functional: the pathway(s) that mediate aggregation by phorbol ester and higher concentrations of thrombin.

2. The pathway(s) involved in signal transmission from the platelet plasma membrane to the cell interior is(are) either defective, unable to be converted to the normal sequence of cellular events or not under the normal control mechanisms.

3. Phosphorylation of the 20 kDa myosin light chain correlated well with platelet shape change in both groups, but there was not a consistent relationship between phosphorylation of the 20 and 47 kDa proteins and aggregation or secretion of dense granule ATP.

4. Decreased phosphorylation of an unidentified 65 kDa band in affected platelets is of unknown functional significance, but may be of major importance in the pathogenesis of the defect.

5. The production of TxB_2 by affected platelets in response to different agonists suggests that the mechanism for the production of TxB_2 is intact, but not under the normal control mechanisms.

6. The similar production of TxB_2 by control and affected platelets stimulated by arachidonic acid suggests that it is the release of arachidonic acid that is abnormal when affected platelets are stimulated by other agonists.

A hypothetical model of the defect is illustrated in Figure 92 (Page 373). It has been constructed from the results available and does not explain all the findings. It is proposed that: (1) the efficiency of the signal transduction from the external receptor through the G_p regulatory protein and phospholipase C is decreased; and (2) the defect can be bypassed to some extent by an increase in the strength of the agonist which can then generate a signal that is able to be transmitted.

The putative ADP receptor is a 100 kDa membrane protein which is closely associated with GPIIb-IIIa.[93a] In the resting platelet, the ADP receptor interferes with the binding of fibrinogen to GPIIb-IIIa. The binding of ADP to its receptor induces a conformational change that permits the binding of fibrinogen to GPIIb-IIIa and induces shape change that is not inhibited by the presence of EDTA or EGTA.[93a] It is proposed that when ADP binds to its receptor on affected platelets, shape change and fibrinogen binding occur, but the amount of TxA_2 generated is not sufficient to activate phospholipase C. However, this does not explain: (1) the failure of primary aggregation, unless activation of phospholipase C, or a subsequent step, is essential for primary aggregation; (2) the abnormally rapid ATP secretion, even if the secretion is mediated by an influx of Ca^{2+} from the external medium

via the Ca^{2+} channel that is presumed to be close to, but separate from, GP11b-111a;[54,394] and (3) the greater generation of TxB_2 by affected platelets unless, in the absence of phospholipase C activation, there is also failure of a negative feedback step which would normally inhibit TxA_2 generation.

Thrombin is a strong platelet agonist which induces rapid release of dense granule contents, including ADP. At low concentrations of thrombin, platelet aggregation is inhibited by preincubation with a high affinity analogue of ADP.[93a] At higher concentrations of thrombin (>0.025 U/ml), aggregation is not inhibited. At these higher concentrations, thrombin also proteolytically degrades the putative 100 kDa ADP receptor and allows GPIIb-IIIa to undergo the conformational change which permits fibrinogen binding. The inability of affected platelets to aggregate in response to lower concentrations of thrombin could be explained by failure of activation of phospholipase C. At higher concentrations, thrombin-induced aggregation may be induced by activation of phospholipase C and/or fibrinogen binding induced by proteolytic cleavage of the 100 kDa protein. The greater sensitivity of affected platelets to inhibition by aspirin is not explained.

The failure of low concentrations of PAF to induce any response except shape change may be caused by an inability to activate phospholipase C. Higher concentrations of PAF are able to activate phospholipase C, inducing phosphorylation of proteins, secretion and generation of TxB_2. This does not explain the failure of primary

aggregation. However, high concentrations of PAF did cause some increase in light transmission in the aggregometer cuvette.

The aggregation of affected platelets by the phorbol ester PMA which directly activates protein kinase C supports the proposal that the defect precedes the activation of protein kinase C. However, the means by which activation of protein kinase C induces aggregation are not known.

The calcium ionophore A23187 releases Ca^{2+} from intracellular stores in the dense tubular system. In affected platelets, it is proposed that the amount of TxB_2 generated is not sufficient to activate phospholipase C. The increase in intracytoplasmic Ca^{2+} concentration is sufficient to activate protein kinase C and myosin light chain kinase, but this alone does not induce secretion.

In conclusion, the results obtained in each of the three major sections of this study do not fit any described genetic or acquired platelet defect or any situation created <u>in vitro</u> and cannot be explained using current knowledge of platelet function. Virtually all knowledge of signal transduction in platelets is based on theoretical assumptions derived primarily from experimental manipulation of normal platelets. The inability of these theories to explain the findings in this study suggests that this genetic platelet defect may hold a key to more complete understanding of the mechanisms involved in normal platelet activation.

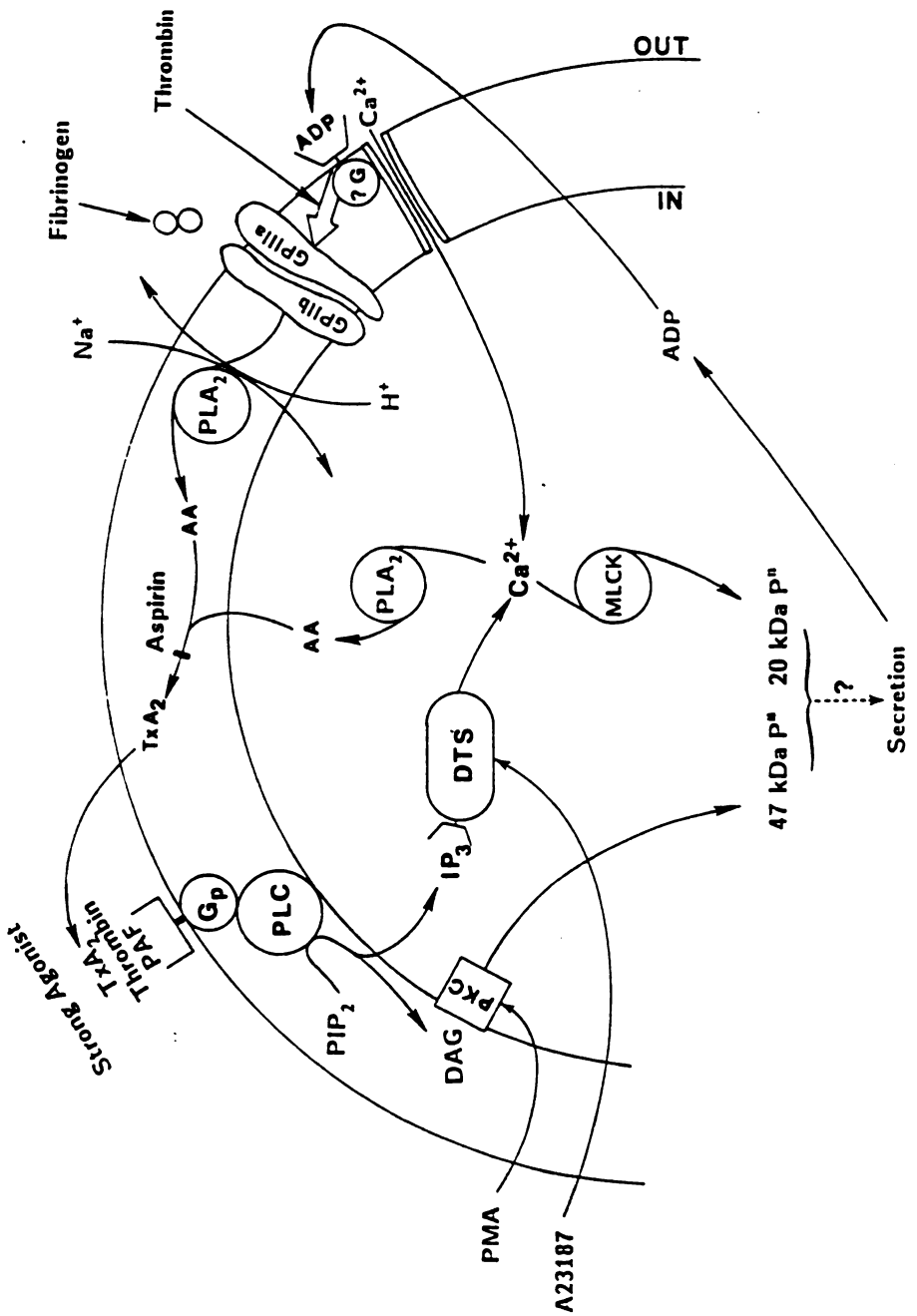


Figure 92. Hypothetical model of platelet activation including those pathways investigated in this study.

------→ = this pathway may involve several intermediate steps and/or other contributing factors

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LIST OF REFERENCES

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