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BASSET HOUND HEREDITARY THROMBOPATHY: MEASUREMENT OF PLATELET CYTOSOLIC CALCIUM, pH AND 1,2-DIACYLGLYCEROL PRODUCTION

By

Jennifer Sue Thomas

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ABSTRACT

BASSET HOUND HEREDITARY THROMBOPATHY:

MEASUREMENT OF PLATELET CYTOSOLIC CALCIUM, pH AND

1,2-DIACYLGLYCEROL PRODUCTION

By

Jennifer Sue Thomas

Basset hound hereditary thrombopathy is an autosomal recessive defect characterized by hemorrhagic diathesis. Affected platelets have normal quantities of membrane GPIIb-IIIa and bind fibrinogen following ADP stimulation. Affected platelets undergo shape change but do not aggregate in response to agonists which depend on phospholipase C and thromboxane production for full platelet activation (ie. ADP, PAF and calcium ionophore A23187). They aggregate in response to agonists which bypass phospholipase C (ie. high concentrations of thrombin and phorbol ester). Secretion of dense granule ATP occurs irrespective of aggregation. Following stimulation, affected platelets produce thromboxane; however, the production is not under normal regulatory control. Affected platelets yield normal phosphorylation of the 20kDa and 47kDa proteins and decreased phosphorylation of a 64-67kDa protein of unknown function and identity. The aim of this dissertation was the further investigation of events in platelet activation and their relationship to Basset hound hereditary thrombopathy. First, it was found that affected platelets had normal resting $[Ca^{++}]_i$. Using

aequorin-loaded platelets, there were no consistent differences between control and affected post-stimulation $[Ca^{++}]_i$. Using fura 2-loaded platelets, there were no consistent differences between control and affected post-stimulation $[Ca^{++}]_i$ in the presence of external Ca^{++}. In the absence of external Ca^{++}, affected platelets had decreased post-stimulation $[Ca^{++}]_i$ in response to PAF, thrombin and ionomycin. Secondly, affected platelets had normal resting pH_i. In response to agonists which do not induce aggregation in affected platelets (ie. ADP, PAF, A23187 and thromboxane mimetic U46619), there was decreased post-stimulation pH_i in affected platelets relative to control platelets. Following stimulation with thrombin, there was no difference in pH_i between affected and control platelets. Finally, 1,2-diacylglycerol levels were normal in affected platelets at rest or following stimulation with ADP, PAF, A23187, thrombin or phorbol ester. In response to U46619/epinephrine, affected platelets had increased 1,2-diacylglycerol production relative to control platelets. Although these findings help localize the platelet defect in Basset hound hereditary thrombopathy, they cannot explain the disorder based on current understanding of normal platelet function.

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
BFU-M	burst forming unit-megakaryocyte
BSS	Bernard Soulier syndrome
$[Ca^{++}]_i$	concentration of cytosolic free ionized calcium
cAMP	cyclic adenosine monophosphate
cAMP-PDE	cAMP phosphodiesterase
CDP-DG	cytidine diphosphate-diacylglycerol
CFU-M	colony forming unit-megakaryocyte
CPM	counts per minute
DG	diacylglycerol
DMSO	dimethyl sulfoxide
DTS	dense tubular system
EDTA	ethylene diamine tetraacetate
EGTA	ethylene glycol tetraacetic acid
FSBA	5'-p fluorosulfonylbenzoyl adenosine
GP	glycoprotein
HETE	hydroxyeicosatetraenoate
IP_2	inositol diphosphate
IP_3	inositol trisphosphate
IP_4	inositol tetrakisphosphate
lysoPA	lysophosphatidic acid
M-CSA	megakaryocyte-colony stimulating activity
M-CSF	megakaryocyte-colony stimulating factor
MLCK	myosin light chain kinase
OCS	open canalicular system
PA	phosphatidic acid
PAF	platelet activating factor
PGE_1	prostaglandin E_1
PGG_2	prostaglandin G_2
PGH_2	prostaglandin H_2
pH_1	phosphatidylinositol
PIP	phosphatidylinositol 4-phosphate
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PMA	phorbol myristate acetate
PPP	platelet poor plasma
PRP	platelet rich plasma
TSF	thrombocytosis-stimulating factor
TxA_2	thromboxane A_2
TxB_2	thromboxane B_2
vWD	vonWillebrand's disease
vWF	vonWillebrand's factor

INTRODUCTION

Platelets play an important part in the maintenance of normal hemostasis. In the past few decades, a great deal of research has focused on platelet function and biochemistry due to the potential role of platelets in the pathogenesis of atherosclerosis, thrombosis and other cardiovascular diseases; major causes of illness and death in Western cultures. Platelets are also frequently studied as a model for cellular physiology and biochemistry because they are readily available and have signal transduction pathways linked to identifiable surface receptors.

Some of the current knowledge about platelet function and biochemistry has resulted from studies on humans or animals with hereditary or congenital platelet disorders. By identifying an enzyme, glycoprotein or other structure which is missing or non-functional in affected platelets, it is possible to come to a better understanding of the role played by that component in normal platelet function. In the 1970's, a veterinarian identified such a disorder in a group of related purebred Basset hounds which has been termed Basset hound hereditary thrombopathy (BHT). Examination of the pedigrees of affected dogs suggest that BHT has an autosomal recessive pattern of inheritance. BHT is characterized by hemorrhagic diathesis which is associated with markedly defective <u>in vitro</u> aggregation.

Platelets from dogs affected with BHT share many abnormal clinical and functional characteristics with platelets from humans affected with Glanzmann's thrombasthenia, leading to the initial hypothesis that BHT was an animal model for this human disorder.[300] Glanzmann's thrombasthenia is a hereditary platelet dysfunction characterized by abnormal glycoprotein IIb-IIIa (GPIIb-IIIa) content or function. GPIIb-IIIa is a surface membrane glycoprotein complex that acts as the platelet fibrinogen receptor. It is the binding of fibrinogen to two adjacent platelets which mediates platelet aggregation. Platelets from dogs affected with BHT, however, were demonstrated to have normal GPIIb-IIIa content using two dimensional gel electrophoresis.[299] Following stimulation, affected platelets were also found to bind ^{125}I-labelled[298] and gold-labelled fibrinogen[119] to the same extent as normal canine platelets, indicating that the GPIIb-IIIa complex is functional and that the defect must lie in some, as yet unidentified, post-fibrinogen binding event(s).

Because BHT platelets do not aggregate in spite of normal fibrinogen binding, it was hypothesized that affected platelets have a defect in signal transduction. Signal transduction is the process by which an external signal is transmitted into the cell to elicit an appropriate physiologic response. As a general rule, signal transduction involves the following sequence of events: (1) binding of an external stimulus (agonist) to a specific surface receptor; (2) regulation of the activity of an effector pathway(s) by the activated receptor; (3) alteration of the production or function of intracellular second messengers by the effector system(s); and (4) mediation by second

messengers of the activity, structure or phosphorylation of cellular proteins. The final result is the modulation of the cellular response.

In an attempt to further characterize the disorder and to localize the biochemical defect in BHT, an extensive examination of affected platelet aggregation and ATP dense granule secretion was performed.[254] For this study, as well as all additional studies, a wide range of agonists were used in order to activate different effector pathways. It was found that affected platelets only aggregate in response to those agonists which cause activation independent of phospholipase C activation and thromboxane production. In affected platelets, secretion of dense granules does not occur in response to all agonists; however, it occurs independently of the ability to aggregate and is dependent on or concurrent with thromboxane production.

In studies concurrent to this dissertation research, the resting and post-stimulation phosphorylation of affected and control platelet proteins was measured to assess the generation of intracellular second messengers.[255] It was found that both affected and control platelets have similar phosphorylation patterns of the major, identified cytosolic proteins. Unexpectedly, it was discovered that affected platelets do not phosphorylate a 64-67kDa protein to the same extent as control platelets. This protein has not been previously described and its functional significance is unknown.[255]

Finally, in additional studies, thromboxane production was measured in affected and control platelets.[256] Thromboxane is produced

from membrane arachidonic acid following platelet activation and acts as a positive feedback stimulus to further enhance platelet activation. It was found that affected platelets have increased production of thromboxane in response to those agonists which stimulate secretion and decreased production of thromboxane in response to those agonists which do not stimulate secretion. This indicates that the pathway for thromboxane production is intact in affected platelets but not under proper regulatory control. It also suggests that secretion in affected platelets is strongly dependent on thromboxane production and that aggregation is not directly correlated to thromboxane production.

The aim of the current dissertation is to further explore the biochemistry of signal transduction in platelets from affected and control canine platelets in order to identify and/or characterize a defective pathway(s) or enzyme(s). At the time of the initiation of this dissertation research, the studies on GPIIb-IIIa,[299] fibrinogen binding[298,119] and aggregation/secretion[254] in BHT had been completed and the study on protein phosphorylation was underway in the laboratory.[255] Thus, it was determined that measurement of resting and post-stimulation cytosolic free ionized calcium ($[Ca^{++}]_i$) was the next important event to be investigated due to the central role of Ca^{++} as a second messenger in platelet activation. Intracellular Ca^{++} is integral in determining the activity of a number of effector enzymes and the organization of cytoskeletal proteins. It was anticipated that there would be decreased Ca^{++} mobilization in affected platelets in response to those agonists which do not cause platelet aggregation.

Following the conclusion of the calcium studies for this dissertation, the concurrent data on protein phosphorylation and the preliminary results on the thromboxane production in affected platelets became available. Therefore, it was determined that the measurement of platelet cytosolic pH_i fluxes was an appropriate area of investigation to best integrate available data. Increased cytosolic pH_i frequently accompanies platelet activation and is associated with activation of phospholipase A_2 (the enzyme responsible for arachidonic acid release and subsequent thromboxane production), release of internal Ca^{++} and organization of the platelet cytoskeleton. In addition, the adrenergic receptor is a 64kDa which is closely associated with, if not actually part of, the Na^+/H^+ antiport which is involved in the regulation of pH_i. If the adrenergic receptor were the same as the poorly phosphorylated 64-67kDa protein in affected platelets, then abnormal alkalinization responses would be anticipated in activated BHT platelets.

Finally, because of a suspected role in the mediation of abnormal responses in BHT, the measurement of phospholipase C activity was judged to be essential for the final portion of the dissertation research. Previous aggregation studies[254] had shown that aggregation and complete platelet activation occur in BHT when phospholipase C is bypassed, indicating that phospholipase C was not being normally activated in response to those agonists unable to cause aggregation. In contrast, the data on protein phosphorylation, Ca^{++} mobilization and thromboxane production suggest that the second messengers generated by phospholipase C were being produced. In order to assess phospholipase C activity, the production of 1,2-diacylglycerol was measured. 1,2-Diacylglycerol is

one of the second messengers generated by the phospholipase C mediated hydrolysis of membrane phosphoinositides.

In summary, this study supports the hypothesis that BHT affected platelets have a defect in several biochemical pathways and it details signal transduction defects that relate to cell dysfunctions.

CHAPTER 1: LITERATURE REVIEW

Platelets occupy a vital role in the maintenance of normal hemostasis in concert with the coagulation system and the vascular wall. In response to a break in the integrity of the vessel wall, platelets form a primary hemostatic plug by adhering to the subendothelial tissue and secondarily recruiting additional platelets. In addition to forming the primary plug, platelets play an important part in the coagulation cascade by providing a phospholipid surface to accelerate the activation of several coagulation factors which convert circulating fibrinogen to insoluble fibrin. The fibrin then enmeshes and stabilizes the primary plug.[184,403]

Platelets respond to a wide variety of both inhibitory and stimulatory substances. Platelet plasma membrane receptors bind to specific external stimuli and subsequently transmit the signal into the cell. This culminates in a distinct pattern of physiologic reactions commonly termed the platelet response. These reactions can be elicited both <u>in vitro</u> and <u>in vivo</u> and include: (1) adhesion of platelets to a foreign surface; (2) shape change from a smooth disc to a sphere with numerous pseudopodial projections; (3) aggregation by the formation of platelet-to-platelet bridges; and (4) the release reaction characterized by the secretion of the contents of cytoplasmic granules and the production of arachidonate metabolites.[212,377,403,478]

Although platelets respond to a complex variety of agonists and antagonists, only a limited number of effector pathways and second messengers are utilized to produce a cellular reaction.[212] None of the physiologic agonists can penetrate the plasma membrane of intact platelets.[478] Instead, exogenous stimuli bind to a specific receptor(s) on the platelet surface. The activated receptor transmits this signal into the interior of the cell by regulating the activity of specific effector pathways such as enzyme systems or membrane ion channels. These effector systems alter intracellular second messengers which then mediate the activity, phosphorylation or structure of intracellular proteins. This alteration in the cellular protein triggers the physiologic response. The process by which an external stimulus is translated into the cellular response is termed signal transduction or processing.[212,377,403] Amplification or inhibition of the signal can occur at any step due to the multiple points of interaction or communication between the different arms of the signal transduction pathway. Activation of platelets causes the production and/or release of substances which act as positive or negative feedback to respectively amplify or inhibit the platelet response.[9,103,212]

Although a large amount of information about platelet activation and function has been published in the past few decades, there are still many unanswered questions. Most of the published information has been limited to studies in platelets from humans, rats or rabbits. The material presented in this literature review will attempt to summarize the pertinent findings. It is assumed that canine platelets act in a similar manner to that published; however, relatively little has been

published on canine platelets. Known deviations in the activity or behavior of canine platelets from human platelets will be described and discussed.

PLATELET PRODUCTION

The average circulating platelet number in normal human blood is 248,000 platelets/ul, with a range of 148,000-348,000/ul.[461] The normal circulating platelet number in dog blood is similar with an average of 300,000 platelets/ul and a range of 200,000 to 500,000/ul.[183] Human platelets in peripheral blood have a mean survival time of six to ten days[403] while dog platelets have a shorter survival time of only four to seven days.[182] Platelets are removed from the circulation by consumption in the hemostatic process, removal by the reticuloendothelial system as part of the normal aging process or destruction as part of a pathologic immune-mediated disorder. In a healthy animal, the number of circulating platelets is relatively constant and is maintained by the production and release of platelets from the precursor megakaryocytes.[182]

Megakaryocytopoiesis

Megakaryocytopoiesis is a developmental cycle involving at least five stages: (1) the commitment of a pluripotential stem cells to megakaryocyte differentiation; (2) megakaryocyte mitotic activity to expand cell numbers; (3) variable numbers of endomitotic divisions to increase nuclear number; (4) cytoplasmic growth with the acquisition of

organelles and platelet specific proteins; and (5) formation of demarcation membranes within the megakaryocyte cytoplasm to release platelets into the circulation.[253,374]

Committed stem cells form two functionally identifiable classes of megakaryocyte precursor cells: (1) the burst forming unit-megakaryocyte (BFU-M); and (2) the colony forming unit-megakaryocyte (CFU-M). BFU-M are differentiated by producing multiple clusters of megakaryocytes which contain larger numbers of megakaryocytes than the CFU-M. The next developmental stage is a small, mononuclear cell which cannot be identified morphologically but which can be identified by the expression of phenotypic platelet specific markers. These cells are a transition cell between the progenitor cell and the megakaryocyte.[144] Megakaryocytes are morphologically identifiable in the bone marrow by their large size and their lobulated nuclei. The DNA content of the megakaryocytes varies, with most cells having ploidy values from 8 to 64N. The size and number of platelets produced from each cell is dependent on the nuclear polyploidization of the megakaryocyte.[253]

Regulation of Megakaryocytopoiesis and Platelet Production

The control of megakaryocytopoiesis and platelet production can be divided into two distinct phases: (1) a proliferative, mitotic phase which involves the BFU-M and the CFU-M and is responsible for maintaining megakaryocyte numbers; and (2) a non-proliferative phase stage which involves the non-mitotic development of megakaryocytes and culminates in the release of platelets.[253,374]

Mitotic division of the megakaryocyte precursors is not directly influenced by circulating platelet numbers; instead, this phase is regulated by, and serves to maintain, megakaryocyte and progenitor cell numbers.[253] CFU-M requires the presence of a factor(s) with megakaryocyte colony stimulating activity (M-CSA) to promote the formation of colonies of megakaryocytes in vitro. Whether this activity is due to the presence of a single megakaryocyte colony stimulating factor (M-CSF) or to a collection of factors with M-CSA has not been determined.[144] Factors affect megakaryocytopoiesis by having M-CSA themselves or by promoting CFU-M proliferation in concert with other substances. These factors include granulocyte-macrophage colony stimulating factor, interleukin 3, interleukin 1, interleukin 6, erythropoietin and thrombopoietin.[144]

The non-proliferative phase of megakaryocyte development, on the other hand, is responsive to circulating platelet numbers. The development is characterized by the appearance of cytoplasmic organelles, the mitotic division of the nucleus without division of the cytoplasm (endomitosis), the appearance of membrane antigens and platelet release. Thrombocytopenic animals contain a substance in their urine and plasma, termed thrombocytosis-stimulating factor (TSF) or thrombopoietin. TSF promotes megakaryocyte development in at least three ways: (1) stimulation of the commitment of progenitor cells to the megakaryocyte line; (2) stimulation of megakaryocyte endomitotic divisions to increase cell size and ploidy and (3) shortening of the megakaryocyte maturation time. Whether TSF is a single cytokine or a

collection of cytokines has not been determined. The site of production of thrombopoietin is not known.[144,182]

PLATELET STRUCTURE

Canine and human platelets are of a similar volume (7.6-8.3 fl)[184] and, in the resting state, they circulate as discoid cells which are 2-3 um in diameter.[403] Platelets are anucleate and contain only a small amount of DNA in the mitochondria.[403] They have limited ability to synthesize proteins.[169] On a dry weight basis, platelets contain approximately 50% protein, 8.5% carbohydrate and 41.5% lipid and other constituents.[184] Microscopic examination of Wright's stained blood smears reveals granular organelles which are dispersed throughout the cytoplasm. Electron microscopic examination of platelets defines the unit (plasma) membrane, the open canalicular system, the dense tubular system, mitochondria, glycogen stores, three types of secretory granules and cytoskeletal elements.[452]

Mitochondria and Glycogen

Resting platelets do not require much energy to maintain their anabolic processes, but stimulated or activated platelets expend large amounts of catabolic energy. ATP is essential to maintain platelet activation with the different platelet responses varying in their energy requirement. The catabolic ATP demands, in order of increasing need, are shape change, aggregation, alpha- and dense-granule secretion, lysosomal granule secretion and arachidonic acid liberation.[171]

Platelets have large stores of glycogen which can be utilized for ATP production.[169,171] Platelets contain relatively few mitochondria and their number and size are smaller than the mitochondria present in muscle cells.[169,266] Although oxidative metabolism produces approximately 80% of the total ATP needed in the resting state, platelets have a well-developed glycolytic system which can fully compensate for any increase in energy demand associated with activation. Simultaneous inhibition of oxidative phosphorylation and glycolysis or glucose starvation of cells severely inhibits platelet responses.

Granules

The platelet cytosol contains three identifiable types of secretory granules: (1) alpha granules; (2) dense granules; and (3) lysosomal granules.

Alpha granules are the most numerous of all the granules. On ultrastructure they appear as membrane bound round or oval structures with a moderately electron-dense matrix.[184,266] They contain a variety of proteins which function in coagulation, inflammation and wound healing. These proteins can be divided into three groups: (1) proteins specific to platelets (platelet factor 4, platelet derived growth factor, β-thromboglobulin); (2) proteins which function in the coagulation cascade (fibrinogen, factor V, factor VIII/von Willebrand's factor); and (3) proteins not specific to platelets which have a variety of functions (thrombospondin, fibronectin, albumin, α_2-plasmin

inhibitor, histidine-rich glycoprotein, high-molecular weight kininogen, α-anti-trypsin, α_2-macroglobulin).[403]

Dense granules are less numerous than alpha granules and appear as round to oval structures with an electron dense core surrounded by an electron-lucent area. They contain primarily adenine nucleotides (ATP and ADP), amines (serotonin, histamine and catecholamines) and bivalent cations (Ca^{++} and Mg^{++}).[184,266] All species have relatively high levels of ADP, ATP and serotonin in their dense granules while the quantity of divalent cations varies. Human platelet dense granules contain mainly Ca^{++} while canine platelet dense granules contain relatively equal quantities of Ca^{++} and Mg^{++}.[267] Within the granules, Ca^{++}, inorganic pyrophosphate, ATP and ADP form insoluble high molecular complexes. Except for serotonin, which is freely exchangeable, the contents of the dense granules are almost totally unexchangeable with their cytoplasmic counterparts.[403]

Lysosomal granules morphologically resemble alpha granules. They contain four types of acid hydrolases: (1) acid proteases (endo- and exopeptidases); (2) acid glycosidases (β-hexosaminidase, β-glucoronidase, α-mannosidase and galactosidase); (3) acid phosphatase; and (4) aryl sulfatases.[403] The release of lysosomal granules requires a strong stimulus and only partial release occurs. The function of these granules is unknown. Platelets phagocytize extracellular material; however, the formation of a phagolysosome and subsequent destruction of the phagocytized material does not occur.[480] Instead, the lysosomes release their contents into the external media where they

may play a role in the clearing of thrombi, the inactivation and degradation of heparin and the inflammatory response.[169,403]

Cytoskeleton

The platelet cytoskeleton consists of four components: (1) the membrane skeleton; (2) the microtubular coil; (3) actin and myosin filaments; and (4) a variety of associated proteins.[130] The cytoskeleton maintains the normal platelet resting discoid shape and participates in the formation of a contractile system necessary for shape change, pseudopod formation, contraction and granule secretion.[184]

The membrane skeleton is located directly under the plasma membrane and is composed of a network of actin filaments. It is a submembranous lining that stabilizes the plasma membrane and helps maintain its shape.[131] The membrane skeleton pool of actin is separate from the cytoplasmic pool that is involved in contraction. Unlike the cytoplasmic pool, the membrane skeleton actin is resistent to Ca^{++}-induced depolymerization. Actin binding protein links the membrane skeleton with the membrane glycoproteins (GP) Ib-IX, Ia, IIa, and a glycoprotein of M_r 250,000 of unknown function.[128,129] The attachment of the membrane skeleton to GPIb-IX determines its location within the membrane, thereby regulating the ability of GPIb-IX to bind to von Willebrand's factor following injury to the vessel wall. Because of its attachment to both GPIb (one of the thrombin receptors) and GPIa (a proposed collagen receptor), it has been proposed that the membrane skeleton also plays a role in signal transduction.[131] During platelet

aggregation, activation of the intracellular Ca^{++}-dependent protease (calpain) causes the hydrolysis of actin binding protein. This releases the membrane skeleton from the membrane glycoproteins and permits reorganization of the cytoskeleton.[132]

The second component of the cytoskeleton, the microtubular coil, consists of a single strand of polymerized tubulin coiled upon itself.[30] The microtubule coil is located under the membrane skeleton and helps maintain the resting discoid shape.[452] During platelet activation, the platelet organelles are compressed into the cell center and surrounded by a constricting rim of microtubules and microfilaments. Some studies indicate that the microtubule coil dissolves upon platelet activation and reassembles in its new location,[406] while others suggest that microtubule disassembly-reassembly is not necessary for platelet activation.[455] In support of the latter statement, taxol treatment of platelets, which stabilizes microtubules and prevent their disassembly, does not inhibit platelet shape change or the centralization of organelles.[452]

The third component of the cytoskeleton is the actin and myosin filaments. Actin comprises approximately 15-20% of the platelet protein.[130] It is divided into two distinct pools, one associated with the membrane cytoskeleton as already discussed and the other associated with the platelet cytosol. In the resting state, 40-50% of the total actin is present in the polymerized state. The remainder is present as monomeric actin complexed to a protein, profilin, which stabilizes the actin. The polymerized actin forms a network which helps maintain the

cell shape and the distribution of the organelles dispersed in the cytoplasm. Following activation, 70-80% of the total actin becomes polymerized.[130] This network of polymerized actin is present as bundles of filaments associated with actin binding protein. Polymerized actin is found in the forming pseudopodia as well as in the cytoplasmic network. The cytoplasmic network is rich in actin and myosin and is associated with the centralizing organelles.[478] Once platelet activation occurs and the membrane skeleton is released from the plasma membrane, the cytoplasmic actin filaments become associated with the membrane GPIIb-IIIa. This association only occurs if aggregation is present. It does not occur if platelets are activated but aggregation is prevented.[308,448]

Myosin is present in the resting platelet primarily as monomers which are diffusely distributed throughout the cytoplasm. During platelet activation, increasing concentrations of cytosolic free calcium activate the Ca^{++}-dependent enzyme, myosin light chain kinase (MLCK). Activated MLCK phosphorylates the 20kDa light chain of myosin, allowing myosin to polymerize and interact with actin.[478] The interaction of myosin with actin activates the myosin-associated Mg^{++}-dependent ATPase[1] and the subsequent generation of a contractile force results from the sliding of the myosin and actin filaments past each other.[228] Following stimulation with most agonists, myosin phosphorylation is closely correlated with shape change.[95] The subsequent actin-myosin association also plays a role in the centralization of cytoplasmic granules and secretion.[142]

Finally, there are a variety of other cytoplasmic structural proteins associated with the cytoskeleton. These proteins can be divided into four basic classes: (1) those that complex monomeric actin and help stabilize it (ie. profilin); (2) those that cross-link actin filaments and regulate the formation of an organized network (ie. actin binding protein and α-actinin); (3) those that stabilize actin filaments (ie. tropomyosin); and (4) those that cap actin filaments and restrict their length (ie. gelsolin and 235K protein).[478]

Dense Tubular System

The dense tubular system (DTS) is an internal membrane system analogous to the sarcoplasmic reticulum in muscle cells. There is no direct communication between the DTS and the external environment, although the DTS does form junctional complexes with the open canalicular system.[450] The role of these complexes is not known. The DTS is derived from the megakaryocyte endoplasmic reticulum and has been demonstrated to act as an internal calcium (Ca^{++}) storage site.[51] Numerous studies have identified an energy-driven Ca^{++}-ATPase on the membranes of the DTS which mediates Ca^{++} uptake and internal sequestration.[107,111,166,265] The DTS also contains a receptor-operated Ca^{++} channel that mediates Ca^{++} release into the cytoplasm following appropriate stimulation. This channel is separate from the Ca^{++}-ATPase transport system and does not require ATP to function. Ca^{++} release via this channel is not induced by increasing concentrations of cytosolic free ionized calcium ($[Ca^{++}]_i$) but it is stimulated by the production of the water soluble second messenger inositol trisphosphate (IP_3) from the

hydrolysis of membrane phosphatidylinositol 4,5-bisphosphate (PIP_2).[2] Finally, the DTS is the major site in the platelet for both cyclooxygenase and thromboxane synthetase activity.[69,227]

Open Canalicular System

The open canalicular system (OCS) consists of tortuous, interconnected invaginations of the plasma membrane within the platelet cytosol which extend from one surface of the platelet to the other.[453] These channels serve as one mechanism by which cytoplasmic granules are released to the external environment during secretion.[454] The OCS also is a route for the internalization of substances from the plasma,[453] a site for the clearance of membrane receptor complexes,[230] and a membrane reserve that can be evaginated onto the platelet surface during platelet activation.[118]

Plasma Membrane

The platelet plasma membrane is a typical lipid bilayer characteristic of many cell types. It's two main functions are to provide: (1) a permeability barrier to limit the transport of substances into and out of the platelet cytosol; and (2) a matrix to support the surface receptors involved in cell interactions and contact.[26] The membrane is composed of 35% lipids, 57% proteins and 8% carbohydrates. The lipids consist of primarily the phospholipids and cholesterol. The phospholipids are arranged in a fluid bilayer with their hydrophilic heads oriented toward the aqueous internal cytoplasm or external

environment and their hydrophobic tails oriented towards the center of the bilayer. The portion of the bilayer in contact with the external environment contains primarily the neutral phospholipids (phosphatidylcholine and sphingomyelin) while the inner portion of the membrane in contact with the cytosol contains primarily the negatively charged phospholipids (phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol).[370]

At physiologic temperatures, the platelet plasma membrane is fluid. Cholesterol is dissolved in the phospholipids and helps modulate the fluidity of the bilayer.[370] Distributed throughout the plasma membrane bilayer are proteins and glycoproteins which function as specific surface receptors, transport systems or enzymes. The hydrophobic portions of the proteins or glycoproteins interact with the center of the bilayer while the hydrophilic portions interact with either the polar surfaces of the bilayer or the aqueous external or internal regions.[370] In the normal physiologic state, these proteins or glycoproteins are able to move laterally over the surface of the membrane; however, they are not easily removed from the membrane except with the use of detergents. Specific platelet membrane glycoproteins reported to be involved in the processes of adhesion, aggregation or activation include GPIa-IIa, GPIb, GPIc-IIa, GPIIb-IIIa, GPIV, GPV, GPIX, GMP-140 and PTA1.[78,215]

GPIa-IIa

GPIa-IIa exists as a heterodimer and is part of a superfamily of adhesion receptors termed the integrins.[215] The complex is related to the very late activation antigen 2 (VLA-2) present on the surface of activated T lymphocytes, with GPIa being analogous to the alpha subunit and GPIIa being analogous to the beta subunit. Studies on purified GPIa-IIa indicate that the complex plays a role in mediating the Mg^{++}-dependent adhesion of platelets to collagen.[400,401] Evidence supporting the role of GPIa in collagen-induced platelet activation comes from studies on the platelets from a person with a deficiency of membrane GPIa. This patient had a mild clinical bleeding disorder characterized in the laboratory as a failure of platelets to respond to collagen stimulation.[282] Recent studies have reported that GPIa is actually two distinct proteins, Ia and Ia*, which can be separated by two-dimensional nonreduced/reduced gel electrophoresis. Ia* may be a granule constituent that is only present on the surface of activated platelets. It is this glycoprotein which is absent in the patient with the bleeding disorder.[35]

GPIb

GPIb is a major sialoglycoprotein expressed on the surface of non-activated platelets. It consists of alpha and beta chains and it forms a non-covalent complex with GPIX. GPIb also forms a complex with the cytoskeletal protein, actin binding protein, linking it to the cytoplasmic membrane skeleton as discussed above.[78,128,129] In

experimental isolation procedures, GPIb consistently coisolates with several poorly defined cellular components. This has lead to the proposal that the GPIb complex plays a role in signal transduction.[78] At physiologic shear rates, the alpha chain of GPIb functions in the von Willebrand factor (vWF)-mediated adhesion to the exposed subendothelium.[197] The exact mechanism by which vWF mediates this adhesion is not known; however, vWF binds readily to heparin-like glycosaminoglycans and collagen, both of which are present in the subendothelium. Binding to the subendothelium induces a change, possibly conformational, in vWF, which allows it to bind to GPIb and subsequently cause contact-mediated platelet activation.[78,215]

An autosomally recessive deficiency of the membrane complex GPIb-IX has been identified in humans and is termed Bernard-Soulier syndrome (BSS).[260] It is characterized by mucocutaneous bleeding of variable severity. It clinically resembles von Willebrand disease (vWD), a disorder associated with the decreased function or production of vWF. Unlike vWD, however, the defect in BSS is not corrected by the addition of normal vWF since affected platelets are not able to bind vWF.[260]

BSS is characterized by the inability of affected platelets to adhere to the exposed subendothelium. <u>In vitro</u> aggregation in response to stimulation with ADP, collagen or epinephrine is normal while aggregation in response to thrombin is delayed.[142] GPIb contains a binding site for thrombin on its alpha chain.[197] The importance of this site in platelet activation in response to thrombin is not clear. As mentioned, the platelets from humans with BSS aggregate in response to

thrombin, although the rate of aggregation is reduced.[260] In addition, studies have shown that the binding of thrombin to GPIb is not directly coupled to activation of the major effector enzymes systems including phospholipase C, protein kinase C or phospholipase A_2.[185]

Platelets from BSS patients and neuraminidase-treated platelets have a reduced surface charge due to decreased sialic acid density. This is responsible for decreased electrophoretic mobility and, presumably, the shortened survival time of these affected or altered platelets.[147,286] Studies on platelets from humans with BSS have shown that there is also altered organization of the phospholipid bilayer, suggesting a role for GPIb in governing membrane fluidity.[481]

GPIc-IIa

GPIc is a relatively minor membrane glycoprotein which forms a heterodimer complex with GPIIa. The complex is a member of the integrin superfamily and is analogous to the very-late-activation antigen 3 (VLA-3) on T-lymphocytes. It mediates adhesion to fibronectin and may interact with cytoskeletal actin filaments.[78,215]

GPIIb-IIIa

The GPIIb-IIIa complex is a major constituent of the platelet plasma membrane and is another member of the integrin superfamily. GPIIb is composed of two disulfide-linked alpha and beta subunits while GPIIIa consists of a single polypeptide chain.[307] The two glycoproteins

exist as a Ca^{++}-dependent heterodimer complex.[63] Studies have identified a number of binding sites for Ca^{++} on GPIIb.[307] In resting platelets at physiologic temperatures, the complex can be reversibly dissociated by short (less than 10 minutes) incubation with the divalent cation chelator ethylenediamine-tetraacetate (EDTA). Longer incubation periods cause irreversible dissociation of the complex.[126]

The GPIIb-IIIa complex contains the activation dependent fibrinogen receptor and, as such, plays an essential role in platelet aggregation.[303] This role of the complex was established based on the following findings: (1) platelets with little or no membrane GPIIb-IIIa cannot bind fibrinogen; (2) platelets can bind approximately 40,000 molecules of fibrinogen, the same as the number of molecules of GPIIb-IIIa; (3) isolated GPIIb-IIIa adsorbed to plastic can bind fibrinogen in the presence of Ca^{++}; and (4) several monoclonal antibodies directed against portions of the GPIIb-IIIa complex can inhibit fibrinogen binding.[478]

Monoclonal antibodies that recognize only the GPIIb-IIIa complex bind to both resting and stimulated platelets, indicating that an intact GPIIb-IIIa complex is present on resting platelets.[261] Following activation, additional GPIIb-IIIa complexes are exposed on the platelet surface. Whether these complexes originate from increased exposure of the open canalicular system[466] or fusion of the alpha granules with the plasma membrane[89] remains to be determined. Recent studies have shown that the membrane GPIIb-IIIa complexes on resting platelets are actively

recycled into the open canalicular system, to secretory granules and back to the surface membrane.[446]

The GPIIb-IIIa complex on resting platelets cannot bind fibrinogen. Some stimulation of the platelet is required to expose the fibrinogen binding site on the complex. This finding is supported by the discovery of monoclonal antibodies that only recognize the activated GPIIb-IIIa complex.[373] The exposure of the fibrinogen binding site may be dependent on a conformational change in GPIIb-IIIa or a rearrangement in the surrounding membrane.[215,307] Using studies on permeabilized platelets, it has been proposed that there are two distinct routes leading to fibrinogen binding site exposure.[371] The first route involves hydrolysis of membrane phosphoinositides, the production of second messengers and the subsequent elevation in $[Ca^{++}]_i$. The second route is Ca^{++}-independent and may involve regulation by G proteins. Alternatively, it has been proposed that aggregin (a 100kDa membrane protein) sterically interferes with the GPIIb-IIIa complex. Proteolytic digestion of aggregin (i.e. chymotrypsin) or the binding of ADP to aggregin induces a conformational change in the protein which leads to the exposure or unmasking of the fibrinogen binding site on GPIIb/IIIa.[82,83]

Once activated, the GPIIb-IIIa complex can bind to a variety of adhesive proteins including fibrinogen, fibronectin, vitronectin, collagen and vWF.[215] GPIIb-IIIa recognizes the peptide sequence Arg-Gly-Asp (RGD) on these adhesive proteins; however, optimal binding of fibrinogen may require recognition of addition sequences.[307,322]

Because of its relative abundance and/or its higher affinity, fibrinogen is the primary mediator of platelet aggregation.[215] In the absence of fibrinogen, alternative adhesive proteins (such as vWF) can bind to GPIIb-IIIa and sustain aggregation.[105]

The GPIIb-IIIa complex has numerous other functions besides the mediation of fibrinogen binding. First, GPIIb-IIIa binds to the cytoskeletal proteins talin and actin filaments following the onset of platelet aggregation.[181,308,429] This interaction connects the external adhesive proteins to the internal contractile proteins generated after activation and facilitates the process of clot retraction. Secondly, the binding of fibrinogen to GPIIb-IIIa initiates Na^+/H^+ exchange and subsequent TxA_2 production in epinephrine-stimulated platelets by some, as yet, undefined mechanism.[20] Thirdly, the GPIIb-IIIa complex itself appears to play role in Ca^{++} influx in both resting and stimulated platelets.[52,316,468,469] Lastly, recent studies have demonstrated activation-induced phosphorylation of GPIIIa which may be involved in the induction of granule secretion.[207,296]

Glanzmann's thrombasthenia is an autosomal recessive disorder identified in human platelets which is characterized by a deficiency or altered function of the GPIIb-IIIa complex.[260] The disorder is clinically characterized by variable severity of mucocutaneous bleeding. Laboratory findings include abnormal clot retraction and failure of platelets from affected individuals to aggregate in response to any of the physiologic agonists. Affected platelets adhere to subendothelial

tissue and bind vWF, but they fail to spread or to recruit additional platelets to form a primary hemostatic plug.[260]

GPIV

GPIV is a major component of the platelet membrane which is also identified as GPIIIb. It has been recently reported to be the receptor for thrombospondin[8] and may play an auxiliary role in aggregation. Platelets release thrombospondin from their alpha granules during secretion which then binds to GPIV in a Ca^{++}-dependent manner. The thrombospondin also binds to fibrinogen and may form bridges which stabilize the platelet aggregates.[297] Other studies identify GPIV as a receptor involved in collagen adhesion.[415]

GPV

The role of GPV in platelet function is not known.[78] In vivo, GPV is cleaved by thrombin to release a water-soluble fraction, leading to the suggestion that it serves as a thrombin receptor. Recent studies have shown, however, that a monoclonal antibody to GPV does not inhibit thrombin activation, thereby negating its role in thrombin-induced platelet activation.[36]

GPIX

GPIX is found tightly bound to GPIb in the platelet membrane but its role in this complex is unknown.[78]

GMP-140

GMP-140 is a membrane protein within the secretory granules which is redistributed to the platelet plasma membrane following platelet activation. Monoclonal antibodies to GMP-140 serve as markers for activation. The role of GMP-140 is not known; however, it appears to be related to a gene family of receptors called selectins. This family includes ELAM-1, a cytokine-inducible receptor on endothelial cells that mediates neutrophil binding.[259] A monoclonal antibody directed against GMP-140 inhibits collagen- and thrombin-induced platelet aggregation, suggesting a role for GMP-140 in mediating platelet aggregation.[297]

PTA1

PTA1 is a heavily sialated glycoprotein of M_r 67kDa which is present on the plasma membrane of platelets and lymphocytes. It is believed that the associated sialic acid residues that protrude from the plasma membrane strongly contribute to the surface charge along with similar residues associated with GPIb. Monoclonal antibodies directed against PTA1 initiate platelet aggregation and secretion. It has been proposed that this platelet activation involves protein kinase C (PKC) and subsequent phosphorylation of the 47kDa protein. During activation by the monoclonal antibody, the PTA1 antigen is phosphorylated. This antigen is phosphorylated during activation by the physiologic agonists collagen and thrombin and the non-physiologic agonist phorbol. The role of PTA1 in platelet activation <u>in vivo</u> is not known.[367]

THE PLATELET RESPONSE

The primary role of platelets in hemostasis is to form a platelet plug in response to injury to the vessel wall. Although platelets respond to a wide variety of stimuli, they do so by eliciting a limited series of physiologic events termed the basic platelet response: adhesion of platelets to the site of injury, shape change, aggregation, and the secretion of granule contents and release of arachidonate metabolites. The number of these responses elicited depends on: (1) the number and type of stimulating agonists; and (2) the concentration of agonist(s) in contact with the platelets.[377,403]

Adhesion

Platelets will adhere to almost any foreign surface. Whether the platelets become further activated depends on the surface involved. *In vitro*, unactivated platelets which adhere to a surface can either detach or remain adhered.[29] *In vivo*, platelets rarely, if ever, come in contact with foreign surfaces since circulating plasma proteins readily adsorb to such surfaces. The degree of adhesion and subsequent activation of the circulating platelets depends on the protein adsorbed. Albumin inhibits adhesion while fibrinogen, vWF, fibronectin, vitronectin, thrombospondin, α_2-macroglobulin and laminin all promote adhesion.[295]

Platelets adhere to surfaces in a process mediated by plasma membrane glycoproteins. At high shear rates, adhesion of platelets to

the subendothelium is probably mediated by membrane GPIb and vWF.[295,354] Studies in vitro indicate that at high flow rates the exposure of vWF to a foreign surface causes an alteration in vWF, allowing it to bind to GPIb which is already exposed on the membrane of resting platelets. This leads to contact-induced platelet activation and the exposure of the fibrinogen binding site on GPIIb-IIIa. Fibrinogen, vWF and the other adhesive proteins are able to bind to the complex and mediate spreading or platelet-to-platelet adhesions.[264] The role of GPIb and vWF in platelet adhesion is substantiated by the absence of platelet adhesion in patients with Bernard-Soulier syndrome.[260]

The role of GPIa in platelet adhesion is less clear. In vivo, fibrillar collagen is the most likely subendothelial element that initiates platelet adhesion since it is a strong inducer of platelet aggregation.[309] As discussed, GPIa has been identified as a collagen receptor. Studies on the platelets of a patient with a congenital deficiency of GPIa have shown decreased adhesion to the subendothelium as well as absence of shape change and aggregation in response to collagen. The abnormal adhesion appears to be due to defect in spreading and not in the initial adherence. These studies suggest that vWF mediates the initial attachment of the platelet to the subendothelium while collagen mediates the subsequent spreading.[282,283] Recent studies have shown that platelets adhere to collagen in both a Mg^{++}-dependent and a bivalent-cation-independent manner. The Mg^{++}-dependent adhesion is mediated by GPIa. The mechanism mediating the bivalent-cation-independent adhesion is not known. It has been proposed that this adhesion is mediated by vWF or some other adhesive protein

which can bind to collagen and act as a bridge connecting collagen to the platelet.[476]

Adhesion requires metabolic energy, a functioning contractile mechanism and an external source of bivalent cations. It causes activation similar to that caused by a strong agonist such as thrombin. Adhesion is not dependent on the formation of thromboxane A_2 (TxA_2) or the release of ADP.[295] The initial phase of adhesion is the contact phase which is followed by the spreading phase where there is increased contact between the platelet membrane and the surface. One group has proposed that there are three steps involved in the process of adhesion: (1) the attachment of vWF to the subendothelium; (2) the binding of vWF to the platelet; and (3) platelet spreading and aggregate formation.[309] This theory is supported by studies using monoclonal antibodies. Antibodies against GPIb block adherence to the subendothelium at low and high shear rates while antibodies against GPIIb/IIIa decrease adherence at high shear rates only. This suggests that GPIb is the primary receptor for adhesion while GPIIb/IIIa mediates spreading since it is only at high shear rates that spreading becomes important.[104]

Shape Change

Shape change is the initial, reversible response of platelets in suspension to a stimulus.[377] Morphologically, it is characterized by the loss of the platelet's discoid shape, transition to a spherical shape, the extension of pseudopods or filopodia and the movement of the internal organelles to the cell center where they are surrounded by a

ring consisting of the constricting microtubular coil and actin filaments.[452] Shape change is accompanied by actin polymerization, both as the formation of bundles within the filopodia and as a network around the centralizing granules.[478] The formation of pseudopods is inhibited by a compound, cytochalasin B, which caps actin filaments. The subsequent addition of thrombin, causes granule centralization because the agonist-induced increase in $[Ca^{++}]_i$ still supports the formation of a contractile cytoskeleton containing actin and myosin. These findings suggest the importance of actin polymerization in the extension of filopodia and the importance of the actin-myosin interaction in granule centralization.[142]

Shape change occurs within a few seconds of stimulation, requires metabolic energy and is independent of the presence of external divalent cations (ie. Ca^{++} and/or Mg^{++}) or fibrinogen.[172,377] Granule centralization is not dependent on the release of arachidonic acid metabolites. Cyclooxygenase inhibition by aspirin blocks granule centralization only in those cases where there is also inhibition of myosin light chain phosphorylation, such as with the weak agonist epinephrine.[142,143] Physiologically, shape change precedes aggregation and secretion; however, shape change may occur without subsequent aggregation and secretion.

Shape change and granule centralization are correlated with the phosphorylation of the 20kDa myosin light chain.[95,142] Myosin light chain phosphorylation is stimulated by increasing cytosolic $[Ca^{++}]_i$ in a calmodulin-dependent manner.[96,163] Phosphorylation of myosin light

chain may also occur independent of an increase in cytosolic Ca^{++}.[153] It has been proposed that this phosphorylation occurs at a site separate from the Ca^{++}-dependent phosphorylation site and is mediated by PKC.[276] Other studies have demonstrated that increasing cytosolic pH (pH_i) and an influx of external Na^+ plays a role in cytoskeletal reorganization and, therefore, may play a role in the initiation of shape change.[231]

Shape change and granule centralization are not a prerequisite for aggregation and secretion. This is best demonstrated by those agonists which are selective activators of PKC (ie. phorbol myristate acetate or synthetic diacylglycerides).[142] These agonists cause some degree of pseudopod formation, possibly due to PKC-mediated stimulation of actin polymerization; however, granule centralization does not occur in spite of the initiation of aggregation.[142] In addition, shape and granule centralization are not limited to activated platelets. Exposure of platelets to cold or to antimitotic drugs (such as vincristine or colchicine) cause shape change due to the disassembly of the microtubule coil. Since there is no associated myosin light chain phosphorylation, there is no granule centralization.[142,452]

Aggregation

In the physiologic setting, aggregation generally follows shape change, though it requires a stronger stimulus than shape change. <u>In vitro</u>, two types of aggregation responses can be demonstrated: (1) reversible aggregation which is not accompanied by secretion; and (2) irreversible aggregation which is accompanied by secretion and the

release of arachidonate metabolites.[377] Aggregation requires fibrinogen (or some other adhesive protein), extracellular divalent cations (Ca^{++} and/or Mg^{++}), exposure of the fibrinogen binding site and platelet-to-platelet collisions.[377,478] The process of aggregation itself may be a passive, non-energy requiring process once shape change and exposure of the fibrinogen binding sites occur.[403]

Aggregation is proposed to occur when a dimeric fibrinogen molecule binds to two adjacent platelets, creating a platelet-to-platelet bridge.[303,478] Binding of fibrinogen to the platelet membrane requires some activation-induced alteration in the GPIIb-IIIa complex which subsequently permits fibrinogen to attach. The binding of fibrinogen to its receptor is specific, saturable and inhibited by the chelation of external Ca^{++} and Mg^{++}. Fibrinogen binding is also inhibited by metabolic inhibitors, prostaglandin E_1 (PGE_1) and pH < 6.5.[303] Fibrinogen is a glycoprotein hexamer consisting of three non-identical pairs of chains (alpha, beta and gamma).[377] It contains two low affinity binding sites on the alpha chains and two high affinity binding sites on the gamma chains. The relative contribution of the different binding sites to <u>in vivo</u> platelet aggregation is not clear; however, studies indicate that the alpha chain bindings sites are not required for irreversible fibrinogen binding.[302] Irreversible fibrinogen binding is defined as the permanent attachment of fibrinogen to the platelet which is unaltered following incubation with EDTA and is dependent on cytoskeletal activation and association with GPIIb/IIIa.[304] While initial, reversible fibrinogen binding is dependent on an intact GPIIb/IIIa complex, irreversible fibrinogen binding is independent of an

intact GPIIb-IIIa complex. Irreversible binding may be mediated by secondary interactions between fibrinogen and GPIIb, GPIIIa or some alternate membrane constituent.[302]

Exposure of the fibrinogen binding site may involve a change in the conformation of the GPIIb-IIIa complex or in the surrounding membrane arrangement.[215] It has been demonstrated that the exposure of the fibrinogen binding site is a Ca^{++}-dependent process.[373] Inositol 1,4,5-trisphosphate (IP_3) and increased $[Ca^{++}]_i$ induce fibrinogen binding site exposure in saponin-permeabilized platelets. This effect is blocked by cyclooxygenase inhibition, suggesting a dependance on the Ca^{++}-dependent production of arachidonic acid metabolites. Stable GTP analogues and diacylglycerol also induce exposure of the fibrinogen binding site in a similar system.[284,371] These studies suggest exposure of the fibrinogen receptor is partially dependent on G proteins and activation of PKC.[377] Epinephrine induces fibrinogen receptor exposure in the absence of any synergistic agonists. Whether this is due to a localized Ca^{++} flux, the action of a G protein or some other second messenger system has yet to be determined.[372]

Alternately, it has been proposed that the ADP receptor, aggregin, plays a role in the exposure of the fibrinogen binding site. This 100kDa membrane protein is located in close proximity to the GPIIb-IIIa complex. In the resting state, aggregin is proposed to sterically interfere with the GPIIb-IIIa complex. Binding of ADP causes a conformational change in aggregin, thereby allowing exposure of the fibrinogen binding site. This conformational change in aggregin can

also be induced by proteolysis by chymotrypsin or high concentrations of thrombin.[81,82,83] The proteolysis induced by thrombin is not a direct cleavage; instead, it is due to the thrombin-stimulated activation of the intracellular protease calpain.[321]

Numerous studies have shown that fibrinogen binding alone is not sufficient to sustain aggregation, demonstrating the need for one or more post-fibrinogen binding events. Two monoclonal antibodies to GPIIb-IIIa (AP3 and Tab) allow fibrinogen binding to platelets but inhibit aggregation and secretion.[281] Platelets exposed to the cold or formalin fixed after stimulation with ADP are able to bind fibrinogen but are unable to aggregate.[305] This suggests that normal membrane fluidity or flow may be necessary for aggregation, possibly as a prerequisite for receptor mobility.

The Release Reaction

The release reaction is characterized by the secretion of the contents of cytoplasmic granules and the formation of arachidonate metabolites. The release reaction comprises two major positive feedback loops that serve to amplify the platelet response: (1) the release of ADP from dense granules; and (2) the release of arachidonate metabolites.[103,377] This amplification is most important in the stimulation of full platelet activation by agonists of intermediate or weak strength. The release reaction does not play a role in the primary responses of adhesion, shape change and reversible aggregation in response to any of the agonists. The contribution of each of the

release pathways to the overall response can be studied in vitro by the addition of: (1) cyclooxygenase blockers to inhibit arachidonate metabolism; and (2) ADP-scavenging enzymes (apyrase, creatinine phosphokinase) or ADP-receptor blockers (ATP) to prevent feedback by released ADP.[377]

Secretion of Granules

Secretion of the contents of both alpha and dense granules occurs following stimulation with sufficient doses of any of the physiologic agonists. Secretion of lysosome enzymes, however, requires stimulation with strong agonists such as high concentrations of thrombin or collagen. Relative to alpha and dense granules, secretion of lysosome enzymes occurs at a slower rate and never involves complete release of the lysosomal contents. Secretion of all types of granules requires energy and may be inhibited by metabolic inhibitors.[359,403] Two potential mechanisms for granule release to the external environment have been proposed. First, following platelet activation the membrane of the storage granules may fuse with the membrane of the open canalicular system.[454] An alternate mechanism, demonstrated in bovine platelets (which do not have a formed OCS) and proposed to also exist in human platelets, involves the fusion of granules directly with the plasma membrane.[310,451] Electron microscopic examination of platelets stimulated using synthetic diacylglycerol has shown swelling of granules, fusion of the swollen granules to each other and the eventual fusion of granules with both the OCS and the plasma membrane.[142]

Secretion has frequently been associated with activation of phospholipase C (PLC) and the subsequent production and/or release of intracellular second messengers.[170,222,382] Following stimulation with agonists of weak or intermediate strength, secretion is fully or partially dependent on the secondary activation of PLC by the released arachidonate metabolites. Secretion in response to these agonists is fully or partially blocked by the use of cyclooxygenase inhibitors.[16,68] Granule secretion is not, however, strictly dependent on PLC activation. This is demonstrated by the addition of thrombin to saponin-permeabilized platelets which causes secretion without any measurable activation of PLC.[226] In addition, inhibition of PLC-mediated diacylglycerol formation by a compound which inhibits G protein function, GDPβS, in saponin-permeabilized platelets does not inhibit thrombin-stimulated dense granule secretion.[57]

Cytosolic Ca^{++} also plays a role in mediating platelet secretion. Permeabilized platelets can be induced to secrete the contents of their granules by increasing the $[Ca^{++}]$ in the external medium which directly increases $[Ca^{++}]_i$.[204,205] The addition of the calcium ionophore, ionomycin, to intact, aspirin-treated platelets causes elevation of $[Ca^{++}]_i$ and subsequent secretion in the absence of measurable TXA_2 formation and in the absence of PLC activation.[339] The addition of calcium ionophore A23187 to platelets from patients with Glanzmann's thrombasthenia causes dense granule secretion independent of both TxA_2 production and aggregation.[232] One role for Ca^{++} is the Ca^{++}-dependent activation of MLCK and subsequent phosphorylation of myosin light chain. As discussed earlier, this leads to interaction of myosin and actin

filaments and the generation of a contractile force which plays an important role in the process of secretion.[142,203] A recent report has shown impaired release of intracellular Ca^{++} and decreased phosphorylation of myosin light chain in the platelets from two humans with secretion defects, once again demonstrating the close association between cytosolic Ca^{++} and secretion.[325]

Secretion can also occur at basal cytosolic $[Ca^{++}]_i$.[334,335,360] This may result from activation of PKC since numerous studies have shown that the phosphorylation of the 47kDa protein is closely associated with secretion.[3,285,291,377,400] One study showed that PKC activation facilitates the membrane fusion necessary for granule secretion.[139]

In vivo, Ca^{++} mobilization and PKC activation most likely work in synergy to promote granule secretion.[159,194,380] One researcher has proposed a two step model for platelet secretion. The initial release of Ca^{++} and myosin light chain phosphorylation cause shape change and granule centralization. The increased $[Ca^{++}]_i$ primes PKC for the next step where activation of PKC then modulates platelet secretion.[377]

The Release of Arachidonate Metabolites

Stimulated platelets release arachidonate from the various membrane phospholipids. The major pathway for arachidonic acid release involves the hydrolysis of phospholipids (primarily phosphatidylcholine but also phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine) at the fatty acyl 2 position by phospholipase A_2

(PLA_2).[122,245,319,320,434] PLA_2 is a Ca^{++}-dependent enzyme[27,397] that is activated by directly increasing $[Ca^{++}]_i$ in permeabilized platelets[151] or by increasing $[Ca^{++}]_i$ in intact platelets using calcium ionophores.[339] One report identified at least two separate forms of PLA_2, one specific for phosphatidylcholine which is dependent on increased $[Ca^{++}]_i$ and one specific for phosphatidylethanolamine which is Ca^{++}-independent.[19]

Factors other than cytosolic Ca^{++} play a role in regulating PLA_2 activity. PLA_2 activity is maximal in an alkaline environment.[27] Activation of Na^+/H^+ exchange by agonist-stimulation causes cytosolic alkalinization which potentiates activation of PLA_2.[6,411] Activators of PKC enhance arachidonic acid release by modulating the activity of PLA_2.[7,122,150,152] In addition, other studies suggest that G proteins serve a regulatory function in PLA_2 activity.[6,7,137,280,396] G proteins are a family of related guanine-nucleotide binding proteins that mediate the interaction between the surface receptor and an intracellular effector system. There are two proposed mechanisms by which G proteins regulate PLA_2: (1) G protein-dependent activation of PLC with the production of (1,4,5)IP_3 and the eventual release of Ca^{++}; or (2) direct G protein activation of PLA_2 by some, as yet, unidentified G protein.[53]

Two other routes for activation-induced arachidonic acid release exist in platelets. The first is PLC-mediated hydrolysis of phosphoinositol with the production of the second messenger 1,2-diacylglycerol (1,2-DG). Arachidonic acid is then released by the sequential action of diglyceride and monoglyceride lipases.[76] The

second pathway involves the phosphorylation of 1,2-DG to phosphatidic acid (PA) and subsequent activation of a Ca^{++}-dependent PLA_2.[38]

In the resting cell, the concentration of arachidonic acid is maintained at a low level by the activity of arachidonate scavenging enzymes which incorporate free arachidonate into membrane phospholipids by the sequential action of the enzymes arachidonyl-CoA synthase and arachidonyl-CoA lysophospholipid acyltransferase. Decreased activity of either of these enzymes could lead to increased levels of free arachidonic acid. One study showed that PKC activation inhibits both of these enzymes [136] while a separate study showed no effect.[173]

Once freed from the membrane phospholipids, arachidonic acid may be oxygenated by either the cyclooxygenase or the lipoxygenase pathway. In the cyclooxygenase pathway, arachidonic acid is converted to prostaglandins G_2 and H_2 (PGG_2/PGH_2). These endoperoxides are metabolized by thromboxane synthetase to produce the unstable metabolite TxA_2.[403] All of these metabolites are potent platelet agonists and are an important part of the amplification mechanism. They cause activation of PLC[379] and Ca^{++} mobilization.[47,50]

The lipoxygenase pathway causes the production of 12-hydroxyeicosatetraenoate (12-HETE) and 15-hydroxyeicosatetraenoate (15-HETE) as well as several other dihydroxylated products.[447] The role of these metabolites in platelet activation is unclear. Some evidence indicates an inhibitory role for these products. 12-HETE inhibits PLA_2 activity, decreasing the release of arachidonic acid from membrane

phospholipids and causing inhibition of aggregation.[369] Other evidence suggests a stimulatory role for the lipoxygenase metabolites. Lipoxygenase inhibitors inhibit both Ca^{++} flux and aggregation in response to ADP, indicating that lipoxygenase metabolites play a stimulatory role in ADP-induced aggregation and Ca^{++} mobilization.[46]

SIGNAL TRANSDUCTION OR PROCESSING

None of the physiologic agonists are able to penetrate the platelet plasma membrane; instead, they bind to surface receptors.[478] Signal transduction is the process by which an external stimulus (ie. agonist binding) is transmitted through the plasma membrane and translated into a cellular response. The binding of an agonist to a surface receptor stimulates specific effector systems, such as enzymes or ion channels, which then regulate the production of cellular second messengers. It is these second messengers which actually modulate the physiologic response by altering the conformation, enzymatic activity or phosphorylation of cellular proteins.[377] Although platelets are stimulated or inhibited by a wide range of substances, there appears to be a finite number of effector systems available for signal transduction. It is often difficult to correlate a specific effector system or second messenger with a specific cellular response since many events are occurring within a very short period of time following platelet activation. In addition, there are multiple points of cross-over or checks and balances between the different effector systems. Enzyme systems which play a major role in signal transduction include the following: (1) adenylate cyclase which controls the production of

the second messenger cyclic AMP (cAMP); (2) PLC which mediates the hydrolysis of membrane phosphoinositides and subsequent production of the second messengers (1,4,5)IP_3 and 1,2-DG; and (3) PKC which phosphorylates a variety of cellular substances. Important second messengers include (1,4,5)IP_3, 1,2-DG and Ca^{++}. Ion channels which play a role in platelet activation include Ca^{++} channels and the Na^+/H^+ antiport.

Adenylate Cyclase and cAMP Production

Cyclic AMP production is regulated by adenylate cyclase while cAMP removal is regulated by cAMP phosphodiesterases. Increased cAMP is caused by agents that stimulate adenylate cyclase (ie. prostacyclin, PGE_1, prostaglandin D_2 and adenosine) and/or inhibit phosphodiesterase activity.[9,403] Decreased cAMP is caused by agents that inhibit adenylate cyclase (ie. epinephrine, ADP and thrombin) and/or stimulate phosphodiesterase activity.[403] As a general rule, increased cAMP is inhibitory to platelet function while decreased cAMP facilitates platelet activation.[9,377] Increased cytosolic cAMP inhibits platelet adhesion, shape change, secretion and aggregation.[376]

Adenylate cyclase produces cAMP from ATP in a reaction coupled to surface receptors by G proteins which mediate the interaction between receptors and effector systems. Adenylate cyclase is regulated by two distinct G proteins: (1) G_s which serves a stimulatory role; and (2) G_i which serves an inhibitory role. G_s and G_i are heterotrimeric proteins with similar beta and gamma subunits but different alpha subunits.

Cholera toxin ADP ribosylates the alpha subunit of G_s, leading to permanent activation. Pertussis toxin ADP ribosylates the alpha subunit of G_i which uncouples G_i from the receptor and prevents agonist-induced inhibition of adenylate cyclase.[53,377] Na^+ and other monovalent cations inhibit both the basal and stimulated activity of adenylate cyclase. The data suggests that the cations interfere with coupling between membrane receptors, G protein and the catalytic portion of adenylate cyclase. Na^+ and GTP are also necessary for maximal inhibition of adenylate cyclase by epinephrine.[405]

Binding of an agonist to its receptor leads to the dissociation of GDP from the heterotrimeric G protein and the binding of GTP to the alpha subunit. Binding of GTP alters the alpha unit in some manner, possibly conformational, and allows it to dissociate from the beta-gamma units. The dissociated alpha subunit is mobile in the membrane where it interacts with the target effector system while the beta-gamma subunits remain relatively stationary. The effect is terminated by the slow hydrolysis of GTP to GDP by the GTPase inherent in the alpha subunit. The alpha subunit then reassociates with the beta-gamma units. Nonhydrolyzable analogues of GTP (ie. Gpp(NH)p or GTPgammaS) bind at the GTP site and cause persistent G protein activation while nonhydrolyzable analogues of GDP (ie. GDPβS) bind at the GDP site and cause persistent inhibition.[53,377]

Although it is clear that cAMP inhibits platelet function, the exact mechanisms involved are unclear. Cyclic AMP activates cAMP-dependent kinase(s) which phosphorylate, and therefore modulate, the

activity of a number of enzymes and proteins.[9] Increased concentrations of cAMP increase the phosphorylation of at least four different proteins of M_r 51kDa (P51), 36kDa (P36), 24kDa (P24) and 22kDa (P22). P51 and P24 are phosphorylated very rapidly, but their roles in the inhibitory action of cAMP is not known. P24 has been identified as the beta subunit of GPIb which plays an important role in vWF mediated adhesion and is the site for the attachment of the membrane skeleton. Whether this phosphorylation plays a physiologic role is not known.[133] P22 has been identified as the protein thrombolamban which is proposed to play a role in regulating the Ca^{++}-ATPase pump which sequesters Ca^{++} into the DTS.[3] Recent studies, however, have shown that P22 does not interact with the Ca^{++}-ATPase present in the platelet membranes[125] and that the 22kDa protein is actually related to a family of GTP-binding proteins. This study has also demonstrated that the cAMP-dependent kinase phosphorylates at least 9 different proteins. The identity and function of these proteins is not known.[458]

Numerous studies have shown that increased cAMP interferes with Ca^{++}-mediated stimulatory responses. cAMP inhibits the agonist-induced rise in $[Ca^{++}]_i$ by limiting influx from the external environment[350,433] and/or suppressing release from internal stores.[43,240,350,433] This inhibition of release may be due to a regulatory effect on the $(1,4,5)IP_3$-induced Ca^{++} release from the DTS.[112,268] The release of internal Ca^{++} by calcium ionophores, which bypass the physiologic Ca^{++} release process, is not blocked by cAMP, indicating a specific role for cAMP in mediating this process.[350] Cyclic AMP also stimulates Ca^{++} sequestration into the DTS.[43,120,167] Others studies, however, have

shown no stimulation of Ca^{++} transport in platelet microsomes or membrane vesicles in response to cAMP.[330,449]

Increased cAMP has numerous other inhibitory effects. First, PKC activity is decreased due to cAMP-mediated inhibition of the potentiating effects of secreted ADP[213] or some other non-ADP-mediated events.[74,213] Secondly, cAMP inhibits PLC-induced hydrolysis of membrane phosphoinositides by some, as yet, unidentified mechanism.[205,344,376,430,472] Thirdly, cAMP stimulates the formation of phosphoinositol 4-phosphate (PIP), possibly by inhibiting phosphatidylinositol kinase. This causes decreased availability of polyphosphoinositides for hydrolysis by PLC and leads to decreased production of the second messengers (1,4,5)IP3 and 1,2-DG.[73,220] In addition, increased cAMP inhibits fibrinogen receptor exposure.[206,376] Finally, cAMP activates a cAMP-dependent kinase which phosphorylates MLCK.[165] If the Ca^{++}-calmodulin complex is present, this phosphorylation does not affect enzyme activity. If the Ca^{++}-calmodulin complex is absent, phosphorylation causes a decrease in MLCK activity and inhibits the subsequent activation of MLCK by Ca^{++}.[88]

Phospholipase C and Membrane Phosphoinositide Hydrolysis

One of the main effector systems coupled to the action of stimulatory agonists is the PLC-mediated hydrolysis of membrane phosphoinositides to produce the second messengers (1,4,5)IP_3 and 1,2-DG. Additional second messengers produced as a result of this reaction

include other inositol phosphates, PA and lysophosphatidic acid (lysoPA).

Phosphoinositide Metabolism

Quantitatively, phosphatidylinositol (PI) is a minor phospholipid, comprising only 5-7% of total platelet phospholipids.[31,252] Of that, approximately 15 and 5% are PIP and PIP_2 respectively.[377] Three features of the phosphoinositides point to an important role in platelet function: (1) a strongly acidic polar head group; (2) a preponderance of arachidonic acid; and (3) a very active metabolic cycle. The phosphoinositides contain a glycerol backbone with a polar head group containing a myo-inositol ring in position 3 and fatty acids bound to positions 1 and 2 on the glycerol backbone. The myo-inositol ring can be esterified with one or two phosphate groups to yield the polyphosphoinositides, PIP and PIP_2.[249] The 1-stearoyl 2-arachidonyl species comprises 71% of the total phosphoinositides, compared to 47% of phosphatidylethanolamine and 10% of phosphatidylcholine.[244] This positions arachidonic acid in a site where it is readily cleaved by PLA_2 to participate in the platelet response. It has been proposed that the platelet enzyme 1-acyl-glycerophosphoryl-inositol acyltransferase is partially responsible for the relative abundance of arachidonic acid at position 2.[195]

The phosphoinositides have a very active metabolism in both the resting and stimulated state. They are in a continual "futile cycle" involving an ATP-dependent interconversion of the different

phosphoinositides.[249] The phosphoinositides exist in a metabolically homogeneous pool within the platelet, with relatively free exchange between the polyphosphoinositides and PI.[431] PI and PIP are phosphorylated by PI-kinase and PIP-kinase respectively. These kinases are Mg^{++}-dependent and are bound to the plasma membrane.[198] Phosphate groups are removed from the PIP and PIP_2 by PI phosphomonoesterases. These enzymes are located in both the cytosol and the membranes. They require Mg^{++} at the physiologic concentrations for maximal activity.[37,338] This "futile cycle" is ongoing in the resting platelet and does not play a direct role in the agonist-stimulated breakdown of membrane phosphoinositides; however, it is important in maintaining the proper equilibrium of the different phosphoinositides for activation to occur.[249] The ratio of PIP_2:PA and PIP:PA remains relatively constant following agonist stimulation, suggesting that the activity of both the kinases and the phosphomonoesterases are tightly regulated in stimulated cells as well as in resting cells.[404]

PI can be formed from 1,2-DG by the following sequence of reactions: (1) phosphorylation of DG in position 3 to yield PA; (2) conversion of PA to cytidine diphosphate-diacylglycerol (CDP-DG) by CTP-DG cytidyl transferase; and (3) addition of an inositol ring to CDP-DG by PI synthase to yield PI.[249,368] This pathway becomes an important mechanism following platelet activation to recycle the 1,2-DG molecule.[249,368]

Phospholipase C Mediated Hydrolysis of PI

The phosphoinositides are primarily positioned on the cytoplasmic side of the plasma membrane, placing them at the correct physical location to play a role in signal transduction.[368] Binding of an agonist to a specific receptor causes the PLC-mediated phosphodiesteric cleavage of the phosphoinositides to yield the corresponding inositol phosphate and 1,2-DG. Physiologic agonists having surface receptors which directly modulate PLC activity include thrombin, PAF, TxA_2 and vasopressin.[58,106,174,269,272,312,337,378] Agonist-induced stimulation of PLC activity is independent of extracellular Ca^{++} and Mg^{++}.[383]

The initial response of agonist binding is a rapid decrease in PIP_2 (within 10 seconds of stimulation) followed by increased formation of PIP_2.[37,277] The decrease in PIP_2 could result from: (1) increased hydrolysis by PLC; (2) decreased phosphorylation of PIP by PIP kinase; or (3) increased dephosphorylation of PIP_2 by phosphomonoesterase.[249,377] There is simultaneously increased production of the inositol phosphates within 5 to 10 seconds of agonist addition,[94,340,341,343,375,378,418] suggesting that the decrease in PIP_2 is primarily due to hydrolysis by PLC.

Phosphoinositide-specific PLC is not a single enzyme but is composed of multiple forms located in the cytoplasm and bound to the platelet membrane. The role of the different subtypes of the PI-specific-PLC is not currently known. They vary in terms of molecular weight, substrate specificity, pH optima and degree of Ca^{++}-

dependence.[18,25,53,238,343] An early study has suggested that the majority of PLC activity is present in the cytosol and requires millimolar concentrations of Ca^{++} for maximal activity.[23] PLC is not activated by Ca^{++} alone, since the calcium ionophore A23187-mediated increase in cytosolic Ca^{++} is unable to initiate the hydrolysis of PIP_2.[337] These findings preceded the identification of: (1) a membrane-bound PLC which shows greater activity for the hydrolysis of the polyphosphoinositides and is active at Ca^{++} levels present within the resting platelet cytosol;[25,248,343] and (2) a soluble PLC which shows preferential hydrolysis of PIP_2 and is activated by G proteins.[17] As a general rule, the identified PLC enzymes optimally hydrolyze PIP_2 at low concentrations of Ca^{++} but hydrolyze PIP_2, PIP and PI equally well at millimolar concentrations of Ca^{++}. This means that initial receptor-mediated activation of PLC induces the preferential hydrolysis of the polyphosphoinositides, causing the release of membrane bound Ca^{++}[37] and the production of the second messengers (1,4,5)IP_3 and 1,2-DG.[377,418] (1,4,5)IP_3 mediates the release of intracellular stores of Ca^{++} (see section on IP_3-mediated events). The increased $[Ca^{++}]_i$ is associated with PLC-mediated hydrolysis of PI in addition to hydrolysis of polyphosphoinositides.

G Protein Regulation of PLC

Brass[53] lists the following five criteria as evidence that a G protein, termed G_p, modulates PLC activity: (1) guanine nucleotides stimulate PIP_2 hydrolysis and/or IP_3 and DG production in permeabilized cells or isolated platelet membranes; (2) guanine nucleotides mimic the

effects of agonists known to stimulate PLC; (3) agonist-induced PI hydrolysis is enhanced by the addition of GTP to permeabilized cells or membranes; (4) the addition of pertussis toxin or GDP analogues inhibit agonist-induced phosphoinositide hydrolysis; and (5) the extent of ADP-ribosylation of G proteins by pertussis toxin correlates with the inhibitory effects of the toxin on agonist-induced phosphoinositide hydrolysis. Each of these criteria have not been shown to operate in all tissues.[53]

Many studies in platelets implicate the important role of G_p-mediated activation of PLC.[53,57,280,399] Partially purified PLC from human platelet membranes is stimulated by the addition of purified G proteins from brain tissue, suggesting a role for G protein regulation.[223] The addition of the GTP analogue guanosine 5'-(γ-thio)triphosphate (GTPγS]) to platelet membrane suspensions or to permeabilized platelets increases PLC activity and 1,2-DG and/or IP_3 production.[17,24,57,160,175] In permeabilized platelets, GTP analogues potentiate the stimulatory effects of thrombin.[175] Addition of a GDP analogue (GDPβS), on the other hand, inhibits the ability of thrombin to stimulate PLC in permeabilized platelets.[57] Caution should be used in the interpretation of these results since some studies have suggested that both GTP- and GDP-analogues have effects on platelet activation independent of G protein-mediated events.[24,210]

As mentioned in the section on adenylate cyclase, pertussis toxin ADP-ribosylates the alpha unit of G_i and subsequently uncouples it from the receptor. Pertussis toxin also inhibits thrombin-induced release of

intracellular Ca^{++} [293] and 1,2-DG production[57] by ADP-ribosylating membrane proteins, suggesting that a pertussis toxin-sensitive G protein similar to G_i functions in thrombin-stimulated PLC activation. This effect is overcome by using higher concentrations of thrombin.[21,57] These findings indicate that low concentrations of thrombin activate PLC by a pertussis toxin-sensitive G protein while high concentrations of thrombin activate platelets by a pathway independent of PI hydrolysis[57] or by a PLC pathway modulated by a pertussis toxin-resistant G protein.[21] Pertussis toxin has minimal to no effect on PAF- or TxA_2 mimetic U44069-stimulated GTPase activity in platelet membranes, indicating that the G protein regulating PLC activity in response to these agonists is pertussis resistent and therefore different from the adenylate cyclase G_i.[174] This finding has been supported by studies utilizing permeabilized platelets in which U46619-stimulated activation of PLC was unaffected by the addition of pertussis toxin. This study has also suggested that the TxA_2 receptors are coupled to PLC by a pertussis-resistent G_p which is different than the pertussis-sensitive G_p coupled to thrombin activation.[58]

Inositol Phosphates: Metabolism and Role as Second Messengers

The PLC-mediated hydrolysis of the phosphoinositides causes the rapid production of a variety of inositol phosphates.[377] Hydrolysis of PIP_2 yields primarily (1,4,5)IP_3 and lesser quantities of (1,2-cyclic 4,5)IP_3.[94,418] (1,4,5)IP_3 is rapidly converted to either: (1) inositol 1,4-bisphosphate [(1,4)IP_2] by a specific 5-phosphomonoesterase; or (2) inositol 1,3,4,5-tetrakisphosphate [(1,3,4,5)IP_4] by a specific 3-

kinase.[84,418] This 3-kinase is stimulated by Ca^{++} in a calmodulin-dependent fashion.[93,348] (1,3,4,5)IP_4 is subsequently converted to inositol 1,3,4-trisphosphate [(1,3,4)IP_3] by a specific 5-phosphomonoesterase.[84] (1,3,4)IP_3 is metabolized by phosphatases to yield either inositol 1,3 phosphate or inositol 3,4 phosphate.[377] (1,2-cyclic 4,5)IP_3 is also hydrolysed by 5-phosphomonoesterase to yield inositol 1,2-cyclic bisphosphate; however, this reaction occurs at a 10-fold slower rate than that of the non-cyclic (1,4,5)IP_3, leading to the relatively greater accumulation of (1,2-cyclic 4,5)IP_3 over time.[84,87,418] (1,2-cyclic 4,5)IP_3 is not phosphorylated by 3-kinase to a cyclic IP_4 metabolite.[84]

The various isomers of inositol bisphosphate/inositol cyclic bisphosphate and inositol monophosphate/inositol cyclic monophosphate are produced by either the PLC-mediated hydrolysis of PIP or PI respectively or the sequential degradation of the polyphosphoinositides/ cyclic polyphosphoinositides. The metabolism of the inositol phosphates is very complex and involves a number of alternate pathways with enzymes differing in requirements for Mg^{++} and sensitivity to lithium inhibition.[377] The inositol cyclic phosphates are metabolized via pathways separate from the non-cyclic inositol phosphates. The two pathways converge when inositol cyclic 1,2 monophosphate is metabolized to inositol non-cyclic monophosphate by a hydrolase. The final step in both metabolic pathways is the dephosphorylation of inositol monophosphate to yield free inositol which recycled for use in the cell.[377]

(1,4,5)IP_3 is a water-soluble molecule that is released into the cytoplasm and implicated in the release of intracellular Ca^{++} from the platelet DTS. The addition of (1,4,5)IP_3 to either permeabilized platelets or platelet membrane vesicles causes the rapid release of non-mitochondrial stores of Ca^{++}.[11,12,55,113,242] A monoclonal antibody against IP_3 blocks the thrombin-induced release of Ca^{++} from membrane vesicles, supporting a role for (1,4,5)IP_3 in Ca^{++} release. Although the mechanism by which (1,4,5)IP_3 causes Ca^{++} mobilization is not known, it has been proposed that it binds to specific sites on the DTS and activates a Ca^{++} channel.[377] This IP_3-gated Ca^{++} channel is distinct from the Ca^{++}-ATPase pump[2] and is not dependent on ATP or phosphorylation of membrane proteins. A recent study, which characterized the (1,4,5)IP_3 binding site on platelet membranes using radiolabelled IP_3, has shown that Ca^{++} release could be blocked without inhibiting IP_3 binding. This suggests that the IP_3-gated Ca^{++} channel has a region regulating Ca^{++} release that is distinct from the binding site.[292] (1,4,5)IP_3 also stimulates Ca^{++} release from plasma membrane vesicles, suggesting an additional role for (1,4,5)IP_3 in facilitating Ca^{++} influx from the external environment.[328]

Numerous other inositol phosphate metabolites are formed during platelet activation. (1,2-cyclic 4,5)IP_3 causes Ca^{++} mobilization in permeabilized platelets, although it is less effective than the non-cyclic (1,4,5)IP_3.[462] Since the relative proportion of (1,2-cyclic 4,5)IP_3 increases with time, it has been proposed that (1,4,5)IP_3 is more important in early Ca^{++}-mobilization in platelet activation while (1,2-cyclic 4,5)IP_3 becomes more important in late Ca^{++} mobilization.[418]

One study which compared intracellular $[Ca^{++}]_i$ with inositol phosphate production has suggested that: (1) $(1,4,5)IP_3$ alone cannot account for the sustained elevation in Ca^{++} seen following agonist stimulation; and (2) other metabolites ,such as $(1,3,4)IP_3$ or $(1,3,4,5)IP_4$, must also play a role in Ca^{++} mobilization.[94] Numerous reports have proposed a role for $(1,3,4,5)IP_4$ in the influx of external Ca^{++} in other cell types; however, its role in platelets is not known.[306] The majority of IP_3 produced during platelet activation is the $(1,3,4)$-IP_3 isomer. In other cells types, $(1,3,4)IP_3$ has been shown to cause Ca^{++} release from internal stores, though it appears to be 30-fold less effective than $(1,4,5)IP_3$. It's role in platelet Ca^{++} mobilization is not known.[418]

The addition of $(1,4,5)IP_3$ to permeabilized platelets causes shape change, aggregation and secretion. The addition of cyclooxygenase inhibitors prevents aggregation and secretion, but not shape change. This suggests that Ca^{++} mobilization induced by $(1,4,5)IP_3$ is unable to induce secretion and aggregation by itself. These effects appear to be partially mediated by the production of endoperoxide metabolites by the Ca^{++}-sensitive PLA_2.[12]

Production of Diacylglycerol and Its Role as a Second Messenger

1,2-Diacylglycerol is a neutral lipid that is rich in stearic and arachidonic acids. Only DG with the 1,2-sn-configuration plays a role in platelet activation. 1,2-DG remains in the membrane where it activates the enzyme PKC.[377]

As discussed above, the initial response of agonist-induced activation of PLC is the preferential hydrolysis of PIP_2 and the production of small quantities of 1,4,5)IP_3 and 1,2-DG. (1,4,5)IP_3 is released into the cytosol where it mediates the release of internal stores of Ca^{++}. Activation of additional Ca^{++}-sensitive PLC hydrolyses both polyphosphoinositides and phosphoinositide. While the quantity of (1,4,5)IP_3 released from the hydrolysis of PIP_2 is sufficient to mobilize Ca^{++}, the majority of 1,2-DG production comes directly from the hydrolysis of PI rather than from conversion of PI to PIP_2 with subsequent hydrolysis of the PIP_2.[435,463] Other potential sources for 1,2-DG production in stimulated platelets include the metabolism of triglycerides, PA, phospholipids other than PI and monoglycerides; however, these sources do not contribute a significant quantity of DG to the overall platelet response.[342]

The concentration of 1,2-DG is low in resting platelets.[342] Following stimulation, 1,2-DG levels increase 2-3 fold by 30 seconds and gradually decrease to basal levels by 5 minutes. 1,2-DG production can be detected as early as 5 seconds.[318] Platelets contain two different pathways to assure that DG does not accumulate. The majority of the DG is rapidly phosphorylated by diacylglycerol kinase to PA. The remainder is metabolized by the sequential action of diglyceride and monoglyceride lipases to yield arachidonic acid and a glycerol backbone.[40,41,76]

Role of PA and LysoPA as Second Messengers

Phosphatidic acid is generated from the phosphorylation of DG by diacylglycerol kinase. One study which compared the time relationship between Ca^{++} mobilization and the production of PA has suggested that PA acts as a Ca^{++} ionophore.[179] The addition of a synthetic diacylglycerol, 1-oleoyl-2-acetoyl glycerol (OAG), to permeabilized platelets causes the release of Ca^{++} from the DTS.[56,65] This release is not affected by cyclooxygenase inhibition and not duplicated by specific activators of PKC. It has been suggested that the increased $[Ca^{++}]_i$ is due to the formation of PA or lysoPA from OAG and that these metabolites enhance Ca^{++} mobilization in response to (1,4,5)IP_3.[56] The addition of high concentrations of PA to intact platelets stimulates platelet aggregation and enhances thrombin-induced aggregation and secretion. PA-induced platelet activation is inhibited by cyclooxygenase blockers, indicating that these effects are mediated by cyclooxygenase products and that PA acts to enhance the activity of PLA_2 by increasing $[Ca^{++}]_i$.[214]

LysoPA is formed from the deacylation of PA by a specific PLA_2. Since PA frequently contains arachidonic acid at the sn-2 position, its deacylation to lysoPA serves as a source of arachidonic acid.[38] LysoPA acts as a potent Ca^{++} ionophore <u>in vitro</u>.[141] When added to intact platelets, lysoPA induces aggregation, secretion, and protein phosphorylation.[140] It causes platelet activation in a manner similar to calcium ionophore A23187. There is initial Ca^{++} mobilization which causes subsequent aggregation, inositol phosphate production and protein

phosphorylation. Cyclooxygenase inhibition decreases the aggregation response and blocks inositol phosphate production, suggesting that PLC activation by lysoPA is dependent on the Ca^{++}-dependent activation of PLA_2 and production of arachidonic acid metabolites.[444]

Ca^{++} AND ITS ROLE IN SIGNAL TRANSDUCTION

Ca^{++} plays a pivotal role in many aspects of platelet activation. Extracellular Ca^{++} is required to maintain the heterodimer GPIIb-IIIa complex on the platelet surface. This complex is essential for fibrinogen binding and aggregation.[63] Intracellular Ca^{++} fluxes are important in the platelet responses of shape change and secretion as already discussed. Platelets contain approximately 20nmol of $Ca^{++}/10^8$ cells. It is primarily located in intracellular organelles or bound to membranes or cytoplasmic proteins.[62] Platelets utilize various mechanisms to actively maintain the resting $[Ca^{++}]_i$ at approximately 100nM, some 10,000 fold less than the surrounding plasma.[51] The agonist-stimulated increase in $[Ca^{++}]_i$ results from release of internal stores or from influx of external Ca^{++}.

Platelet Storage Sites for Ca^{++}

Non-exchangeable Ca^{++} Pools

The vast majority of platelet Ca^{++} is stored within the dense granules. This Ca^{++} is released into the external environment following appropriate stimulation. The quantity of Ca^{++} stored in, and

subsequently secreted from, the dense granules is species-dependent.[266] This pool of Ca^{++} is poorly exchangeable with the other pools of intracellular Ca^{++} or extracellular Ca^{++} in the resting platelet.[62,273]

Exchangeable Ca^{++} Pools

Unstimulated platelets contain two exchangeable pools of Ca^{++}. One is a slowly exchangeable pool that contains 54% of the exchangeable Ca^{++}, is not affected by incubation with the Ca^{++} chelator ethylene glycol tetraacetic acid (EGTA) and is most likely intracellular. The second pool contains 46% of the exchangeable Ca^{++}, is removed by incubation with EGTA and is most likely bound directly to or near the platelet surface.[59]

Further examination of the surface-bound pool of Ca^{++} has shown that the surface of unstimulated platelets contain two classes of binding sites: a high affinity class with approximately 57,000 sites and a low affinity class with approximately 460,000 sites.[59] Platelets from patients with Glanzmann's thrombasthenia have reduced numbers of both type of bindings sites, supporting the role of GPIIb-IIIa as a major surface Ca^{++}-binding site on unstimulated platelets.[60] Following stimulation with ADP or epinephrine, there is an increase in the amount of Ca^{++} bound to the platelet surface and examination of the membrane demonstrates the appearance of additional binding sites. These sites develop in both normal and Glanzmann's thrombasthenic platelets, suggesting that these new sites are not located on GPIIb-IIIa but represent some other protein or glycoprotein.[59,60] In spite of the

presence of 1mM external Mg^{++}, removal of the Ca^{++} from the platelet surface causes decreased aggregation and secretion in response to weak agonists. This indicates that external Ca^{++} bound to GPIIb/IIIa is required for maximal responses to stimulation with weak agonists.[60]

In resting platelets, the intracellular pool of exchangeable Ca^{++} behaves as if it is present in two distinct sites: (1) a rapidly exchangeable pool ($t_{1/2}$=17 minutes) located in the cytosol; and (2) a slowly exchangeable pool ($t_{1/2}$= 300 minutes) located in the mitochondria and the DTS. The size of the rapidly exchangeable pool is independent of the external $[Ca^{++}]$. The size of the slowly exchangeable pool varies with the external $[Ca^{++}]$. Studies using an inhibitor of mitochondrial Ca^{++} uptake have shown no alteration in the size of the slowly exchanging pool, implicating the DTS as the primary site of the slowly exchangeable pool.[51]

Maintenance of Resting $[Ca^{++}]$

Resting platelets maintain an average $[Ca^{++}]_i$ of approximately 100nM. Examination of single resting platelets show that the internal Ca^{++} is located in non-homogeneous zones within the cytoplasm, with a continuous Ca^{++}-gradient of increasing concentrations approaching the plasma membrane.[428] Platelets maintain these resting levels by limiting influx, stimulating sequestration into the DTS and promoting efflux.[51]

Ca^{++} Influx

The rate of Ca^{++} influx into non-stimulated platelets is maximal at an external Ca^{++} concentration 20-fold less than that present in normal plasma.[51] Ca^{++} influx occurs through Ca^{++}-selective channels by passive movement down a concentration gradient.[51] Examination of unstimulated platelets from patients with Glanzmann's thrombasthenia has shown a decreased rate of Ca^{++} influx, without any significant effect on the total size of the cytosolic exchangeable pool of Ca^{++} or on $[Ca^{++}]_i$. This implicates the GPIIb-IIIa complex in passive Ca^{++} influx. Whether the GPIIb-IIIa complex itself serves as the channel or whether the complex is needed for the functioning of a distinct Ca^{++} channel in intact platelets has not been determined;[52] however, studies using GPIIb-IIIa incorporated into phospholipid vesicles have shown that GPIIb-IIIa acts as a passive Ca^{++} channel.[347]

Ca^{++} Efflux

The control of Ca^{++} efflux in platelets is not clear. In the resting platelet, Ca^{++} efflux is not directly linked to Ca^{++} influx and does not occur by simple Ca^{++}/Ca^{++} exchange. Ca^{++} efflux is: (1) dependent on a source of energy; (2) stimulated by calmodulin; and (3) competitively inhibited by the cations Gd^{+++} and La^{+++}.[51] In most cells, Ca^{++} efflux across the plasma membrane is controlled by a Ca^{++}-ATPase pump and/or a Na^{+}/Ca^{++} exchange mechanism which exchanges extracellular Na^{+} for intracellular Ca^{++}. The driving force for this exchange is the inwardly directed electrochemical Na^{+} gradient which is

maintained by an energy dependent Na^+-K^+-ATPase on the plasma membrane.[365] There is question over the existence of a Na^+/Ca^{++} in intact platelets, though one study did show the presence of a Na^+/Ca^{++} exchanger in platelet plasma membrane vesicles.[329] In intact platelets, an increase in cytoplasmic Na^+ and/or a decrease in external Na^+ causes an increase in $[Ca^{++}]_i$[365] or in the quantity of Ca^{++} in the intracellular exchangeable pools,[54] indicating that the membrane Na^+ gradient does affect resting Ca^{++} homeostasis. This Na^+/ Ca^{++} exchanger appears to be relatively dormant in the resting platelet, but may be activated by increased $[Ca^{++}]_i$.[365] There is also controversy over the location of the Ca^{++}-ATPase pump in platelets. Early studies detected Ca^{++}-ATPase activity only in internal membrane fractions associated with the DTS and/or the OCS;[107,111,166,265] however, recent reports have demonstrated the presence of two distinct platelet Ca^{++}-ATPases. One of the Ca^{++} pumps appears to be located in the internal membranes while the other appears to be located in the plasma membranes.[114,115,116]

Ca^{++} Sequestration

Numerous studies have demonstrated the presence of Ca^{++}-ATPase activity in the membranes of the platelet DTS[111,265] and/or within specialized structures associated with the OCS which take up and release Ca^{++} in a manner similar to calciosomes in non-muscle tissues.[107,166] The platelet Ca^{++}-ATPase identified on the internal membranes is similar to the ATPase in the sarcoplasmic reticulum of muscle cells, although there are some functional and structural differences.[100,102,107,149] It requires Mg^{++}, membrane phospholipids and ATP for optimum function and

it is stimulated by increasing [Ca^{++}] up to 50uM. Ca^{++}-ATPase-driven Ca^{++} sequestration within the DTS is stimulated by a number of processes including calmodulin,[102,356] increased cytosolic cAMP[43,120,167] and PKC activation.[471,472,473] Inhibition of Ca^{++}-ATPase activity occurs following agonist-induced stimulation of PLC and the subsequent hydrolysis of membrane PIP_2. The mechanism of this inhibition has not been determined.[330]

Agonist-Stimulated Ca^{++} Fluxes

A variety of agonists, including thrombin, PAF, vasopressin, endoperoxides and ADP, stimulate an increase in $[Ca^{++}]_i$.[331] Historically, a number of techniques have been used to measure Ca^{++} flux in platelets. Currently, the most common techniques used to measure platelet $[Ca^{++}]_i$ include: (1) the fluorescent Ca^{++} chelator dyes fura 2, indo 1 and quin2; and (2) the luminescent photoprotein aequorin. The fluorescent dyes are believed to be more indicative of the average $[Ca^{++}]_i$ while aequorin is believed to respond to local zones of Ca^{++} flux.[79,97,188]

Agonist-stimulated elevations in $[Ca^{++}]_i$ arise from increased influx across the plasma membrane, release of Ca^{++} bound to the plasma membrane or release of Ca^{++} from the DTS. The internal Ca^{++} flux measured in the presence of external Ca^{++} is composed of both internal mobilization and influx from the external environment. The internal Ca^{++} flux measured in the absence of external Ca^{++} represents Ca^{++} release from internal stores. It should be kept in mind that the

absence of external Ca^{++} may affect the coupling of the agonist/receptor to the internal Ca^{++} discharge mechanism, secondarily inhibiting the release of internal stores of Ca^{++}.[331]

When Ca^{++} is absent from the external media, the agonist-induced increase in $[Ca^{++}]_i$ is attenuated and transient when compared to the change in $[Ca^{++}]_i$ following stimulation in the presence of external Ca^{++}.[154,155,192,243,313,331] When platelets are stimulated in the presence of external Ca^{++}, the peak $[Ca^{++}]_i$ is generally larger and prolonged in nature. This indicates a role for the influx of external Ca^{++} in sustaining the rise in $[Ca^{++}]_i$.[313]

The use of stop-flow cytometry in combination with the fluorescent dyes to measure $[Ca^{++}]_i$ have enabled researchers to study the sub-second kinetics of Ca^{++} flux in stimulated platelets. In the presence of external Ca^{++}, stimulation of platelets by thrombin, PAF, vasopressin, or the TxA_2 mimetic U46619 causes an increase in $[Ca^{++}]_i$ which is delayed in onset by 200-400ms. In the presence of EGTA, which binds external Ca^{++}, this response is delayed by an additional 60-100ms, implying that the influx of external Ca^{++} precedes the release of Ca^{++} from internal stores and is not dependent on intracellular Ca^{++} release. In addition, the delay between agonist addition and the initiation of a Ca^{++} flux indicates that there are one or more biochemical steps between agonist binding and the release and/or influx of Ca^{++}. These biochemical steps most likely involve the production of intracellular second messengers. Contrary to the other agonists tested, ADP stimulation causes an increase in Ca^{++} which is delayed in onset by only

20ms in the presence of external Ca^{++}. When EGTA is present in the buffer, ADP causes Ca^{++} fluxes which are similar to those initiated by the other agonists in similar conditions,[351,352] suggesting a similar mechanism(s) for the release of internal Ca^{++}. The lack of significant delay between ADP-binding and Ca^{++} influx in the presence of external Ca^{++} suggests that the ADP receptor is closely associated with or coupled to the plasma membrane Ca^{++} channel, possibly via a G protein-mediated mechanism.[331,352] A role for an, as yet unidentified, G protein in regulating influx through a plasma membrane Ca^{++} channel is also suggested by studies using Al^{+++} and Fl^{-1}. These substances, which directly stimulate G proteins, cause an elevation in $[Ca^{++}]_i$ when added to platelets.[331]

The mechanism for the agonist-induced Ca^{++} influx across the plasma membrane is unclear. There are two major types of calcium channels in the membranes of cells: (1) voltage-dependent channels; and (2) receptor-operated channels. Studies using organic Ca^{++} channel antagonists and/or membrane depolarization agents have shown that voltage-dependent Ca^{++} channels do not play a role in the agonist-induced Ca^{++} influx,[109,243,349,477] leading to the conclusion that the Ca^{++} influx is mediated by receptor-operated channels.[14,42,154,192] A role for second messengers is indicated by the lag period in the kinetic studies discussed above. Second messengers implicated in cells other than platelets include (1,4,5)IP_3 and (1,3,4,5)IP_4; however, kinetic studies have shown that influx precedes internal release. This argues against a role for IP_4 which is formed after the release of (1,4,5)IP_3.[352] (1,4,5)IP_3 causes the release of Ca^{++} from platelet

plasma membrane vesicles, suggesting a role in Ca^{++} influx.[328] PIP_2 may also mediate the influx of Ca^{++} through the receptor-operated channels, since it is more potent than (1,4,5)IP_3 in inducing Ca^{++} mobilization from platelet microsomes.[243,289]

The plasma membrane receptor-operated Ca^{++} channel may be closely associated with the GPIIb-IIIa complex. Monoclonal antibodies against the GPIIb-IIIa complex inhibit Ca^{++} influx, but not internal mobilization, following stimulation with weak agonists; however, Ca^{++} influx in response to strong agonists is not affected. It has been proposed that the antibody causes stearic hindrance of an adjacent Ca^{++} channel since platelets from patients with Glanzmann's thrombasthenia, which are lacking the complex, have normal Ca^{++} fluxes. This Ca^{++} channel is important in influx in response to weak agonists while strong agonists appear to stimulate influx via a different route.[316] It has also been proposed that it is the binding of fibrinogen to the GPIIb-IIIa complex or the incorporation of the GPIIb-IIIa complex into the cytoskeleton which opens a pathway for Ca^{++} influx and is blocked by the monoclonal antibody.[469]

As discussed in the section on the inositol phosphates, (1,4,5)IP_3 and (1,2 cyclic 4,5)IP_3 are major stimuli for the receptor-mediated release of Ca^{++} from the DTS.[331] Intracellular Ca^{++} may also be released from the membranes themselves. Studies have shown that the hydrolysis of membrane PIP_2 correlates with a decrease in chlortetracycline fluorescence in stimulated platelets, indicating a release of membrane-bound Ca^{++};[64] however, it is possible that in the

physiological state the Ca^{++} binding sites on PIP_2 are actually occupied by Mg^{++} which is present in relatively higher concentrations in the platelet cytoplasm.[377]

Ca^{++}-Dependent Processes During Platelet Activation

Ca^{++} plays a role in a number of effector pathways during platelet activation. These effector pathways include: (1) Ca^{++}-calmodulin dependent protein kinase; (2) Ca^{++}-dependent proteases; and (3) the enzymes PLC, PLA_2 and PKC.[212] PLC and PLA_2 have been discussed above. PKC will discussed in a later section. Intracellular Ca^{++} also plays an important part in the organization of cytoskeletal proteins.[377]

Ca^{++}-Calmodulin Dependent Protein Kinase

Calmodulin, a Ca^{++}-binding protein present in high concentrations in the platelet cytoplasm, is a primary intracellular Ca^{++} receptor. Once formed, the Ca^{++}-calmodulin complex interacts with a variety of calmodulin-binding proteins which then modulate the cellular response. Ten different calmodulin binding proteins have been identified in platelet preparations and include MLCK, phosphorylase kinase, caldesmon, calmodulin-dependent kinase and phosphoprotein phosphatase.[436] The role of many of these proteins in platelet activation is not known.

MLCK is an enzyme whose activity is regulated by the Ca^{++}-calmodulin complex.[96,142,163,164] Activation of MLCK causes the phosphorylation of the 20kDa myosin light chain.[177,353] The initiation

of shape change correlates with phosphorylation of this 20kDa protein.[95] Myosin phosphorylation is associated with an increase in the actin-activated ATPase activity, the association of myosin with the cytoskeleton and the assembly of myosin into filaments.[377] The association of myosin with actin and the activation of the ATPase leads to the generation of a contractile force which an important role in the centralization of cytoplasmic granules, secretion and clot retraction.[142]

MLCK activity is modulated by substances other than the Ca^{++}-calmodulin complex. Increased cAMP causes the phosphorylation of MLCK which, in the absence of Ca^{++}-calmodulin, inhibits MLCK activity and decreases phosphorylation of the myosin light chain.[88] In the absence of the Ca^{++}-calmodulin complex, PKC phosphorylates MLCK at two different sites and inhibits subsequent calmodulin binding.[178] The physiologic significance of this finding is not clear since it has not been demonstrated to occur in intact platelets.[377]

Calcium-Dependent Protease

Platelets contain two types of Ca^{++}-dependent neutral proteases, more commonly known as calpains, which have similar substrate specificities but different Ca^{++} requirements. Calpain I hydrolyses its substrates at Ca^{++} concentrations of only 1 to 10uM while calpain II requires Ca^{++} concentrations of 0.1 to 1mM.[366] Calpain I is an 80kDa protein which remains intracellular during platelet activation. Upon platelet aggregation, it is cleaved to a 76-78kDa protein which appears

to be the active form.[357] Calpain II is associated with the intracellular membranes in the resting state. Following activation, it becomes exposed on the external portion of the membrane.[366]

The role of Ca^{++}-dependent protease in platelet activation is not clear. Calpains are activated when platelet aggregation is stimulated by a variety of agonists. If aggregation is prevented by not stirring the platelet suspension, the protease is not activated even though the platelets are still activated.[134] Ca^{++}-dependent protease hydrolyses actin binding protein and P235, permitting reorganization of the platelet cytoskeleton during platelet aggregation.[132] Other studies have shown that calpain activation may occur without aggregation, although calpain activation does not induce platelet secretion and aggregation.[110]

Calpain may also have a role in the exposure of fibrinogen binding sites. One recent report has shown that calpain cleaves aggregin in thrombin-stimulated platelets, allowing exposure of the fibrinogen binding site.[321] Another study utilizing a calpain inhibitor has suggested that calpain is involved in the activation of PLC and TxA_2 synthetase.[426] Opposite results were demonstrated in a different study using calpain inhibitors in which calpain had no effect on PLC or adenylate cyclase activity.[61]

Role of Ca^{++} in the Cytoskeleton

Ca^{++} has important, though sometimes opposing, roles in the organization and composition of the platelet cytoskeleton.[377] In resting platelets, the majority of the actin is maintained as monomers by association with the protein profilin. The interaction between actin and profilin is stabilized by Ca^{++}.[235] Gelosin is another protein which interacts with actin to regulate actin filament length. In the resting platelet, gelosin is poorly complexed to actin; however, when $[Ca^{++}]_i$ increases during activation, gelosin tightly complexes to actin. When gelosin is added to preformed actin filaments, the association of gelosin with actin causes the shortening of filaments. When added to actin monomers, the association of gelosin with actin increases the rate of formation of actin filaments by stabilizing the small oligomeric nuclei.[234] Since nuclei formation is the rate limiting step in the formation of actin filaments,[377] the increasing interaction of gelosin and actin during periods of elevated $[Ca^{++}]_i$ may enhance the actin polymerization that occurs during platelet activation.[216] The organization and structure of the cytoskeleton in resting and stimulated platelets is partially controlled by the Ca^{++}-dependent interaction of various proteins (ie. actin binding protein, α-actinin and P 235) with actin.[377] The role of calpain and MLCK in cytoskeletal organization were discussed above.

THE ROLE OF PROTEIN KINASE C IN PLATELET ACTIVATION

Protein Kinase C

Protein kinase C is a Ca^{++}-dependent enzyme which requires the phospholipid phosphatidylserine for its activity.[194] In the resting cell, the majority of PKC is located in an inactive form in the cytoplasm. Following cell activation, most of the PKC is transferred from the cytoplasm to the membrane.[208,465] The binding of PKC to the membrane is inhibited by the removal of Ca^{++} [355] and appears to be due to the synergistic action of both Ca^{++} and 1,2-DG.[380,465] Although enzyme translocation from the cytoplasm to the membrane accompanies platelet activation, studies have shown that PKC activation can occur without translocation. The role of the cytoplasmic, activated PKC has not been determined.[117,301]

Several species of PKC have been identified in mammalian tissue. To date, two subtypes PKC have been identified in human platelets.[427,441] The role of the different subtypes in platelet function is not known.

Diacylglycerol and Phorbol Activation of PKC

In platelets, 1,2-DG activates PKC by increasing the affinity of PKC for Ca^{++}, allowing enzyme activation at Ca^{++} concentrations present in resting cells.[193,414] It has been proposed that phosphatidylserine binds Ca^{++} and subsequently creates a surface in the membrane for PKC

binding. The addition of sn-1,2-DG then causes a change, possibly conformational, in PKC, leading to reversible enzyme activation.[138]

In vitro, PKC can be activated by synthetic diacylglycerols and phorbol esters independently of PLC-induced formation of 1,2-DG. Synthetic diacylglycerols are chemically modified sn-1,2-diacylglycerols which intercalate into the membranes of intact platelets to activate PKC.[194,224] Phorbol esters appear to have a similar structure to the diacylglycerols and to be able to bind PKC, causing activation.[72]

Although phorbol ester- and synthetic DG-induced activation of PKC are frequently used in vitro to simulate the in vivo activation of PKC, there are some significant differences. First, phorbol esters cause permanent activation of PKC[377] by inducing the proteolytic cleavage of the 80kDa PKC into an active 51kDa protein enzyme which is Ca^{++}- and phospholipid-independent.[416,417] Secondly, the synthetic diacylglycerols are more dependent on secreted ADP for platelet activation. The addition of apyrase to synthetic DG-stimulated platelets inhibits aggregation and protein phosphorylation. This inhibition is significantly less in phorbol stimulated platelets.[10] Thirdly, it has been proposed that phorbol esters primarily cause platelet aggregation by a direct effect on altering platelet membranes rather than by activating PKC.[187] Finally, the addition of synthetic diacylglycerols, but not phorbol, to platelets is associated with an increased $[Ca^{++}]_i$ due to its metabolism to the second messengers PA and lysoPA.[56]

Phosphorylation of Proteins by PKC

In platelets, the major substrate of PKC that has been identified is a 47kDa cytoplasmic protein.[362] In resting platelets, this protein consists of two or three poorly phosphorylated components as identified by two-dimensional electrophoresis and isoelectric focusing. Following stimulation, seven to nine phosphorylated components have been identified.[180,239]

Though traditionally associated with secretion, a variety of specific functions for the phosphorylated 47kDa protein have been proposed. Purified 47kDa from unstimulated platelets inhibits actin polymerization while phosphorylated 47kDa protein from stimulated platelets has no inhibitory effects. This suggests a role for the 47kDa protein in the cytoskeletal reorganization that accompanies platelet activation.[158] PKC activation is associated with actin incorporation into the cytoskeleton and the subsequent formation of pseudopods.

Another proposed role for the 47kDa protein is that of a lipocortin. Lipocortins are a group of proteins which inhibit PLA_2. It has been suggested that phosphorylation of the 47kDa protein suppresses its anti-PLA_2 activity, allowing PLA_2 activation to occur.[424] Recent studies have shown that gamma thrombin causes phosphorylation of the 47kDa protein without the measurable production of arachidonic acid that would be expected if PLA_2 activation had occurred.[91] This suggests that substances other than the 47kDa protein control PLA_2 activity.

A third function proposed for the 47kDa protein is that of 5-phosphomonoesterase, an enzyme that cleaves the 5 position phosphorous from the Ca^{++}-mobilizing (1,4,5)IP_3, (1,2cyclic 4,5)IP_3 and (1,3,4,5)IP_4 to yield relatively inactive metabolites. Gel electrophoresis and peptide mapping comparisons of purified 5-phosphomonoesterase and the 47kDa protein of platelets indicate that they are identical proteins.[85] It has been suggested that PKC-mediated phosphorylation of the 47kDa protein increases its activity as a 5-phosphomonoesterase, leading to the termination of the stimulatory Ca^{++} signal generated by the inositol phosphates;[85] however, there has been no demonstration of enzymatic activity by purified platelet 47kDa protein.[377] In addition, inhibition of PKC activation by staurosporine blocks thrombin-stimulated 47kDa phosphorylation without causing measurable alteration in the production of the inositol phosphates.[443]

Substrates for PKC other than the 47kDa protein have been identified in platelets. PKC phosphorylates the light (20kDa) chain of myosin in phorbol ester-stimulated platelets[199] at a site distinct from that phosphorylated by the Ca^{++}-calmodulin dependent MLCK.[275] Thrombin-stimulated platelets phosphorylate myosin at two separate sites. One site is phosphorylated rapidly and is mediated by MLCK. The second site is more slowly phosphorylated by PKC.[276] The physiologic significance of this PKC-mediated phosphorylation of myosin has yet to be determined. Phorbol treatment of platelets is also associated with phosphorylation of three high molecular weight cytoskeletal proteins: (1) actin binding protein; (2) talin (P235); and (3) myosin heavy chain. This supports a

role for PKC in the regulation of contractile or cytoskeletal events in platelet activation.[236]

The Stimulatory Effects of PKC in Platelet Activation

The role of PKC in platelet activation is very complex and unclear since PKC has been reported to have both positive and negative feedback effects.[377] Numerous reports have demonstrated that Ca^{++} and PKC activation act synergistically to induce full platelet activation.[139,142,194] In aspirin-treated platelet rich plasma, the addition of phorbol esters 10-15 seconds prior to the addition of a variety of agonists (including epinephrine, ADP, U46609, collagen, PAF or vasopressin) potentiates both secretion and aggregation.[381] The combination of subthreshold concentrations of synthetic diacylglycerol and calcium ionophore induces secretion.[194] Finally, thrombin-induced aggregation and secretion are potentiated by subthreshold concentrations of phorbol ester.[470] Low concentrations of a variety of agonists which mobilize Ca^{++} also potentiate phorbol-induced aggregation and secretion. It has been suggested that the initial mobilization of Ca^{++} causes the translocation of PKC to the membrane where it is primed for activation by phorbol ester or 1,2-DG.[380]

The mechanism by which PKC potentiates platelet activation is not clear. PKC causes alterations in the membrane GPIIb-IIIa complex, leading to exposure of the fibrinogen binding site and subsequent fibrinogen binding.[371] PKC also activates Na^{+}/H^{+} exchange, causing

cytoplasmic alkalinization which then potentiates platelet activation.[386]

PKC activation enhances the Ca^{++}-dependent arachidonic acid release following stimulation with calcium ionophore A23187.[6,122,150,152] Addition of phorbol ester by itself does not cause any measurable release of arachidonic acid. Phorbol esters and synthetic diacylglycerol, however, cause a dose- and time-dependent potentiation of arachidonic acid release in response to A23187, even in platelets treated with aspirin and creatine kinase/creatinine phosphate to prevent secondary PLC activation.[122] This suggests that PKC promotes the Ca^{++}-dependent PLA_2 release of arachidonic acid by either increasing the enzyme sensitivity to Ca^{++} or making more phospholipid substrate and/or PLA_2 available. One study has proposed that this potentiation is partly mediated by Na^+/H^+ exchange and cytosolic alkalinization[6] while a different study has shown that Na^+/H^+ exchange is not involved.[150] PKC may enhance arachidonic acid release through a G protein-mediated stimulation of PLA_2.[5,7] Phorbol-induced PKC activation inhibits both arachidonyl-CoA synthase and arachidonyl-CoA lysophosphatide acyltransferase, causing decreased incorporation of arachidonic acid into membrane phospholipids and increased availability of arachidonic acid for the production of endoperoxides and TxA_2.[136]

The Inhibitory Effects of PKC on Platelet Activation

PKC has numerous inhibitory functions in platelet activation.[377] It has been proposed that the determination of whether the overall

effect of PKC activation is inhibitory or stimulatory depends on the type of agonist used and the incubation time.[209] Secretion induced by all agonists tested is inhibited by long (5 minutes) periods of phorbol incubation. In non-aspirin treated platelet rich plasma, short (10 seconds) periods of pretreatment with phorbol ester inhibit TxA_2 production and secretion in response to stimulation with weak agonists (ADP, epinephrine and PAF) which are partially dependent on TxA_2 production for full activation. It has been proposed that this effect is due to inhibition of the metabolism of arachidonic acid to TxA_2. If TxA_2 production is blocked by pretreatment of platelets with aspirin, phorbol pretreatment potentiates secretion in response to these same agonists in a TxA_2-independent manner.[209]

PKC activation has multiple inhibitory effects on Ca^{++} mobilization. Treatment of platelets with phorbol or 1,2-DG does not induce increased $[Ca^{++}]_i$ as measured using the fluorescent dye indicators (ie. quin2, fura 2 and indo 1); however, a Ca^{++} peak is detected using aequorin.[437] This suggests that phorbol or 1,2-DG stimulates a localized zone of $Ca^{++}{}_i$ elevation, possibly associated with the plasma membrane. Pretreatment of platelets with phorbol esters inhibits elevations in $[Ca^{++}]_i$ induced by thrombin, ADP, epinephrine, TxA_2 mimetic U46619, PAF and vasopressin.[117,240,279,371] Phorbol ester or diacylglycerol treatment of platelets inhibits influx through the plasma membrane receptor-operated Ca^{++} channel.[13,311,433] Activation of PKC also inhibits the agonist-induced release of internal stores of Ca^{++}, possibly by modulating the formation and/or degradation of $(1,4,5)IP_3$.[433] Following stimulation with thrombin or ADP, activation

of PKC stimulates resequestration of the released Ca^{++}. This sequestration occurs independent of any effect on cAMP or (1,4,5)IP_3.[471,472,473] Finally, PKC activation potentiates Ca^{++} efflux from the cell following stimulation with thrombin, possibly by stimulating the plasma membrane Ca^{++}-ATPase pump.[315,331]

In addition to a negative effect on Ca^{++} mobilization, PKC activation inhibits phosphoinositide hydrolysis as measured by decreased production of PA, 1,2-DG or inositol phosphates in response to ADP, PAF, U46069, thrombin or collagen.[39,380,421,442] One study has proposed that PKC inhibits the G protein coupled to PLC.[5] By itself, stimulation with phorbol esters or synthetic diacylglycerol has no effect on platelet levels of inositol phosphates, indicating no direct effect of PKC on the hydrolysis of the phosphoinositides.[442] Treatment with either phorbol or 1,2-DG actually increase the levels of the polyphosphoinositides, suggesting that PKC modulates the activity of the enzymes involved in the 'phosphoinositide cycle' (ie. the specific kinases or phosphatases).[442]

Finally, PMA inhibits the production of cAMP in response to PGE_1, prostaglandin D_2 and forskolin. PMA also blocks the inhibition of cAMP production by thrombin and epinephrine while it has no effect on ADP-induced inhibition of cAMP production. These findings suggest that the overall effect of PKC activation is inhibition of adenylate cyclase; however, the effects depend on the agonist used.[460] Previous reports have shown that a primary role of PKC is to inhibit the agonist-induced

inhibition of adenylate cyclase by phosphorylating the inhibitory G protein (G_i) associated with adenylate cyclase.[28,196]

THE ROLE OF Na^+/H^+ EXCHANGE IN CELL ACTIVATION

Regulation of intracellular pH is vital to the proper functioning of all cells. The typical cell has a large interior-negative membrane potential, causing the passive influx of H+ ions into and/or efflux of negatively charged ions (ie OH^- or HCO_3^-) out of the cell. To maintain resting cytosolic pH (pH_i), cells must have a mechanism(s) to actively extrude H^+ or to actively accumulate HCO_3^-.[246] A variety of cells respond to stimulation by increasing pH_i. This also requires a process for actively extruding H^+ and/or accumulating HCO_3^-. One mechanism involves anion channels which exchange Cl^- for HCO_3^- in either a Na^+-dependent or -independent manner. Relatively few investigations have examined the role of anion channels in platelets. Cl^-/HCO_3^- exchange has been shown to play a role in the regulation of pH_i by some investigators[294] while others have found no effect of anion exchange on resting or stimulated pH_i.[77] In recent years, Na^+/H^+ exchange is the primary mechanism implicated in the modulation of platelet pH_i.[386]

Characteristics of Na^+/H^+ Exchange

Although the principle function of Na^+/H^+ exchange is to regulate pH_i, it has other important roles in cellular metabolism. The exchanger is involved in signal transduction in response to those agonists which affect cell function by altering pH_i. Since the exchanger transports

Na^+, it controls cell volume regulation. Na^+/H^+ exchange is also involved in the net transport of solutes across epithelia such as the renal proximal tubule or the small intestine.[246]

The stoichiometry of the Na^+/ H^+ antiport is 1:1. Since the drive for the transport of H^+ out of the cell comes from an inwardly directed electrochemical Na^+ gradient, the antiport is not directly dependent on the production of energy. The Na^+ gradient must be maintained by an energy-requiring, plasma membrane Na^+-K^+-ATPase system.[246] Na^+/H^+ exchange is electroneutral, independent of the membrane potential[386] and fairly specific for Na^+ and Li^+. It is inhibited by amiloride or its derivatives.[237] In vitro, this exchange is commonly inhibited using four different experimental manipulations: (1) the addition of amiloride derivatives to directly block the exchange; (2) replacement of external Na^+ to eliminate the inwardly directed Na^+ gradient; (3) increasing extracellular H^+ concentration to block H^+ efflux; and (4) treatment of cells with a Na^+-K^+-ATPase inhibitor to decrease the inwardly directed Na^+ gradient.

The Na^+/H^+ antiport is relatively quiescent in the resting platelet; however, addition of amiloride derivatives to non-stimulated platelets causes a slow decrease in pH_i of 0.05.[475] This indicates that the antiport is responsible for the continuous removal of metabolically generated protons. The activity of the antiport is greatly increased when the cytoplasm is acidified.[237] This suggests that the antiport has two binding sites: (1) a site that exchanges Na^+ for H^+; and (2) a site where H^+ binding modifies the activity of the antiport.[386] Stimulation

with either phorbol esters, 1,2-DG or thrombin causes an increase in pH_i which requires external Na^+ and is inhibited by amiloride derivatives or PKC inhibitors.[387,391,392] The latter findings indicate that PKC also activates the antiport, possibly by altering the setpoint of the exchanger so that it responds to a more alkaline pH_i by increasing its H^+ extrusion.[386] The antiport is activated by other agonists, such as ADP or epinephrine, in a process independent of PKC activation. Epinephrine causes a cytoplasmic alkalinization which is blocked by amiloride derivatives, suggesting that the increase in pH_i is mediated by Na^+/H^+ exchange. This alkalinization is also inhibited by an antibody which blocks fibrinogen binding, indicating a dependence on fibrinogen receptor expression.[20] Finally, preliminary findings in a recent study suggest that the Na^+/H^+ exchanger is regulated by two different G proteins: (1) a G protein stimulated by epinephrine which increases Na^+/H^+ exchange; and (2) a G protein stimulated by fluoride which inhibits Na^+/H^+ exchange.[389]

Na^+/H^+ Exchange and Platelet Activation

Cell activation is often accompanied by increased pH_i. Cytosolic alkalinization represents a balance between the metabolic generation of protons, the buffering capacity of the cytoplasm and the extrusion of H^+ from the cell.[475] Platelet alkalinization and activation of Na^+/H^+ exchange occurs following stimulation with thrombin, PAF, ADP, epinephrine, vasopressin, TxA_2 mimetic U46619 and calcium ionophore A23187.[45,390,394,409,413,423,475] Artificial alkalinization of the platelet cytosol by use of the monovalent cation ionophores, monensin or

nigericin, or by addition of NH_4Cl does not, by itself, induce platelet activation as assessed by the measurement of Ca^{++} mobilization, PA production, aggregation or protein phosphorylation.[385,398,475] Artificial alkalinization does sensitize platelets to subsequent activation by thrombin.[384] This suggests that cytoplasmic alkalinization enhances the platelet responses to the subsequent addition of agonists.

The addition of amiloride derivatives or the removal of external Na^+ prevent Na^+/H^+ exchange. These manipulations also inhibit aggregation in response to ADP, PAF, collagen, phorbol esters, vasopressin and calcium ionophore A23187,[45,388,390,423] suggesting that a functional Na^+/H^+ exchange mechanism is essential for full aggregation responses. Other groups dispute this requirement for Na^+/H^+ exchange. One study has shown that ADP-induced aggregation is inhibited only when using amiloride concentrations much higher than required for inhibition of the antiport. They have proposed that the inhibition demonstrated in the previous studies is due to secondary effects of amiloride.[135] A second study has shown that amiloride derivatives inhibit agonist-induced aggregation in doses 100 times greater than those required for inhibition of the antiport, once again pointing to secondary inhibitory effects of amiloride.[176]

Na^+/H^+ Exchange and Ca^{++} Mobilization

A considerable amount of information suggests that Ca^{++} mobilization and Na^+/H^+ exchange are interrelated. Increased $[Ca^{++}]_i$

precedes cytosolic alkalinization and may activate the antiport. First, the addition of the non-physiologic calcium ionophores, which increase membrane permeability and cause increased $[Ca^{++}]_i$, stimulates Na^+/H^+ exchange.[390] Secondly, removal of Ca^{++} from the external media inhibits alkalinization in response to PAF stimulation. This decreased alkalinization corresponds with a decrease in $[Ca^{++}]_i$ secondary to impaired Ca^{++} influx.[45] Finally, simultaneous measurements of pH_i and Ca^{++} flux demonstrate that there is an initial decrease in pH_i following stimulation and that the subsequent increase in pH_i lags behind the increase in $[Ca^{++}]_i$.[99,398,475] In contrast, a recent study has shown that Ca^{++} mobilization occurs simultaneously with activation of the antiport. It was proposed that the initial decrease in measured pH is actually due to platelet shape change which interferes with the measurement of the fluorescence of the dye used to measure pH_i.[393]

Other evidence suggests that activation of the Na^+/H^+ antiport modulates the Ca^{++} fluxes. Blockage of the antiport by the addition of amiloride derivatives or the removal of external Na^+ markedly reduces the release of Ca^{++} from internal stores and the influx of external Ca^{++} in response to thrombin.[145,384,395] This inhibition is not due to decreased hydrolysis of the membrane phosphoinositides by PLC and can be overcome by artificial alkalinization of the cytosol.[395] Ca^{++} mobilization in response to ADP, U46619 and PAF is also partially dependent on activation of Na^+/H^+ exchange.[45,394] It is not known how pH_i affects Ca^{++} influx across the plasma membrane. (1,4,5)IP_3-induced Ca^{++} release from internal stores is potentiated by an alkaline pH_i.[55] It has been proposed that an alkaline pH_i either enhances the binding of

(1,4,5)IP_3 to its receptor or interferes with the breakdown of (1,4,5)IP_3 to its inactive metabolites.[386]

In contrast, other studies have demonstrated that Ca^{++} mobilization occurs independent of Na^+/H^+ exchange and cytosolic alkalinization. Ca^{++} mobilization in response to thrombin occurs in spite of cytosolic acidification and/or inhibition of Na^+/H^+ exchange.[176,358,398,474] Simultaneous flow cytometric measurements of thrombin-induced changes in cytosolic pH_i and Ca^{++} flux indicate that the initial acidification phase correlates with the increasing $[Ca^{++}]_i$ while the subsequent alkalinization appears to be independent of the Ca^{++} changes.[99]

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

Removal of external Na^+ inhibits platelet aggregation and secretion following stimulation with the weak agonists ADP, epinephrine and low concentrations of thrombin. These agonists are dependent on the formation of arachidonic acid metabolites to induce secondary aggregation and secretion. These effects can be duplicated by treating platelets with aspirin to inhibit cyclooxygenase, suggesting a role for extraplatelet Na^+ in the arachidonic acid release pathway.[86] Blockage of Na^+/H^+ exchange by the addition of amiloride derivatives, the removal of extraplatelet Na^+ or the acidification of external pH also inhibits arachidonic acid mobilization using these same agonists.[412] Inhibition of Na^+/H^+ exchange blocks the activation of PLC by weak agonists but not by high concentrations of thrombin, indicating that PLC activity is not

directly affected by Na^+/H^+ exchange. Instead, it has been proposed that weak agonists cause Na^+/H^+ exchange-mediated activation of PLA_2. PLA_2 then mobilizes a very small pool of arachidonic acid which can only be detected using gas chromatography/mass spectrometry. The subsequent production of cyclooxygenase metabolites acts as an amplification loop to activate PLC.[409] It has also been proposed that weak agonists stimulate PLA_2 activity by a combination of local alkalinization, due to the activation of the Na^+/H^+ exchange, and the release of a small pool of membrane Ca^{++}.[411] In vitro evaluation of PLA_2 shows that enzyme activity is potentiated by increasing pH up to a maximum of 8.0 in the presence of Ca^{++}.[27] Mobilization of arachidonic acid in response to PAF is also partially dependent on Na^+/H^+ exchange.[413]

Stimulation of phosphoinositide hydrolysis by epinephrine is blocked by either: (1) inhibition of PLA_2 or cyclooxygenase activity; (2) inhibition of Na^+/H^+ exchange; or (3) prevention of fibrinogen binding by removal of fibrinogen, addition of a monoclonal antibody against GPIIb-IIIa or incubation with EDTA. Similarly, epinephrine-induced cytosolic alkalinization is inhibited not only by prevention of Na^+/H^+ exchange by amiloride derivatives, but also by a monoclonal antibody to the GPIIb-IIIa complex.[20] These findings suggest that epinephrine stimulates PLC activity in a manner dependent on PLA_2 activation, fibrinogen binding and cytosolic alkalinization. More importantly, these results indicate that weak agonists initiate exposure of fibrinogen binding sites and subsequent fibrinogen binding concomitant with activation of Na^+/H^+. The mechanism by which fibrinogen binding mediates cytosolic alkalinization is not clear. Some

data suggests that the α_2-adrenergic receptor is closely associated with, if not actually a part of, the Na^+/H^+ exchanger.[410] It has been proposed that binding of agonist to the adrenergic receptors causes a local alkalinization but that the production of measurable alkalinization also requires fibrinogen binding.[410]

Summary of Na^+/H^+ Exchange

There are at least two pathways for agonist-induced activation of Na^+/H^+ exchange in platelets. The first occurs following the addition of strong agonists such as thrombin. The binding of agonist to a receptor activates PLC and stimulates the production of the second messengers (1,4,5)IP_3 and 1,2-DG. (1,4,5)IP_3 mobilizes Ca^{++} while 1,2-DG activates PKC which, in turn, stimulates Na^+/H^+ exchange. The second pathway occurs following activation by weak agonists such as epinephrine. The binding of agonist activates Na^+/H^+ exchange by some undefined mechanism that also requires fibrinogen binding. This local alkalinization, in combination with a local Ca^{++} elevation, activates PLA_2. The release of prostaglandin endoperoxides and TxA_2 acts as a positive feedback loop to activate PLC and stimulate subsequent platelet activation.[386,410] While strong agonists cause the greatest activation of Na^+/H^+ exchange, cytosolic alkalinization is not essential for platelet activation to occur in response to these agonists. The increase in pH_i just enhances the process of signal transduction. This enhancement of signal processing is much more important in the response of platelets to weak agonists like ADP or epinephrine.[386,410]

SUMMARY OF THE EFFECTS OF INDIVIDUAL PLATELET AGONISTS

Platelets respond to a large number of physiologic and non-physiologic agonists. The following summary will discuss individually the agonists used in the research for this dissertation. It should be kept in mind that *in vivo*, platelets are stimulated by multiple agonists simultaneously. Numerous studies have demonstrated synergistic responses of different platelet agonists. The addition of subthreshold doses of agonists which do not cause platelet activation alone can induce the full platelet response when added in combination.[377]

Agonists are divided into three groups depending on their ability to cause release and irreversible aggregation. Weak agonists (ie. epinephrine and ADP) require the release of cyclooxygenase metabolites and primary aggregation to induce irreversible aggregation and secretion. Secretion is almost completely inhibited by cyclooxygenase blockers. Agonists of intermediate strength (ie. PAF, vasopressin, prostaglandin endoperoxides/TXA_2 mimetics) induce secretion independent of prior aggregation or release of arachidonate metabolites. Treatment of platelets with cyclooxygenase inhibitors or agents which remove extracellular ADP reduce secretion and aggregation. Strong agonists (ie. high concentrations of thrombin) stimulate secretion and aggregation in a manner that is not affected by cyclooxygenase inhibitors.[377] Low concentrations of strong agonists act like intermediate or weak agonists, but high concentrations of weak or intermediate agonists do not act like strong agonists.

Adenosine Diphosphate (ADP)

ADP is considered a weak physiologic agonist. The binding of ADP to specific platelet receptors has two separate effects: (1) inhibition of the activation of adenylate cyclase by stimulatory agonists; and (2) stimulation of shape change, fibrinogen binding site exposure and aggregation.[377] Studies using 5'-p-fluorosulfonylbenzoyl adenosine (FSBA), an affinity analogue of ADP, have shown that ADP covalently binds to a 100kDa protein, called aggregin, on the plasma surface.[81,82,83] This binding is associated with inhibition of shape change and aggregation but not inhibition of adenylate cyclase activity.[123,324] These findings suggest that there are two distinct membrane receptors for ADP, one coupled to adenylate cyclase and the other coupled to the stimulation of aggregation and shape change. Two separate receptors have yet to be identified.

It has been proposed that aggregin sterically inhibits the fibrinogen binding site on the membrane GPIIb/IIIa complex. Binding of ADP to this receptor causes a conformational change in aggregin which permits exposure of the fibrinogen binding site and subsequent fibrinogen binding to the GPIIb-IIIa complex.[81,82,83] The use of FSBA to block ADP binding at the receptor level also blocks fibrinogen binding and aggregation in response to the prostaglandin endoperoxides or to epinephrine while shape change following stimulation with the endoperoxides is not affected. This suggests that small quantities of ADP are essential in epinephrine- and endoperoxide-stimulated platelets to allow exposure of the fibrinogen binding site.[82] Fibrinogen binding

induced by high concentrations of thrombin is not affected by FSBA, indicating that thrombin uses a different mechanism for exposure of the fibrinogen binding sites.[82]

As discussed above, it has been proposed that ADP initiates platelet activation by stimulating the release of a small amount of arachidonic acid by a Na^+/H^+ exchange-dependent activation of PLA_2. The released arachidonic acid is metabolized by cyclooxygenase to yield the prostaglandin endoperoxides and TxA_2 which amplify the platelet response by subsequently activating PLC.[409,411] Inhibition of Na^+/H^+ exchange blocks the release of arachidonic acid but not the occurrence of primary aggregation.[412] These findings support the theory that binding of ADP to aggregin exposes the fibrinogen binding site, an event essential for the process of primary aggregation. The process of secondary or irreversible aggregation is dependent on the generation of arachidonate metabolites in a Na^+/H^+ exchange-dependent manner.

When platelets are treated with cyclooxygenase inhibitors to block the endoperoxide/TxA_2-mediated activation of PLC, the addition of ADP induces a rapid increase in $[Ca^{++}]_i$.[92,124,290,394] This indicates that Ca^{++} mobilization in response to ADP is partially independent of the release of arachidonate metabolites. Kinetic studies have shown that, in the presence of external Ca^{++}, this mobilization occurs without the lag phase found following stimulation by other agonists.[351,352] The rapid response negates the role of a receptor-mediated production of a diffusible second mediator to induce the Ca^{++} influx. Instead, the response suggests more direct coupling between the receptor and the

plasma membrane Ca^{++} channel, possible via a G protein-mediated mechanism.[331] The increase in $[Ca^{++}]_i$ is partially inhibited when platelets are incubated in EGTA-buffer, indicating a role for the influx of external Ca^{++} as well as the release of internal stores of Ca^{++} in the overall Ca^{++} response. Kinetic studies have shown that, in the absence of external Ca^{++}, ADP-stimulated platelets have a significant lag period between the addition of agonist and the initiation of a measurable change in $[Ca^{++}]_i$. The lag period suggests a role for the production of a diffusible second messenger in the internal Ca^{++} response.[351,352] Ca^{++} mobilization in response to ADP is not affected by incubation with FSBA. Therefore, the receptor mediating Ca^{++} mobilization and adenylate cyclase activation by ADP is not aggregin, the receptor that regulates ADP-induced fibrinogen binding and aggregation.[324]

Whether or not ADP activates PLC independent of the generation of arachidonic acid metabolites is under debate. One study has demonstrated that ADP stimulation of cyclooxygenase-inhibited platelets mobilizes Ca^{++} without inducing phosphoinositide metabolism.[124] A separate study has shown that cyclooxygenase-inhibition by aspirin blocks the production of the inositol phosphates in platelets stimulated by ADP. This finding suggests that PI hydrolysis is initiated secondary to the production of endoperoxides/TxA_2 and not directly by ADP.[409] On the other hand, it has been demonstrated that ADP stimulation of cyclooxygenase-inhibited platelets does not induce measurable PI hydrolysis while it does cause increased production of (1,4,5)IP_3 and 1,2-DG. The lack of measurable PI metabolism is attributed to a low

level of PLC activation, allowing PIP_2 resynthesis from PIP to compensate for the PIP_2 hydrolyzed by PLC.[92]

Epinephrine

Epinephrine is a physiologic stimulant which is classified as a weak agonist. It has two separate effects on platelet responses: (1) induction of aggregation and/or potentiation of aggregation induced by other agonists; and (2) inhibition of adenylate cyclase.[377] Studies using the selective antagonist radioligand ^3H methyl-yohimbine have shown that these effects are mediated through a single receptor, the α_2-adrenergic receptor.[168,271] Though mediated by a single receptor, aggregation and inhibition of adenylate cyclase are two separately controlled events. Observations in a family with a defect in the number of α_2-adrenergic receptors have shown that fewer receptors are required for inhibition of adenylate cyclase than for stimulation of aggregation.[326] In addition, pretreatment of platelets with epinephrine for 5-30 minutes decreases the ability of epinephrine to subsequently initiate or potentiate aggregation; however, pretreatment with epinephrine does not affect the ability of epinephrine to inhibit adenylate cyclase.[270]

Platelets also have β_2-adrenergic receptors which, unlike the α_2-adrenergic receptors, inhibit platelet function by increasing the adenylate cyclase-mediated production of cAMP.[464] The relative effects of epinephrine on platelet function are species dependent and determined by the densities of α_2- and β_2-adrenergic receptors.[202]

Epinephrine inhibits adenylate cyclase in a G_i protein-dependent manner. Binding of epinephrine to the α_2-adrenergic receptor induces a GTP-dependent dissociation of the alpha subunit of G_i from the beta-gamma subunits of G_i with the subsequent inhibition of adenylate cyclase activity. Pertussis toxin ADP ribosylates G_i, preventing the receptor-mediated dissociation and the subsequent inhibition of adenylate cyclase.[53] Inhibition of adenylate cyclase by epinephrine is dependent on the presence of monovalent cations, preferably Na^+.[53,405,410]

In contrast to most other physiologic agonists, epinephrine does not induce platelet shape change.[383] The ability of epinephrine, by itself, to cause aggregation is questionable. In buffers containing physiologic [Ca^{++}], epinephrine does not cause any aggregation, leading one group to propose that epinephrine only potentiates activation by other agonists and is not a true aggregating agent.[219] Other observations have shown that epinephrine causes full aggregation in a medium containing low [Ca^{++}] and added fibrinogen.[201]

The mechanism by which epinephrine potentiates aggregation is not clear. It has been proposed that epinephrine activates platelets by the Na^+/H^+ exchange-dependent activation of PLA_2 in a manner similar to ADP.[409,410,411] There are, however, some differences. Fibrinogen binding appears essential for this activation to occur since it is blocked by monoclonal antibodies against the GPIIb-IIIa complex.[20] Some evidence suggests that epinephrine alone induces exposure of the fibrinogen binding sites and subsequent fibrinogen binding.[372,445] Other studies have demonstrated that binding of FSBA to the ADP receptor

inhibits epinephrine-induced fibrinogen binding, suggesting an essential role for ADP in epinephrine induced platelet activation.[83,123] Previous studies have used ADP-mobilizing enzymes (ie apyrase or creatine phosphokinase) which may have left very small trace amounts of ADP in the preparation. Investigations have shown that epinephrine increases the avidity of the ADP receptor for ADP, making trace amounts of ADP effective in initiating exposure of the fibrinogen receptor.

By itself, epinephrine does not cause an elevation in $[Ca^{++}]_i$ as measured using the fluorescent chelator dyes quin2 or indo 1. It does cause a measurable increase in $[Ca^{++}]_i$ using aequorin in buffers containing Ca^{++}, but not in buffers containing EGTA.[70,75,219,438] This suggests that epinephrine causes a local membrane-associated, influx of external Ca^{++} without any direct effects on the release of internal Ca^{++}.

When added in combination with other agonists, epinephrine potentiates Ca^{++} influx, Ca^{++} release from internal stores, protein phosphorylation of both the 20kDa and 47kDa proteins, Na^+/H^+ exchange and PA formation.[75,219,290,402] The exact mechanism(s) for the epinephrine-induced potentiation of platelet activation is not known. This potentiation is independent of PLC activation[290] since epinephrine alone cannot activate PLC.[383] The stimulatory effects on PLC, as indicated by PA production, are not inhibited by the prevention of Na^+/H^+ exchange, suggesting that some pathway besides Na^+/H^+ exchange must exist to mediate the epinephrine effects on platelet activation.[402]

The potentiation of Ca^{++} mobilization is independent of both fibrinogen binding and TxA_2 production.[317]

Platelet-Activating Factor (PAF)

PAF is a phospholipid and a physiologic agonist of intermediate potency.[377] Although a PAF receptor has not been isolated, binding studies using ^3H-labelled PAF have shown that canine platelets have a high affinity PAF receptor on their platelet surface. In addition, the specific binding of PAF to canine platelets indicates that the receptor is of similar number, capacity and affinity to that found in human platelets.[186] Pretreatment with PAF inhibits the ability of platelets to respond to subsequent stimulation with PAF or other agonists. Washing the platelets to remove external agonist does not restore sensitivity, suggesting that the agonist induces an uncoupling of the receptor from the effector system.[272] At high concentrations, PAF acts as a detergent and non-specifically alters membranes.[364]

Binding of PAF to its receptor mediates the hydrolysis of the membrane phosphoinositides with the production of the second messengers (1,4,5)IP_3 and 1,2-DG.[106,221,375,383] PAF also causes mobilization of Ca^{++}. Measurements of $[Ca^{++}]_i$ in Ca^{++}- and EGTA-containing buffers have shown that the majority of the increase in $[Ca^{++}]_i$ comes from influx of external Ca^{++}.[155,432] Kinetic studies have demonstrated a lag period between PAF-binding and Ca^{++} mobilization, suggesting a requirement for the production of second messengers in the generation of the Ca^{++} signal.[351,352] Cyclooxygenase inhibition only mildly decreases the

increase in $[Ca^{++}]_i$, therefore the Ca^{++} flux is relatively independent of TxA_2 production.[155]

The role of secreted ADP and of cyclooxygenase-mediated production of prostaglandin endoperoxides/TxA_2 in PAF-induced aggregation and secretion is unclear. Shape change and primary reversible aggregation occur independent of ADP and arachidonic acid metabolism. Irreversible aggregation is strictly dependent on ADP release while its dependence on TxA_2 is variable.[250] In response to low concentrations of PAF, activation of the cyclooxygenase pathway is essential for irreversible aggregation and secretion.[68,407] In response to high concentrations of PAF, the findings differ. Some studies have shown that PAF-induced aggregation is minimally affected by treatment with cyclooxygenase inhibitors while secretion of dense granules is partially inhibited.[250,257,258] Other evidence indicates that both aggregation and secretion are partially blocked by inhibition of cyclooxygenase.[155] Finally, incubation of platelets with a specific TxA_2 antagonist blocks both secretion and irreversible aggregation, suggesting that arachidonic acid metabolism is required for full platelet activation to occur in response to PAF. This same study has shown that inhibition of Na^+/H^+ exchange causes partial inhibition of TxA_2 production and subsequent secretion, suggesting that PAF stimulates the release of arachidonic metabolites by two mechanisms, one dependent on Na^+/H^+ and one independent of Na^+/H^+ exchange.[413]

As noted with ADP and epinephrine, PAF inhibits the activation of adenylate cyclase by stimulatory agonists. This inhibition is dependent

on external Na^+ and the presence of guanine nucleotides, suggesting a G_i-mediated mechanism similar to that discussed under the epinephrine-induced inhibition of adenylate cyclase.[162,459]

Thrombin

Thrombin is a strong physiologic agonist which utilizes two distinct mechanisms to activate platelets: a receptor-mediated and a proteolytic process.[263] Thrombin interacts with the platelet surface at two, and probably more, separate sites. It binds to GPIb in a hormone-like manner and causes the proteolytic cleavage of GPV.[36,157,197] The platelets from patients with Bernard Soulier syndrome have delayed, but maximal, responses to thrombin.[185,260] This finding suggests that GPIb and GPV are not essential for the initiation of thrombin-mediated activation. Instead, these glycoproteins increase the rate of activation. Functional studies have also shown that platelets have two types of thrombin receptors. One is resistant to inactivation by chymotrypsin and mediates PLC activation, PKC activation, Ca^{++} mobilization, aggregation and secretion. The second receptor is inactivated by the protease chymotrypsin and is resistant to activation by gamma thrombin, a derivative of alpha thrombin which lacks clotting activity. This site mediates adenylate cyclase inhibition and arachidonic acid mobilization.[262]

Thrombin initiates platelet activation by stimulating PLC activity[21,34,90,262,346] as a result of both receptor-mediated and proteolytic processes.[263,346] The hydrolysis of the

polyphosphoinositides occurs very rapidly and is the triggering event for platelet activation.[37,378] Investigations in saponin-permeabilized platelets have shown that thrombin can cause platelet aggregation and secretion by some unidentified mechanism which is independent of phosphoinositide hydrolysis and PLC activation.[57,226]

Thrombin also stimulates Ca^{++} mobilization in platelets.[438] Studies comparing Ca^{++} mobilization in the presence and absence of external Ca^{++} have shown that the majority of the increase in $[Ca^{++}]_i$ comes from influx of external Ca^{++}. The influx is partially blocked by cyclooxygenase inhibition while the release of Ca^{++} from internal stores is unaffected.[192] Removal of external Ca^{++} partially inhibits aggregation with no effect on secretion.[243] Kinetic studies have shown a delay between the binding of thrombin to its receptor and the initiation of Ca^{++} mobilization. This suggests that the receptor coupling to both the plasma membrane Ca^{++} channel and the internal stores of Ca^{++} is not direct and requires the generation of second messengers.[352,477]

Observations on the effect of the ADP receptor antagonist FSBA have demonstrated that thrombin causes fibrinogen receptor exposure, irrespective of the presence of external ADP. It has been proposed that thrombin causes the cleavage of aggregin, leading to exposure of the fibrinogen binding site and subsequent aggregation.[83] This cleavage is not due to direct proteolytic action by thrombin itself; rather, it is secondary to the activation of calpain by the thrombin-stimulated increase in $[Ca^{++}]_i$.[321]

Stimulation of platelets with thrombin induces activation of Na^+/H^+ exchange and marked cytosolic alkalinization.[98,392] This activation of Na^+/H^+ exchange is not a direct action of thrombin; instead, it is due to the PLC-mediated production of second messengers and secondary activation of PKC.[386] Cytosolic alkalinization is not essential for platelet activation following stimulation with high concentrations of thrombin.[4,99,176,358,398,474] Stimulation of platelets with thrombin also causes the production of arachidonic acid metabolites secondary to the agonist-induced increase in $[Ca^{++}]_i$. The release of these metabolites is not essential for platelet activation since cyclooxygenase inhibition does not prevent aggregation, secretion or PLC activation following stimulation with thrombin.[345] Finally, thrombin inhibits adenylate cyclase activation by a mechanism which is receptor-mediated and dependent on G protein activation.[21,262]

Calcium Ionophores A23187 and Ionomycin

Calcium ionophores A23187 and ionomycin are non-physiologic agonists that transport Ca^{++} across cell membranes and release Ca^{++} from internal stores.[121,457] Observations in platelets in a Ca^{++}-free environment indicate that ionophores can release sufficient internal stores of Ca^{++} to initiate secretion and aggregation, though the presence of external Ca^{++} potentiates the response.[121,313,438,457] This elevation in $[Ca^{++}]_i$ is not affected by inhibition of cyclooxygenase or the removal of ADP, therefore it is a primary effect of calcium ionophores.[339]

Ca^{++} ionophores activate both PLA_2 and PLC as assessed by the measurement of the production of TxA_2, arachidonic acid, PA and the hydrolysis of membrane phosphoinositides.[34,314,339] The activation of PLA_2-mediated arachidonic acid release is Ca^{++}-dependent and requires micromolar $[Ca^{++}]_i$.[314] The activation of PLC is not strictly due to increased $[Ca^{++}]_i$.[225,339] Studies have shown that PLC activation in response to the calcium ionophores is due to the Ca^{++}-dependent activation of PLA_2, the production of arachidonic acid metabolites and the release of dense granule ADP which act as positive feedback loops to activate PLC.[337,339,444]

Although cyclooxygenase inhibition prevents TxA_2 production and PLC activation in platelets stimulated by calcium ionophores, it does not completely block platelet activation. In platelets treated with cyclooxygenase inhibitors, secretion and aggregation still occur following stimulation with the calcium ionophores; however, the responses are inhibited.[339,444] These findings suggest that the Ca^{++} ionophores partially activate platelets via some Ca^{++}-dependent pathways which are independent of the positive feedback loops involving TxA_2 and PLC. Calcium ionophores cause phosphorylation of both the 20kDa myosin light chain and the 47kDa protein.[225,361] Phosphorylation of both proteins can occur in the presence of inhibitors of cyclooxygenase, suggesting Ca^{++}-mediated activation of their respective kinases.[225]

Endoperoxide/Thromboxane Mimetic U46619

Thromboxane A_2 and the prostaglandin endoperoxides are potent physiologic agonists which cause the platelet responses of shape change, secretion and aggregation. They also amplify the platelet response following stimulation with other agonists. Unfortunately, the short half life of both TxA_2 (90 seconds) and PGH_2 (5 minutes) preclude their use for in vitro studies.[156] This has lead to the development of a number of stable mimetics. The stable TxA_2/PGH_2 mimetic U46619 induces shape change, aggregation and secretion.[278] Canine platelets differ from other species in their response to U46619. When U46619 alone is added to canine platelets, it induces only shape change. Full aggregation requires the prior addition of a subthreshold dose of epinephrine to U46619.[66]

The platelet TxA_2/PGH_2 receptor has been isolated and characterized.[108] Binding studies have shown that it is located on the plasma membrane and/or the DTS membranes.[363] Recent observations suggest that there are actually two subtypes of the TxA_2/PGH_2 receptor which mediate separate functions. One subtype mediates shape change while the other mediates aggregation.[107]

Pretreatment of platelets with U46619 causes decreased responsiveness to subsequent stimulation with additional doses of U46619 but not to stimulation with other agonists.[233] The decreased responsiveness results from: (1) initial receptor desensitization due to

the uncoupling of receptor from G protein; and (2) delayed downregulation and loss of receptor sites.[274,419]

Binding of U46619 to the TxA_2/PGH_2 receptor activates PLC independent of arachidonic acid metabolism and external Ca^{++}.[337,379] Activation of platelet PLC by U46619 is accompanied by relatively poor release of arachidonic acid[337] since the desensitization and downregulation of TxA_2/PGH_2 receptors may inhibit the binding, and therefore the positive feedback effects, of endogenously produced endoperoxides and TxA_2.[379] Activation of PLC by U46619 is regulated by a pertussis toxin-resistant G protein[58] and is not affected by inhibition of Na^+/H^+ exchange.[409]

In U46619-stimulated platelets, removal of external ADP is accompanied by partial inhibition of aggregation and PLC activation.[337,379] Evidence using the ADP analogue FSBA indicates that external ADP is required to stimulate exposure of the fibrinogen binding site. The addition of FSBA to U46619-stimulated platelets blocks fibrinogen binding and subsequent aggregation without affecting shape change.[83]

U46619 stimulates Ca^{++} mobilization that is not affected by cyclooxygenase inhibition.[47,50] Ca^{++} mobilization occurs in both the presence and absence of external Ca^{++}, though the response is greater when external Ca^{++} is present. Kinetic studies have shown a time delay between the binding of agonist and the increase in $[Ca^{++}]_i$, indicating the presence of one or more biochemical steps between binding and the

release and/or influx of Ca^{++}.[352] A recent study has shown that inhibition of Na^+/H^+ exchange decreases Ca^{++} mobilization in response to U46619. It was proposed that this inhibition occurs at the level of (1,4,5)IP_3-mediated release from internal stores. Prevention of Na^+/H^+ exchange inhibits cytosolic alkalinization, negating the potentiating effect of increased pH_i on (1,4,5)IP_3-mediated Ca^{++} release.[394]

Finally, studies on platelet membrane preparations have shown that U46619 inhibits both basal and PGE_1-stimulated adenylate cyclase activity in a G protein-dependent manner. It has not been determined whether this inhibition occurs in intact platelets.[15]

Phorbol Ester

Phorbol esters are non-physiologic agonists which cause both aggregation and secretion.[377] The endogenous second messenger 1,2-DG increases the affinity of PKC for Ca^{++} and phospholipid, causing PKC activation.[71,72] Phorbol esters have a structure similar to 1,2-DG and are believed to intercalate into the membrane and cause activation of PKC. In contrast to 1,2-DG, the phorbol esters are not readily metabolized by the cell and cause permanent PKC activation.[377]

The aggregation and secretion patterns following stimulation with phorbol esters are unlike patterns obtained following stimulation with most other agonists. Phorbol esters do not cause shape change, but they cause aggregation that is relatively slow and occurs after a variable delay period.[381,479] Aggregation requires external Ca^{++}, although

concentrations lower than those needed by other agonists are required.[187] Phorbol-induced aggregation is not dependent on the production of arachidonic acid metabolites and is not significantly affected by cyclooxygenase inhibition.[187,456] Partial inhibition of aggregation occurs following the addition of apyrase to remove external ADP or following stimulation of cAMP production.[10,187] This inhibition is much more pronounced in platelets stimulated by the synthetic 1,2-DG than by phorbol esters, highlighting a difference between phorbol- and endogenous 1,2-DG-induced platelet activation.[10] Secretion occurs well after the onset of aggregation and is not accompanied by granule centralization on electron microscopic examination.[456]

The specific effects of phorbol on signal transduction were covered in the section on PKC and will not be repeated.

BASSET HOUND HEREDITARY THROMBOPATHY

Basset hound hereditary thrombopathy (BHT) is an inherited platelet disorder in purebred Basset hounds. Although a similar disorder has not been identified in humans or other animals, BHT presents a unique opportunity to increase our understanding of normal platelet function. The disease was first identified in the 1970's when a practicing veterinarian requested some advice about a group of Basset hounds with hemorrhagic diathesis. Clinically, affected dogs present with signs typical of a platelet disorder. Findings include petechiation, easy bruising, mucosal hemorrhages and excessive bleeding associated with estrous cycles and the shedding of deciduous teeth. The

initial investigation showed that affected dogs have prolonged bleeding times and complete absence of aggregation, either primary or secondary, in response to stimulation with ADP.[32,191] The examination of the pedigrees and *in vitro* aggregation studies on a group of 92 related Basset hounds suggested an autosomal recessive pattern of inheritance.[32]

Further investigations on 45 of these dogs identified numerous similarities with Glanzmann's thrombasthenia in humans.[32] Normal laboratory findings identified in both disorders include coagulation factor parameters, vWF levels, platelet number and morphology on light microscopy and platelet morphology on transmission electron microscopy. Abnormal findings include prolonged bleeding time, decreased *in vitro* retention in glass bead columns and absence of aggregation to any concentration of ADP tested. One difference is clot retraction time which is normal in platelets from dogs with BHT but markedly prolonged or absent in Glanzmann's thrombasthenic platelets. Clot retraction depends on the attachment of both cytosolic actin filaments and extracellular fibrin to the GPIIb-IIIa complex on the surface of the platelet pseudopods. The subsequent generation of a contractile force by the interaction of myosin and actin pulls the pseudopods in toward the cell, also pulling in the attached fibrin.[142]

In addition to failure of primary aggregation in response to ADP, platelets from dogs affected with BHT have abnormal release of dense granule ATP. When platelets from affected dogs are stimulated with 10uM ADP, they change shape and release ATP from the dense granules. The release occurs six times faster than the release in control dog

platelets. Sequential transmission electron microscopy have confirmed the aggregation and secretion defects. It was proposed that the lack of aggregation is due to a defect in either GPIIb-IIIa complex itself or its functional ability to bind fibrinogen.[300]

Due to a clinical resemblance to Glanzmann's thrombasthenia, contact activation was measured in platelets dogs with BHT and control dogs using whole mount electron microscopy. Affected platelets attach poorly to a foreign surface and have decreased contact-induced shape change when compared to control canine platelets. The addition of ADP causes a five-fold increase in adherent platelets and a four-fold increase in spread forms. Aggregate formation is not noted. These findings are similar to those observed in platelets from a human with Glanzmann's thrombasthenia.[251]

Based on the hypothesis that the defect in BHT may represent an inability of affected platelets to bind fibrinogen, the initial investigations focused on the examination of membrane GPIIb-IIIa content and ability to bind fibrinogen. Membrane GPIIb-IIIa content was evaluated using three different techniques: (1) crossed immunoelectrophoresis; (2) two-dimensional nonreduced-reduced electrophoresis; and (3) O'Farrell two-dimensional electrophoresis. There was no difference between affected and control platelets in membrane glycoprotein content using either crossed immunoelectrophoresis or two-dimensional electrophoresis. There was also no difference in membrane glycoproteins using O'Farrell's two-dimensional electrophoresis; however, some low molecular weight lipids and/or

sialoglycoproteins were missing in samples from BHT platelets. The significance of these missing components is not known.[299]

Normal platelet GPIIb-IIIa content does not prove that the complex is functional and able to bind fibrinogen. Fibrinogen binding was therefore measured in affected and control canine platelets. The platelets were stimulated with ADP in the presence of ^{125}I-fibrinogen and the quantity of surface bound radioactivity measured. There was no significant difference between the two groups of dog platelets using this technique.[298] Fibrinogen binding was also evaluated using scanning electron microscopy. Platelets from normal and affected dogs were stimulated with ADP, allowed to adhere and spread and labelled with either albumin-gold or fibrinogen-gold. The binding of fibrinogen-gold was quantitatively and morphologically identical in affected and control platelets.[119]

These investigations demonstrated that BHT platelets are able to bind fibrinogen; however, they also suggest that fibrinogen binding alone is not sufficient to maintain aggregation. Instead, there appears to be one or more post-fibrinogen binding events necessary for aggregation. The necessity for some, as yet, undefined post-fibrinogen binding events has been reported by other groups. One study has demonstrated that formalin fixed, ADP stimulated platelets are able to bind fibrinogen but are unable to aggregate.[305] Another group has described two monoclonal antibodies that inhibit ADP stimulated aggregation without inhibiting fibrinogen binding.[281]

Another study by Catalfamo et al[67] on affected platelets from BHT dogs has reported no fibrinogen binding in response to ADP and impaired rate of fibrinogen binding in response to thrombin. The reason for the discrepancy between the results from this study and the results by Patterson et al[298] and Estry et al[119] discussed above is likely due to the difference in experimental techniques used. Fibrinogen binding in the study by Catalfamo et al[67] was measured using a fibrinogen-coated bead agglutination technique.[80] This technique depends on the formation of bead agglutination after the platelets bind to the beads. It is possible that this technique actually measures the ability of platelets to aggregate rather than their ability to bind fibrinogen. If platelets bind to the fibrinogen coated beads but do not bind to each other, it is difficult to imagine how the beads can agglutinate.

At this point it was determined that BHT is not an animal model for Glanzmann's thrombasthenia, but represents a unique disorder involving a defect in the undefined post-fibrinogen events essential for platelet aggregation. This suggests the importance of transmembrane signal processing and intracellular biochemical changes in the platelet response of aggregation.

The importance of cAMP as an inhibitory second messenger in platelets lead one group to investigate its role in BHT.[48,49] They found that basal levels of cAMP are increased 30-90% in affected platelets relative to control dog platelets. The addition of forskolin, a substance which directly activates adenylate cyclase, causes marked increase in concentrations of cAMP in affected platelets but not in

normal dog platelets.[49] These findings were further investigated by evaluation of cAMP phosphodiesterase (cAMP-PDE), the enzyme responsible for the removal of cAMP. Resting levels of cAMP-PDE activity are similar in affected and control dog platelets. Affected platelets are more sensitive to the inhibitory effects of forskolin and MIX, a competitive inhibitor of cAMP-PDE. The post-stimulation concentrations of cAMP require greater time to return to non-stimulated levels in affected platelets than in control platelets. In addition, the addition of epinephrine or PGI_2 respectively decrease or increase the forskolin stimulated levels of cAMP. In combination, these findings suggested that the G protein regulation of adenylate cyclase is intact in affected and the defect resides in the regulatory control of cAMP-PDE.[48]

Aggregation, ATP dense granule secretion and aspirin sensitivity were examined in platelets from dogs affected with BHT. The aggregation study showed that affected platelets: (1) change shape but do not undergo primary aggregation in response to ADP, PAF and calcium ionophore A23187; (2) have full aggregation responses to high concentrations of thrombin although aggregation is delayed in onset and significantly slower than in control canine platelets; and (3) have phorbol ester-stimulated aggregation responses similar to those in control platelets. The ATP secretion study showed that affected platelets: (1) secrete ATP in response to stimulation with ADP, thrombin, phorbol ester and high concentrations of PAF; and (2) do not secrete ATP in response to calcium ionophore A23187 and low concentrations of PAF. Aspirin treatment of affected and control platelets demonstrated: (1) almost total inhibition of secretion in

response to ADP in both affected and control platelets; and (2) partial inhibition of secretion in response to PAF, thrombin and PMA that is more pronounced in affected platelets. These results suggest that at least one effector pathway is intact in BHT and can elicit full platelet activation. This pathway is stimulated by PMA and high concentrations of thrombin and may involve PKC. In addition, the results of aspirin inhibition indicate that affected platelets are more sensitive to cyclooxygenase inhibition than control platelets and have a greater reliance on the release of arachidonic acid metabolites.[254]

Another research group also measured aggregation responses in platelets from dogs with BHT.[67] They reported similar results following ADP and thrombin stimulation. They also showed that stimulation of affected platelets with either collagen or arachidonic acid causes shape change but not primary aggregation. In contrast to the results discussed above, this group reported that aggregation occurs in response to high concentrations of A23187 in gel-filtered,affected platelets. The concentration of A23187 used in this study has been shown in our laboratory to actually cause cell lysis in gel-filtered platelets.

Phosphorylation of platelet proteins was measured in platelets from dogs with BHT in response to a variety of agonists.[255] There is no significant differences between affected and control platelets in the phosphorylation patterns of the 20kDa myosin light chain and the 47kDa protein. Shape change correlates closely with phosphorylation of myosin light chain; however, there is no consistent correlation between aggregation and/or secretion and the phosphorylation of either the 20 or

47kDa proteins. Interestingly, there is significantly decreased phosphorylation of a 64-67kDa protein in affected platelets. The identity and significance of this protein is not known.[255]

Finally, TxA_2 production, as indicated by production of the stable metabolite TxB_2, was measured in affected and control platelets in response to stimulation with a wide variety of agonists.[256] The basal concentrations of TxB_2 in platelet rich plasma are markedly greater in affected samples than in control samples. Stimulation with ADP, thrombin or high concentrations of PAF causes rapid production of significantly greater concentrations of TxB_2 in affected samples. Stimulation with A23187 or low concentrations of PAF causes the production of significantly decreased concentrations of TxB_2 in affected samples when compared to control samples. As expected, PMA stimulation does not produce significant increases in TxB_2 concentrations in either group. It is interesting to note that those agonists which cause dense granule secretion also cause increased production of TxB_2 in affected samples when compared to control samples. On the other hand, those agonists which do not cause dense granule secretion produce significantly decreased quantities of TxB_2 in affected samples relative to control samples. These findings suggest that the mechanism for the production of arachidonic acid metabolites is intact in affected platelets; however, the release of arachidonic acid metabolites does not appear to be under the normal regulatory control. The addition of exogenous arachidonic acid to affected and control platelets produce similar quantities of TxB_2, indicating that the pathway for the

production of TxA_2 from arachidonic acid is intact and under normal regulation in affected platelets.

In conclusion, the platelet disorder in BHT does not resemble any thrombopathy reported to date. The findings discussed above suggest that there is a defect in some post-fibrinogen binding event(s) leading to failure of platelet aggregation following stimulation with ADP, PAF, calcium ionophore A23187 or low concentrations of thrombin. In contrast, high concentrations of thrombin or PMA cause full platelet activation. Identified abnormalities in BHT platelets include decreased phosphorylation of a 64-67kDa protein, abnormal secretion of ATP from dense granules, greater dependance on the production of arachidonic acid metabolites, altered regulation of TxA_2 production and increased cytosolic cAMP concentrations. None of these findings can adequately explain the defect in BHT based on current understanding of the process of platelet activation, necessitating further investigations of the biochemical pathways involved in platelet signal transduction.

MEASUREMENT OF RESTING AND STIMULATED PLATELET CALCIUM

INTRODUCTION

Calcium ions are intracellular messengers which play a central role in the transmission of signals within living cells.[44] In platelets, Ca^{++} is important in the physiologic responses of shape change, secretion and aggregation.[377]

The concentration of cytosolic free ionized calcium ($[Ca^{++}]_i$) within resting platelets is maintained at approximately 100nM.[51,331,335] Spatial distribution studies on individual platelets have demonstrated that the intracellular free ionized Ca^{++} is not distributed homogeneously within the cytoplasm. Instead, it exists in two different gradients: (1) a continuous gradient with increasing $[Ca^{++}]_i$ towards the plasma membrane; and (2) discontinuous gradients with plateaus in restricted regions of the endoplasm. It has been suggested that the relatively high concentration of Ca^{++} under the plasma membrane results from compartmentation of ion movement by the cytoskeleton while the discontinuous gradients are associated with the internal storage sites.[428] Following stimulation, the gradients disappear and there is a uniform increase in $[Ca^{++}]_i$.

Platelets actively maintain resting $[Ca^{++}]_i$ by: (1) promotion of Ca^{++} efflux across the plasma membrane; (2) energy-requiring sequestration of Ca^{++} into intracellular storage sites; and (3) limitation of the influx of external Ca^{++}. The process of Ca^{++} efflux in platelets is not well understood; however, it is affected by changes in the membrane Na^+ gradient, implying a role for a Na^+/Ca^{++} exchange mechanism.[54,365] Recent studies have also shown that Ca^{++} efflux is regulated by a plasma membrane Ca^{++}-ATPase pump[114,115,116] which is distinct from the Ca^{++}-ATPase present on the internal membranes. The plasma membrane pump is related to the plasma membrane Ca^{++}-ATPase present on erythrocytes and hepatocytes.[116] Ca^{++} sequestration is regulated by a Ca^{++}-ATPase located on the DTS which is related to the Ca^{++} pump located on the sarcoplasmic reticulum of muscle cells.[107,111,265] It has a higher affinity for Ca^{++} and a lower requirement for ATP than the plasma membrane pump.[114] Ca^{++} influx occurs by passive diffusion down a concentration gradient through Ca^{++}-selective channels in the plasma membrane. The rate of influx is maximal at Ca^{++} concentrations 20-fold less than the levels normally found in plasma.[51]

A wide variety of agonists induce an increase in $[Ca^{++}]_i$.[331,438] Agonist-stimulated Ca^{++} fluxes result from the prolonged influx of external Ca^{++} and/or the rapid release of Ca^{++} from exchangeable internal storage sites.[428] Ca^{++} influx occurs along receptor-operated, not voltage-dependent, channels in the plasma membrane.[14,154,243,349,476] These channels may be closely associated with, if not actually part of, the membrane GPIIb-IIIa complex.[316,469]

Kinetic studies have shown that there is a lag period between the addition of agonist and the onset of a measurable increase in $[Ca^{++}]_i$, suggesting a role for one or more biochemical steps between agonist binding and the receptor-operated influx of Ca^{++}. These same studies have also demonstrated that Ca^{++} influx precedes internal Ca^{++} mobilization, suggesting that Ca^{++} influx and internal release are two distinct events.[351,352] The internal release of Ca^{++} is mediated by the production of second messengers generated by the agonist-stimulated hydrolysis of membrane phosphoinositides.[331] (1,4,5)IP_3 is the primary second messenger implicated in the receptor-operated release of Ca^{++} from the DTS.[11,12,242]

Historically, a variety of experimental techniques have been used to measure Ca^{++} fluxes, intracellular storage pools and cytosolic $[Ca^{++}]_i$ in platelets. These include: (1) chlortetracycline; (2) $^{45}Ca^{++}$; (3) fluorescent chelator dyes; and (4) luminescent photoproteins. Chlortetracycline is a fluorescent compound which, after binding Ca^{++}, increases its fluorescence and becomes associated with the platelet membranes. Unfortunately, its intracellular location is not known. Although useful for measuring Ca^{++} flux in some circumstances, chlortetracycline does not reflect changes in $[Ca^{++}]_i$ and may not always accurately reflect Ca^{++} metabolism in the platelet.[44,97] $^{45}Ca^{++}$ is used to estimate the size and exchange rate in the surface bound and intracellular Ca^{++} pools; however, it also does not reflect $[Ca^{++}]_i$.[377]

Currently, the most common techniques utilized to measure platelet $[Ca^{++}]_i$ include a family of fluorescent dyes and the luminescent

photoprotein aequorin. The fluorescent dyes are polycarboxylic Ca^{++} chelators related to EGTA and include the compounds quin2, fura 2 and indo 1. The acetoxymethyl (AM) ester form of these dyes is lipophilic and readily penetrates the plasma membrane of the platelets. Once in the cytosol, the ester is cleaved by cytosolic esterases to form the free acid which is then trapped within the cell. The free acid binds to divalent cations in 1:1 stoichiometry. The Ca^{++}-bound dye increases its fluorescence relative to the unbound form. The change in fluorescence can be measured and is proportional to the corresponding change in $[Ca^{++}]_i$.[79,97,148,331] Quin2 was the first such dye synthesized. Unfortunately, due to its low extinction coefficient and fluorescent quantum yield, large cytosolic concentrations of quin2 are required to produce fluorescence signals significantly greater than background autofluorescence and noise. This high concentration of quin2 buffers intracellular free Ca^{++}. In addition, the cleavage of quin2-AM generates potentially harmful concentrations of formaldehyde, protons, and acetate ions.[79,97,333] Finally, quin2 has a relatively high affinity for Ca^{++} and rapidly becomes saturated, losing resolution at high concentrations of Ca^{++}.[148] Quin2 does not accurately measure $[Ca^{++}]_i$ above 2-3uM, though it is effective at measuring levels in the normal resting range.[79,148]

These problems with quin2 lead to the generation of the newer dyes fura 2 and indo 1. These dyes have three advantages over quin2: (1) they produce 30-fold greater fluorescence; (2) they have improved selectivity for Ca^{++} over other divalent cations; and (3) they yield a change in either the emission or the excitation fluorescence wavelength

following Ca^{++} binding. The former two features significantly lower the quantity of cytosolic dye needed to produce a measurable signal, decreasing Ca^{++} buffering and the generation of deleterious substances. The latter feature permits calculation of $[Ca^{++}]_i$ based on the ratio of fluorescence of the wavelengths of Ca^{++}-bound and -unbound fura 2 and indo 1. The use of the ratio method to calculate $[Ca^{++}]_i$ partially negates the effects of cell size, intracellular dye concentration or instrument inefficiency which affect the calculation of $[Ca^{++}]_i$ using the single wavelength technique.[79,97,148,425] Finally, the newer dyes have greater resolution, allowing the accurate calculation of $[Ca^{++}]_i$ up to 5-10uM.[79]

The luminescent photoprotein aequorin has also been frequently used to measure $[Ca^{++}]_i$ in platelets. Aequorin is a polypeptide of 20,000 daltons which contains a bound chromophore. When this complex binds to free Ca^{++}, the chromophobe becomes oxidized and releases CO_2 and a photon of light of wavelength 469 nm. The amount of light produced is quantitated by a photomultiplier tube and used to calculate the $[Ca^{++}]_i$. The response of aequorin to $[Ca^{++}]_i$ is exponential over the range of 10^{-7} to 10^{-4}M, therefore a small increase in $[Ca^{++}]_i$ in a local region gives a greater increase in the luminescence signal than if that same amount of Ca^{++} were evenly distributed throughout the cytosol.[44,97,188] It has been suggested that aequorin detects local zones of Ca^{++} flux, possibly associated with the plasma membrane, while the fluorescent chelator dyes detect average $[Ca^{++}]_i$.[188,190]

At the initiation of the research for this dissertation, it had

been determined that the platelet defect in Basset hound hereditary thrombopathy is not related to abnormalities in membrane GPIIb/IIIa structure or function.[119,298,299] Instead, the defect in BHT appears to be associated with post-fibrinogen binding events involved in the process of signal transduction. It was decided to measure the resting and post-stimulation $[Ca^{++}]_i$ due to the pivotal role of Ca^{++} as an intracellular second messenger in signal transduction. $[Ca^{++}]_i$ was measured using both aequorin and fura 2 since the two experimental techniques measure different aspects of Ca^{++} metabolism. Fura 2 was chosen over indo 1 because fura 2 has been more commonly used to measure $[Ca^{++}]_i$ in platelets in suspensions. Platelets were stimulated using a similar range of agonists as those used for the aggregation/secretion study[254] to determine if there was any association between Ca^{++} flux and the abnormalities identified in the aggregation and/or secretion of affected platelets.

MATERIALS AND METHODS

Experimental Subjects

For the measurement of $[Ca^{++}]_i$ using fura 2-loaded platelets, the affected group consisted of five Basset hounds with BHT. There were one male and four female dogs. The control group consisted of one male and two female Basset hounds without evidence of BHT. Due to the unavoidable loss of dogs and the replacement with different dogs, the experimental groups for the measurement of $[Ca^{++}]_i$ using aequorin-loaded platelets were different. The affected group consisted of four Basset

hounds with BHT which were a subset of the affected group for the fura 2 studies. There were one male and three female dogs. The control group consisted of two female and one male Basset hounds with no evidence of BHT. Only one of the female dogs had been used in the fura 2 study.

All dogs were free from signs of clinical disease other than those associated with BHT. They did not receive any medication during the study and had been vaccinated against rabies, distemper, leptospirosis, adenovirus, parainfluenza and parvovirus. They were housed in kennels in the Veterinary Clinical Center at Michigan State University, were fed a commercial dry dog chow (Hill's Pet Products, Topeka, KS) and received regular exercise.

Reagents

Adenosine diphosphate (ADP) was purchased from Sigma Chemical Company (St. Louis, MO). Stock solution (2.6mM) was mixed in 20mM Tris-saline, aliquoted and stored at -20°C. Platelet-activating factor (PAF) was purchased from Calbiochem Corporation (San Diego, CA). Stock solution (1.0 mg/ml) was mixed in ethanol and stored at -20°C. Thrombin was purchased from Sigma Chemical Company, mixed in distilled water and stored in aliquots at -20°C until use. Phorbol-12-myristate-13-acetate (PMA) was purchased from Calbiochem. Stock solution (1.56 mM) was mixed in ethanol and stored at -20°C. Ionomycin was purchased from Calbiochem. Stock solution (2mM) was mixed in dimethyl sulfoxide (DMSO), aliquoted and stored at 4°C. Fura 2-AM was purchased from Calbiochem. Stock solution (1mg fura 2-AM in 249ul anhydrous DMSO) was

aliquoted and stored in a dessicator jar at -20°C. Aequorin was purchased from the Mayo Clinic Foundation (Rochester, MN). Stock solution (1mg aequorin in 333ul Ca^{++}-free water containing 7mM EGTA) was aliquoted into plastic cuvettes and stored at -20°C. Sepharose 4-B was purchased from Sigma Chemical Company. Digitonin was purchased from Sigma Chemical Corporation, mixed in de-ionized distilled water to a final concentration of 10mg/ml and stored at 4°C. Prostaglandin E_1 (PGE_1) was purchased from Sigma Chemical Company. Stock solution (1mM) was mixed in ethanol and stored at -20°C. All other chemicals were of reagent grade.

Stock solutions were diluted at the time of use and maintained on ice. ADP was diluted with 0.9% NaCl. Thrombin was diluted with distilled water. PAF, ionomycin and PMA were diluted with balanced salt solution (136mM 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). Final concentrations of ethanol or DMSO in the platelet solutions did not exceed 0.5% v/v.

Platelet Collection

To limit variability in experimental results, blood must be collected from healthy dogs which are not excited or receiving any medication.[32] For all procedures, blood was obtained by jugular venipuncture using an 18 gauge needle into a plastic syringe containing 3.8% trisodium citrate at a ratio of 9ml of blood to 1ml anticoagulant. The dogs are conditioned to calmly accept this procedure and minimal physical restraint is required. If the collection was disrupted, the

original sample was discarded and a second sample collected.

To obtain platelet rich plasma (PRP), the blood was separated by centrifugation 3-5 times at 1,324 x g for 60 seconds, removing a small quantity of PRP between each spin. After removal of sufficient quantities of PRP, platelet poor plasma (PPP) was obtained by centrifugation of the remainder of the blood at 1,324 x g for 13 minutes. For all platelet isolation procedures, plastic (polystyrene, polyethylene or polypropylene) labware was used.

Measurement of $[Ca^{++}]_i$ Using Fura 2

$[Ca^{++}]_i$ was measured by modification of the technique described by Daniel et al.[97] PRP was acidified by the addition of 9ul 1M citric acid/ml PRP. The suspension was incubated at room temperature for 5 minutes prior to centrifugation for 15 minutes at 800 x g. The supernatant was discarded and the platelet pellet resuspended in 4ml of autologous PPP acidified by the addition of 36ul 1M citric acid. Fura 2-AM was added from stock solution to yield a final concentration of 4uM and the platelet sample incubated at 37°C for 30 minutes. The suspension was centrifuged at 800 x g for 15 minutes, the supernatant discarded and the pellet resuspended in 1 ml Ca^{++}-Mg^{++}-free Tyrode's buffer (137mM NaCl, 2.7mM KCl, 5.5mM glucose, 0.35mM Na_2HPO_4, 10mM HEPES and 0.2% bovine serum albumin, pH 7.4). The platelets were gel-filtered by passing the suspension over a 10ml Sepharose 4B column equilibrated with the same buffer. The eluted platelet suspension was manually counted using the Unopette system and a Neubauer hemocytometer and

diluted to 1 x 10^8 platelets/ml using the same buffer. $MgCl_2$ (final concentration 0.8mM) was added and the suspension divided into 1.5ml aliquots into plastic spectrophotometer cuvettes, then covered by parafilm. The cuvettes were maintained at room temperature until 10 minutes prior to the measurement of $[Ca^{++}]_i$ at which time they were warmed to 37°C. Five minutes prior to measuring $[Ca^{++}]_i$, either 1mM $CaCl_2$ or 1mM EGTA was added along with a teflon coated magnetic stir bar. Fluorescence was measured in a Perkin-Elmer MPF 66 spectrofluorometer using an excitation wavelength of 339nm and an emission wavelength of 502nm. The platelet suspension was stirred and resting fluorescence recorded. Agonist (60ul) was added and maximum post-stimulation fluorescence recorded. Agonists used in this study included ADP, PAF, thrombin, PMA and ionomycin. The $[Ca^{++}]_i$ was calculated based on the formula: $[Ca^{++}] = K_d [(F-F_{min})/(F_{max}-F)]$ where K_d is the dissociation constant of fura 2 (assumed to be 224nM), F is the measured fluorescence of the sample, F_{max} is the sample fluorescence following addition of 4mM Ca^{++} and lysis by 50uM digitonin, and F_{min} is the sample fluorescence following addition of 6mM EGTA/75mM Tris base to the lysed platelet suspension. Autofluorescence of the platelet suspension was determined by measuring fluorescence in similarly prepared platelet samples not incubated with fura 2-AM. None of the agonists used had significant autofluorescence at the wavelength settings used for these experiments. Leakage of dye from the samples was determined by centrifuging representative platelet samples for 1 minute at 11,600 x g. The fluorescence of the supernatant was measured in the presence and absence of external Ca^{++} to determine the Ca^{++} sensitivity of the leaked dye. The external dye was found to be

composed of both Ca^{++}-sensitive and Ca^{++}-insensitive forms. The leakage rate was relatively small and the resting $[Ca^{++}]_i$ relatively constant over the time span of the experiments. For these reasons, it was decided to not correct for external dye in the calculations.

Measurement of $[Ca^{++}]_i$ Using Aequorin

The platelets were prepared using a modification of the technique described by Yamaguchi et al.[467] PRP was acidified by the addition of 9ul 1M citric acid/ml PRP. The sample was incubated at room temperature for 5 minutes prior to centrifugation for 15 minutes at 800 x g. The supernatant was discarded and the platelet pellet gently resuspended in 10 ml HEPES buffered saline (140mM NaCl, 2.7mM KCl, 0.1% bovine serum albumin, 0.1% glucose and 3.8mM HEPES, pH 7.6) containing 1uM PGE_1 and 5mM EGTA. The sample was incubated for 5 minutes at room temperature prior to centrifugation at 800 x g for 15 minutes. The supernatant was discarded, the pellet gently resuspended in 90ul of the same buffer at which time 10ul stock aequorin solution was added. DMSO was added over a period of 7.5 minutes at a rate of 1ul DMSO/1.5 minutes to yield a final concentration of 6% by volume. The suspension was incubated for 2 minutes at room temperature, diluted to 1 ml using HEPES buffered saline without PGE_1 or EGTA and gel-filtered by passing the sample over a 10ml Sepharose 4B column equilibrated with the same buffer. The gel-filtered platelets were manually counted using the Unopette system and a hemocytometer and diluted to a final concentration of 1.5×10^8 platelets/ml using the same buffer. $MgCl_2$ (final concentration 0.8mM) was added and the sample divided into 1ml aliquots in plastic

aggregometer cuvettes, then covered with parafilm. Five minutes prior to the measurement of $[Ca^{++}]_i$, the cuvettes were warmed to 37°C, a teflon coated magnetic stir bar inserted and either 1mM $CaCl_2$ or 1mM EGTA added. The cuvettes were placed into a Platelet Ionized Calcium Aggregometer (PICA, Chronolog, Haverton, PA) and stirred at 900 rpm. After maintaining a steady resting baseline, 40ul of agonist was added and both aggregation and peak dye luminescence were recorded on a dual channel Omniscribe recorder (Houston Instruments, Austin, TX). The total platelet aequorin luminescence (L_{max}) was determined by lysis of a four-fold diluted platelet sample containing 1mM Ca^{++} by 150ul of Triton X-100. The $[Ca^{++}]_i$ was calculated from the maximum peak of the aequorin luminescence signal as described in the PICA instruction manual, using the calibration curve of log L/L_{max} vs log $[Ca^{++}]$. The calibration curve was supplied with each lot of aequorin and was constructed using a medium containing 150mM KCl, 5mM PIPES and 1mM $MgCl_2$.

Statistical Analysis

For the fura 2 studies, the resting and post-stimulation $[Ca^{++}]_i$ in response to each agonist was measured on three separate occasions in all dogs. For the aequorin studies, the resting and post-stimulation $[Ca^{++}]_i$ in response to each agonist was measured on two separate occasions in all dogs. For each dog, the $[Ca^{++}]_i$ were averaged for each concentration of agonist to yield a representative value which was used in the statistical comparisons. All data was analyzed using a computer software statistical/graphics program (Statgraphics, STSC, Rockville, MD). Due to the small sample sizes, it was found that the distribution-

free non-parametric were not effective. For that reason, all data was compared using the standard parametric tests. Control and affected values were compared using the student's t test for independent samples while the stimulated and resting values within a group were compared using the t test for paired samples.

RESULTS

The agonists used for the calcium studies were the same ones used in the aggregation and dense granule secretion study on affected platelets which had been previously reported[254] and included PAF, ADP, thrombin, PMA and a calcium ionophore. To evaluate Ca^{++} fluxes, the calcium ionophore ionomycin was used instead of A23187 because A23187 has fluorescent properties at the wavelengths used for the fura 2 studies which interfered with sample fluorescence.

Results will be presented according to agonist. The agonist concentrations listed in the tables are the final concentrations in the platelet samples. The concentrations of agonists were chosen based on aggregation studies on similarly prepared control platelets. When only one concentration of agonist was used, it was a concentration found to give maximal aggregation response. When two concentrations of agonist were used, the higher concentration was found to yield maximal aggregation response while the lower concentration was found to yield sub-maximal to no aggregation response. It was found that ionomycin has a relatively narrow range of activity separating non-activation, activation and cell lysis. The difference in the concentrations used

for the aequorin and fura 2 studies reflect a different sensitivity of the two different platelet preparations to ionomycin activation.

When loaded with fura 2, the canine platelets had increased aggregation responses to agonist stimulation, as indicated by lower threshold for agonist concentration and increased aggregation rate and maximum percent aggregation, when compared to similarly manipulated platelets not loaded with fura 2. Compared to non-loaded platelets, the fura 2-loaded platelets also had an increased incidence of spontaneous shape change following the initiation of suspension stirring. Increased aggregation and secretion responses have also been reported in human platelets loaded with fura 2.[218] Similar aggregation findings were not noted in aequorin-loaded platelets, although the platelets did have an increased incidence of spontaneous shape change when compared to non-loaded platelets.

The $[Ca^{++}]_i$ listed in all tables is the maximal or peak post-stimulation $[Ca^{++}]_i$ measured in the presence or absence of external Ca^{++}. As mentioned above, the peak $[Ca^{++}]_i$ in the presence of external Ca^{++} is indicative of Ca^{++} flux due to both release from internal stores and influx. The $[Ca^{++}]_i$ in the presence of external EGTA is solely indicative of Ca^{++} release from internal stores. The figures of fluorescence or luminescence are representative tracing for both control and affected platelets.

The quantity of external fura 2 that had leaked from the platelets during the course of the experiment was assessed by centrifugation of

platelet suspensions at set time periods and measurement of the fluorescence of the supernatant. The leakage rate for control platelets was 3.7%/hr while the leakage rate for affected platelets was 3.5%/hr. The fluorescence of the supernatant showed minor increases in fluorescence following the addition of excess Ca^{++}, suggesting that it was composed of both Ca^{++}-sensitive and -insensitive forms.

Resting $[Ca^{++}]_i$

There was no significant difference between resting $[Ca^{++}]_i$ in control or affected platelets using aequorin in the presence or absence of external Ca^{++}. The measured $[Ca^{++}]_i$ in the presence of external Ca^{++} was significantly larger than the measured $[Ca^{++}]_i$ in the absence of external Ca^{++}, but there was no significant difference between affected and control platelets. The results are summarized in Table 1.

Table 1. Resting $[Ca^{++}]_i$ (uM) in control and affected aequorin-loaded platelets.

External Buffer	Control (n = 3)	Affected (n = 4)
1mM Ca^{++}	3.6 ± 0.4	3.2 ± 0.3
1mM EGTA	2.2 ± 0.3	2.0 ± 0.2

Values = mean (uM) ± SD; n = number of dogs tested.

Using fura 2-loaded platelets, there was also no significant difference between resting $[Ca^{++}]_i$ in affected and control platelets in either Ca^{++}-or EGTA-containing buffer. As with aequorin, the $[Ca^{++}]_i$ in the presence of external Ca^{++} was greater than the $[Ca^{++}]_i$ in the absence of external Ca^{++} in both groups of platelets. The resting $[Ca^{++}]_i$ was compared at the beginning and the end of each day's series of experiments to assess the effect of the leakage of dye from the cells. There was no significant difference in either affected or control platelets, suggesting a minimal effect of dye leakage on the calculated $[Ca^{++}]_i$. The results are presented in Table 2.

Table 2. Resting $[Ca^{++}]_i$ (nM) in control and affected fura 2-loaded platelets.

External Buffer	Control (n = 4)	Affected (n = 5)
1mM Ca^{++}		
Initial $[Ca^{++}]_i$	133.9 ± 13.5	146.0 ± 19.9
Final $[Ca^{++}]_i$	130.6 ± 16.0	140.0 ± 9.8
1mM EGTA		
Initial $[Ca^{++}]_i$	86.6 ± 14.6	78.7 ± 9.9
Final $[Ca^{++}]_i$	75.7 ± 7.1	73.7 ± 9.1

Values = mean (nM) ± SD; n = number of dogs tested.

Adenosine Diphosphate (ADP)

While there was no significant difference between affected and control aequorin-loaded platelets in the peak $[Ca^{++}]_i$ following stimulation with high concentrations (10uM) of ADP in either Ca^{++}- or EGTA-containing buffer, there was a marked trend for the values to be greater in the control dogs. In Ca^{++}-containing buffer, the post-stimulation $[Ca^{++}]_i$ was significantly increased over resting values in both groups of platelets. Although a distinct post-stimulation, luminescent peak was measurable in EGTA-containing buffer, the difference between resting and post-stimulation $[Ca^{++}]_i$ was not statistically significant. The results are summarized in Table 3. There was no consistent difference in the appearance of the luminescence tracings in control or affected platelets; however, 50% of the control samples had a biphasic curve. This response was never noted in affected samples. See Figure 1 for representative tracings.

Table 3. Peak $[Ca^{++}]_i$ (uM) following stimulation with 10uM ADP in control and affected aequorin-loaded platelets.

External Buffer	Control (n = 3)	Affected (n = 4)
1mM Ca^{++}	6.0 ± 1.1 #	4.6 ± 0.4 #
1mM EGTA	2.5 ± 0.4	2.2 ± 0.4

Values = mean (uM) ± SD; n = number of dogs tested; # = significantly different from resting, at $p < 0.05$.

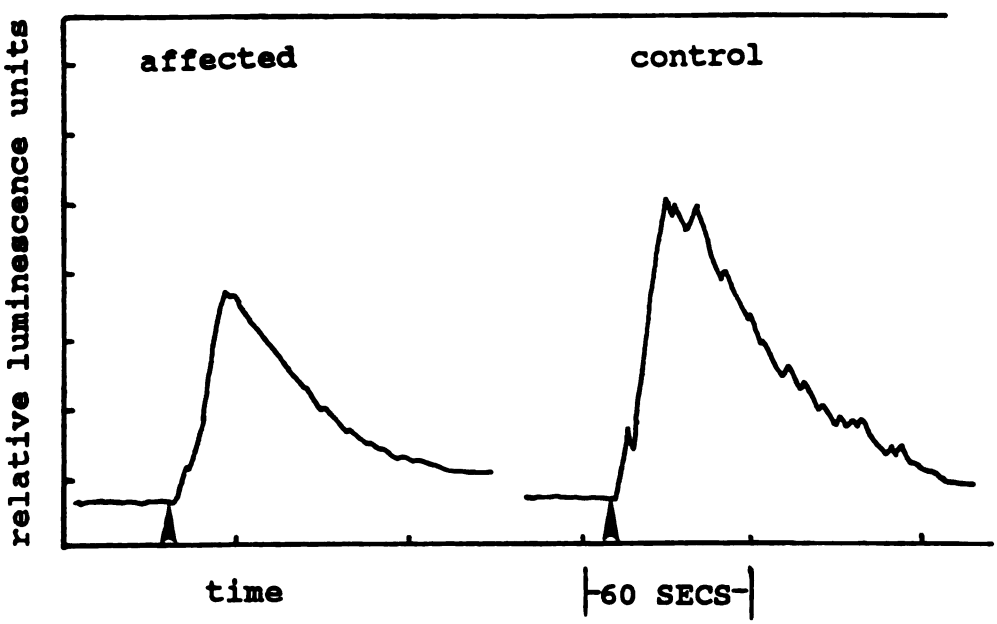


Figure 1. Representative luminescence tracings for the response of aequorin-loaded control and affected platelets to 10uM ADP in the presence of external Ca^{++}. Arrow = addition of agonist.

Using fura 2 as the Ca^{++}-indicator, there was significantly greater post-stimulation $[Ca^{++}]_i$ in affected platelets in response to both low (2uM) and high (10uM) concentrations of ADP when Ca^{++} was present in the external buffer. This difference was no longer found when EGTA was present in the external buffer. In this situation, there was a trend for lower post-stimulation $[Ca^{++}]_i$ in affected platelets, but the difference was not statistically significant. The stimulated $[Ca^{++}]_i$ was significantly increased over resting $[Ca^{++}]_i$ in all experimental groups. See Table 4 for a summary of the results.

Table 4. Peak $[Ca^{++}]_i$ following stimulation with ADP in control and affected fura 2-loaded platelets.

Buffer	Agonist	Control (n = 4)	Affected (n = 5)
1mM Ca^{++}	10uM ADP	311.0 ± 19.1 #	399.5 ± 26.9 * #
	2uM ADP	279.3 ± 66.7 #	341.6 ± 23.3 * #
1mM EGTA	10uM ADP	125.2 ± 15.4 #	114.0 ± 14.4 #
	2uM ADP	116.7 ± 25.8 #	100.6 ± 12.5 #

Values = mean (nM) ± SD; n = number of dogs tested; * = significantly different from control, $p < 0.05$; # = significantly different from resting, $p < 0.05$.

In EGTA-containing buffer, the appearance of the fluorescence curves in response to both concentrations of ADP was similar in affected and control platelets. The curves were characterized by a sharp increase in fluorescence with a slower decline to resting levels. In Ca^{++}-containing buffer, the ADP-induced fluorescence curves differed in shape between affected and control platelets. The curves in affected platelets had a broader peak with a slower decline toward resting when compared to the curves in control platelets. See Figure 2 for representative tracings.

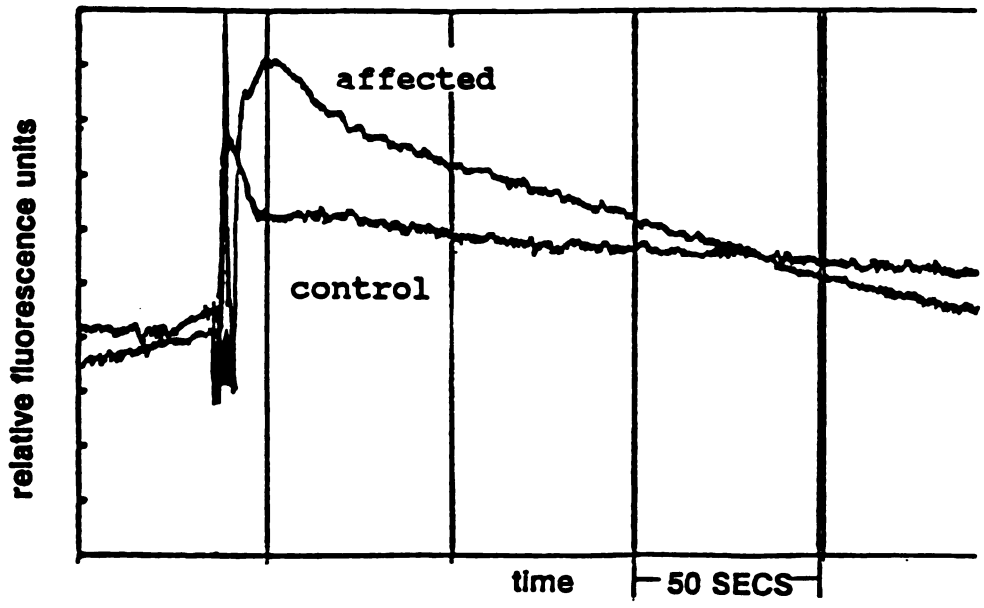


Figure 2. Representative fluorescence tracings of fura 2-loaded control and affected platelets in response to 10uM ADP in the presence of external Ca^{++}. Arrow = agonist addition.

Platelet-Activating Factor (PAF)

Using aequorin as the Ca^{++} indicator, there was no significant difference between control and affected platelets in peak $[Ca^{++}]_i$ following stimulation with either high (1.0uM) or low (0.01uM) concentrations of PAF in the presence or absence of external Ca^{++}. There was a trend for greater post-stimulation $[Ca^{++}]_i$ in control platelets in response to either concentration of PAF in EGTA-containing buffer or low concentrations of PAF in Ca^{++}-containing buffer. The post-stimulation $[Ca^{++}]_i$ was significantly increased relative to resting values in affected and control platelets following the addition of all concentrations of PAF tested. See Table 5 for a summary of the results.

Table 5. Peak $[Ca^{++}]_i$ (uM) following stimulation with PAF in control and affected aequorin-loaded platelets.

Buffer	Agonist	Control (n = 3)	Affected (n = 4)
1mM Ca^{++}	1.0 uM PAF	16.7 ± 2.9 #	16.6 ± 2.8 #
	0.01 uM PAF	9.1 ± 1.8 #	7.4 ± 1.0 #
1mM EGTA	1.0 uM PAF	5.2 ± 0.8 #	4.2 ± 0.5 #
	0.01 uM PAF	5.0 ± 0.9 #	4.0 ± 0.6 #

Values = mean (uM) ± SD; n = number of dogs tested; # = significantly different from resting, $p < 0.05$.

There was no consistent difference in the appearance of the luminescent tracings. See Figure 3 for representative tracings.

In contrast, using fura 2 as the Ca^{++} indicator, the post-stimulation $[Ca^{++}]_i$ was significantly increased in affected platelets relative to control platelets following the addition of high concentrations of PAF in the presence of external Ca^{++}. There was no difference between the two groups of platelets in response to low concentrations of PAF. In EGTA-containing buffer, the results were reversed. The peak post-stimulation $[Ca^{++}]_i$ was significantly decreased in affected platelets relative to control platelets in response to high and low concentrations of PAF. In both the presence and absence of external Ca^{++}, the post-stimulation $[Ca^{++}]_i$ was significantly increased over resting levels in response to all concentrations of PAF in control

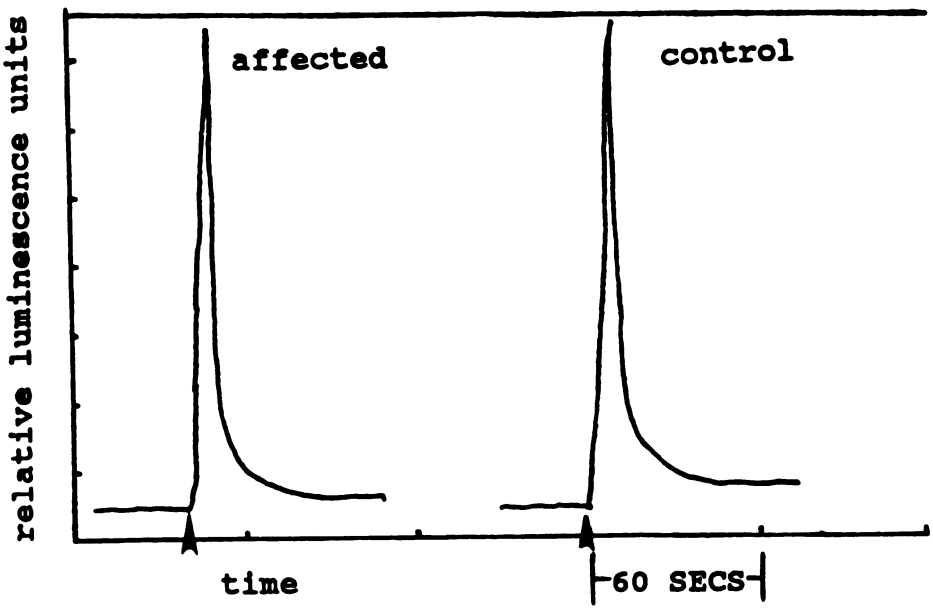


Figure 3. Representative luminescence tracings of aequorin-loaded affected and control platelets in response to luM PAF in the presence of external Ca^{++}. Arrow = agonist addition.

and affected platelets. See Table 6 for a summary of the results. There was no difference in the appearance of the fluorescence curves between control and affected platelets. In the presence of external Ca^{++}, the fluorescence curves were characterized by a sharp peak which quickly decreased to a level greater than resting. When external Ca^{++} was removed, the curves were characterized by a sharp increase which then decreased gradually toward resting levels. See Figure 4 for representative tracings.

Table 6. Peak $[Ca^{++}]_i$ in response to stimulation with PAF in control and affected fura-2 loaded platelets.

Buffer	Agonist	Control (n = 4)	Affected (n = 5)
1mM Ca^{++}	1.0 uM PAF	676.8 ± 98.7 #	842.9 ± 47.8 * #
	0.01 uM PAF	485.9 ± 74.5 #	465.2 ± 71.1 #
1mM EGTA	1.0 uM PAF	248.6 ± 25.5 #	176.6 ± 9.0 * #
	0.01 uM PAF	218.1 ± 18.2 #	164.8 ± 17.9 * #

Values = mean (nM) ± SD; n = number of dogs tested; * = significantly different from control, $p < 0.05$; # = significantly different from resting, $p < 0.05$.

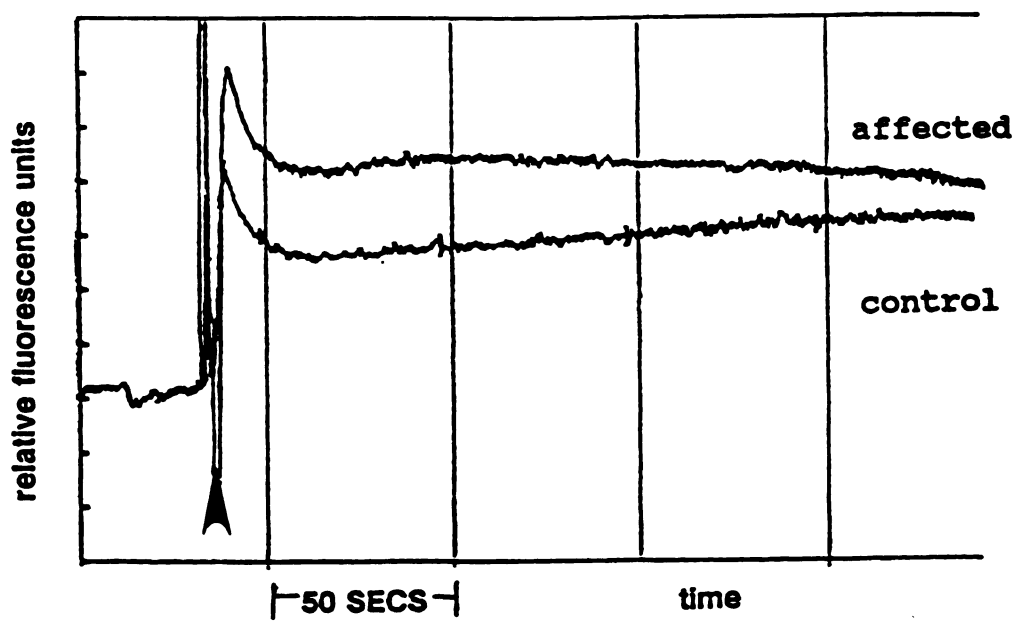


Figure 4. Representative fluorescence tracings of fura 2-loaded control and affected platelets in response to 1uM PAF in the presence of external Ca^{++}. Arrow = addition of agonist.

Thrombin

Using aequorin-loaded platelets, there was no significant difference between control and affected platelets in the post-stimulation $[Ca^{++}]_i$ following the addition of high (0.25U/ml) or low (0.01U/ml) concentrations of thrombin in either the presence or absence of external Ca^{++}. There was a trend for the post-stimulation $[Ca^{++}]_i$ to be greater in the control platelets than in the affected platelets. In all cases, post-stimulation $[Ca^{++}]_i$ was significantly elevated over resting concentrations. See Table 7 for a summary of the results. The luminescence tracings were similar in appearance in both control and affected platelets. See Figure 5 for representative curves.

Table 7. Peak $[Ca^{++}]_i$ (uM) following addition of thrombin in control and affected aequorin-loaded platelets.

Buffer	Agonist	Control (n = 3)	Affected (n = 4)
1mM Ca^{++}	0.25U/ ml Thrombin	13.4 ± 3.3 #	12.1 ± 0.9 #
	0.01U/ ml Thrombin	6.1 ± 0.7 #	5.5 ± 1.0 #
1mM EGTA	0.25U/ ml Thrombin	5.5 ± 1.4 #	3.9 ± 0.4 #
	0.01U/ ml Thrombin	5.1 ± 1.5 #	3.5 ± 0.3 #

Values = mean (uM) ± SD; n= number of dogs tested; # = significantly different from resting, $p < 0.05$.

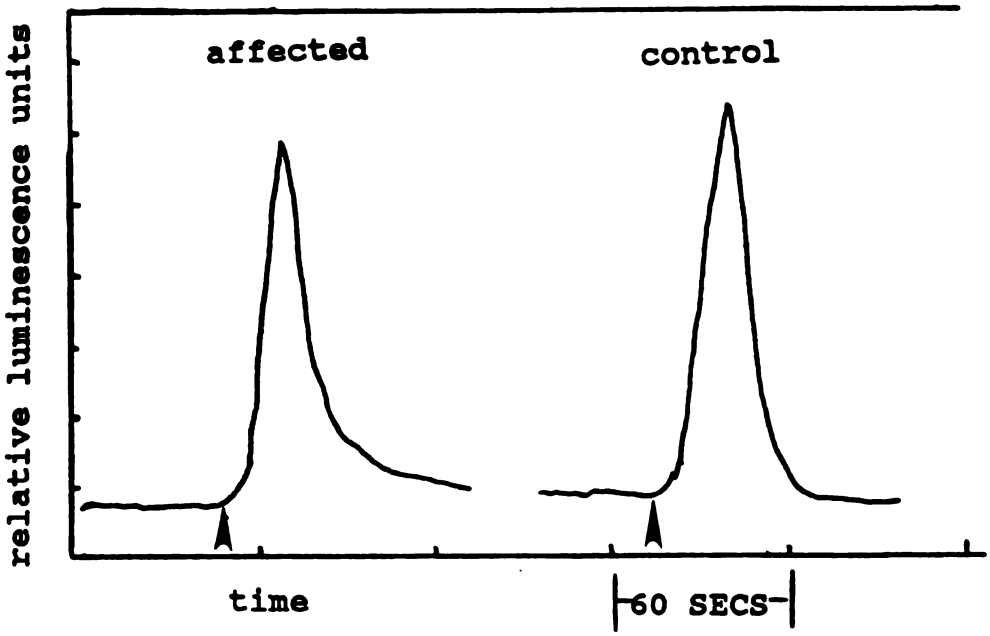


Figure 5. Representative luminescence tracings of aequorin-loaded control and affected platelets in response to 0.25U/ml thrombin in the presence of external Ca^{++}. Arrow = addition of agonist.

Using fura 2 as the Ca^{++} indicator, there was no significant difference in the post-stimulation $[Ca^{++}]_i$ between control and affected platelets following the addition of either concentration of thrombin. In EGTA-containing buffer, there was significantly decreased post-stimulation $[Ca^{++}]_i$ in affected platelets relative to control platelets following stimulation with high concentrations of thrombin. Following the addition of low concentrations of thrombin, there was a trend for higher post-stimulation $[Ca^{++}]_i$ in control platelets; however, the difference was not statistically significant. In both groups, all post-stimulation values were significantly increased relative to resting $[Ca^{++}]_i$. See Table 8 for a summary of the results.

Table 8. Peak $[Ca^{++}]_i$ (nM) following thrombin stimulation in control and affected fura 2-loaded platelets.

Buffer	Agonist	Control (n = 4)	Affected (n = 5)
1mM Ca^{++}	0.25U/ ml Thrombin	744.4 ± 88.7 #	696.2 ± 53.2 #
	0.01U/ ml Thrombin	292.1 ± 42.6 #	299.4 ± 31.2 #
1mM EGTA	0.25U/ ml Thrombin	235.3 ± 5.5 #	161.8 ± 15.3 * #
	0.01U/ ml Thrombin	155.4 ± 27.9 #	124.3 ± 20.9 #

Values = mean (nM) ± SD; n = number of dogs tested; * = significantly different from control, $p < 0.05$; # = significantly different from resting, $p < 0.05$.

The appearance of the fluorescence curves in control and affected platelets was similar in the EGTA-containing buffers. In response to high concentrations of thrombin, there was a sharp fluorescence peak which gradually decreased to baseline levels. Following low concentrations of thrombin, the fluorescence peak was broader but otherwise similar. In Ca^{++}-containing buffer, the fluorescence curves appeared slightly different in response to high concentrations of thrombin. The affected platelets reached a similar peak to that of control platelets; however, the level of fluorescence remained relatively stable in control platelets while it tended to gradually decrease in affected platelets. Following stimulation with low concentrations of thrombin, the fluorescence curves were similar in appearance. See Figure 6 for representative fluorescence curves.

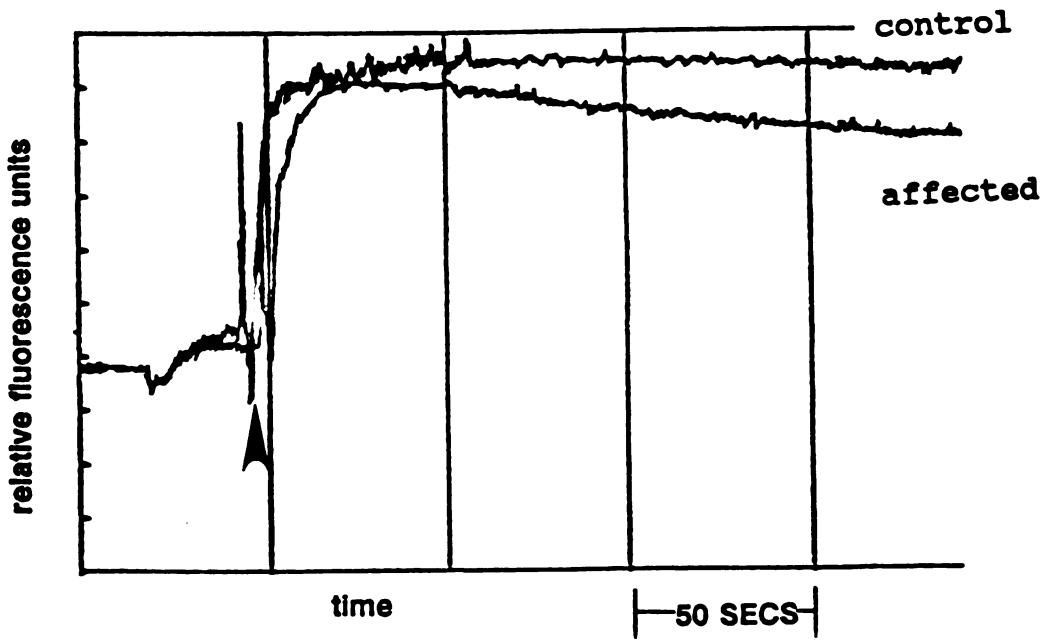


Figure 6. Representative fluorescence tracings of fura 2-loaded affected and control platelets in response to 0.25U/ml thrombin in the presence of external Ca^{++}. Arrow = addition of agonist.

Ionomycin

Using aequorin-loaded platelets, there was no significant difference between control and affected platelets in the post-stimulation $[Ca^{++}]_i$ in response to high (2uM) or low (0.5uM) concentrations of ionomycin in the presence of external Ca^{++}. In both groups, the post-stimulation $[Ca^{++}]_i$ was significantly increased over resting values. In EGTA-containing buffer, the peak $[Ca^{++}]_i$ was significantly decreased in affected platelets relative to control platelets following the addition of high concentrations of ionomycin. The post-stimulation $[Ca^{++}]_i$ of both affected and control platelets was

significantly increased over resting values. There was no significant difference between affected and control dogs in post-stimulation $[Ca^{++}]_i$ following the addition of low concentrations of ionomycin. Though there was a trend for the stimulated $[Ca^{++}]_i$ to be increased relative to resting values, the difference was not statistically significant. See Table 9 for a summary of the results. There was no consistent difference between affected and control platelets in the appearance of the luminescence tracings in any of the experimental groups in response to ionomycin. See Figure 7 for representative tracings.

Table 9. Peak $[Ca^{++}]_i$ (uM) following addition of ionomycin in control and affected aequorin-loaded platelets.

Buffer	Agonist	Control (n = 3)	Affected (n = 5)
1mM Ca^{++}	2.0 uM ionomycin	10.1 ± 2.0 #	10.6 ± 2.1 #
	0.5 uM ionomycin	5.6 ± 1.3 #	5.3 ± 0.8 #
1mM EGTA	2.0 uM ionomycin	3.2 ± 0.3 #	2.6 ± 0.3 * #
	0.5 uM ionomycin	2.7 ± 0.3	2.3 ± 0.3

Values = mean (uM) ± SD; n = number of dogs tested; * = significantly different from control, $p < 0.05$; # = significantly different from resting, $p < 0.05$.

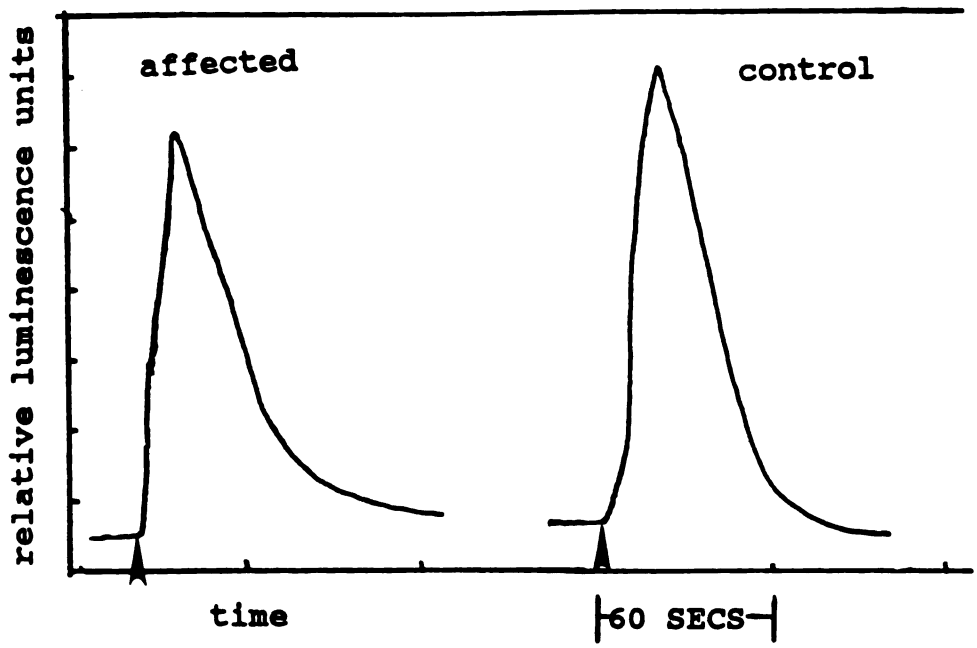


Figure 7. Representative luminescence tracings of aequorin-loaded control and affected platelets in response to 2uM ionomycin in the presence of external Ca^{++}. Arrow = addition of agonist.

Using fura 2-loaded platelets in the presence of external Ca^{++}, there was no significant difference between control and affected platelets in the maximum post-stimulation $[Ca^{++}]_i$ in response to high (5uM) or low (2uM) concentrations of ionomycin. When compared to resting values, the post-stimulation $[Ca^{++}]_i$ was significantly increased in both affected and control platelets. In EGTA-containing buffer, the post-stimulation $[Ca^{++}]_i$ was significantly decreased in affected platelets in response to high concentrations of ionomycin. There was no significant difference between the two groups following the addition of low concentrations of ionomycin. All post-stimulation values for $[Ca^{++}]_i$ were significantly increased over resting levels. See Table 10 for a summary of the results.

Table 10. Peak $[Ca^{++}]_i$ (nM) in response to ionomycin in control and affected fura 2-loaded platelets.

Buffer	Agonist	Control (n = 4)	Affected (n = 5)
1mM Ca^{++}	5.0 uM Ionomycin	817.6 ± 252.2 #	609.4 ± 47.5 #
	2.0 uM Ionomycin	315.8 ± 37.8 #	318.0 ± 19.7 #
1mM EGTA	5.0 uM Ionomycin	141.6 ± 1.3 #	119.3 ± 14.7 * #
	2.0 uM Ionomycin	110.4 ± 9.0 #	96.9 ± 8.8 #

Values = mean (nM) ± SD; n = number of dogs tested; * = significantly different from control, $p < 0.05$; # = significantly different from resting, $p < 0.05$.

There was no significant difference in the appearance of the fluorescence tracings between affected and control platelets. In the presence of external Ca^{++}, there was a relatively rapid increase to peak fluorescence levels which were maintained throughout the test period. In the absence of external Ca^{++}, there was a similar increase to peak fluorescence levels; however, the fluorescence levels gradually decreased over the test period. See Figure 8 for representative curves.

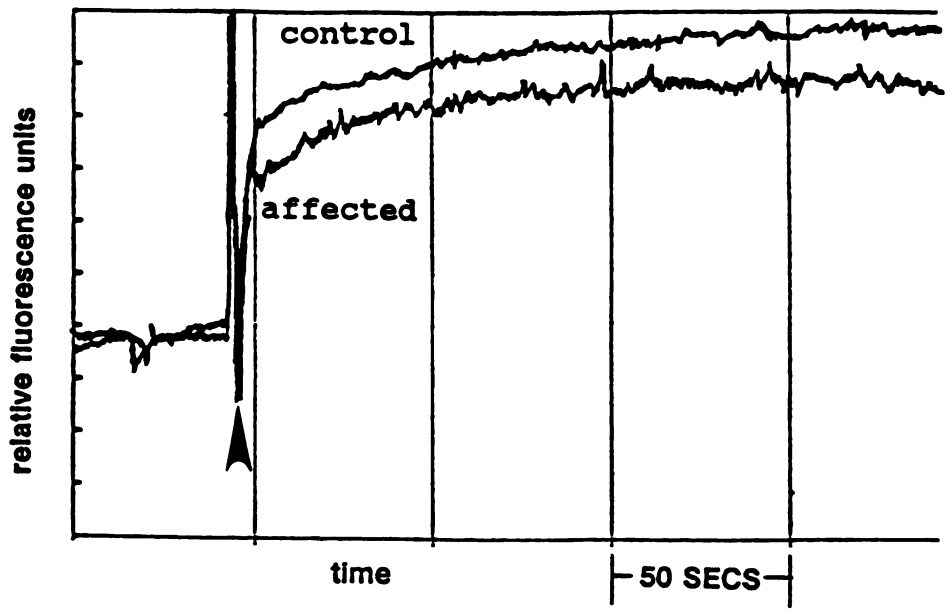


Figure 8. Representative fluorescence tracings of fura 2-loaded control and affected platelets in response to 5uM ionomycin in the presence of external Ca^{++}. Arrow = addition of agonist.

Phorbol Myristate Acetate (PMA)

Using aequorin as the Ca^{++} indicator, the post-stimulation $[Ca^{++}]_i$ in response to 3uM PMA was significantly less in the affected platelets than in control platelets in the presence of external Ca^{++}. The post-stimulation $[Ca^{++}]_i$ in both groups was significantly increased over resting concentrations. In EGTA-containing buffer, there was no significant difference between the two groups of platelets, although there was a general trend for higher post-stimulation $[Ca^{++}]_i$ in control platelets. Though a luminescence peak was always detectable, the

calculation of $[Ca^{++}]_i$ yielded values that were not statistically different from resting concentrations. See Table 11 for a summary of the results. There was no difference in appearance of the luminescence tracings between affected and control platelets. See Figure 9 for representative tracings.

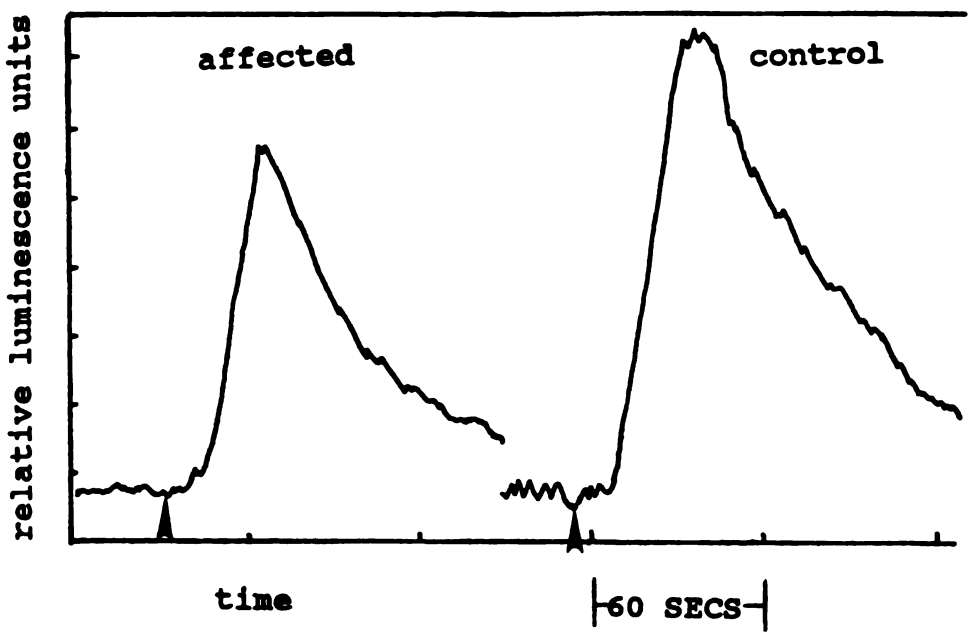


Figure 9. Representative luminescence tracings of aequorin-loaded control and affected platelets in response to 3uM PMA in the presence of external Ca^{++}. Arrow = addition of agonist.

Table 11. Peak $[Ca^{++}]_i$ (uM) in response to 3uM PMA in control and affected aequorin-loaded platelets.

Buffer	Control (n = 3)	Affected (n = 4)
Ca^{++}	6.6 ± 1.1 #	4.7 ± 0.5 * #
1mM EGTA	2.7 ± 0.4	2.3 ± 0.4

Values = mean (uM) ± SD; n = number of dogs tested; * = significantly different from control, $p < 0.05$; # = significantly different from resting, $p < 0.05$.

In contrast to the increased $[Ca^{++}]_i$ using aequorin, the PMA-induced post-stimulation $[Ca^{++}]_i$ was decreased compared to resting values in both control and affected fura 2-loaded platelets in the presence of external Ca^{++}. The decrease was of similar magnitude in both affected and control platelets. In an EGTA-containing buffer, the post-stimulation $[Ca^{++}]_i$ was also decreased relative to resting levels in response to PMA in both groups; however, the decrease was only statistically significant in the control platelets. See Table 12 for a summary of the results. There was no difference between the affected and control platelets in the appearance of the fluorescent tracings. In the presence of external Ca^{++}, the curves were characterized by a moderate decrease in fluorescence which was maintained for the time period recorded. In the absence of external Ca^{++}, the decrease in fluorescence was less distinct and, in some cases, absent. See Figure 10 for representative tracings.

Table 12. Peak $[Ca^{++}]_i$ in response to 3uM PMA in control and affected fura 2-loaded platelets.

External Buffer	Control (n = 4)	Affected (n = 5)
1mM Ca^{++}	101.4 ± 6.8 #	97.5 ± 15.6 #
1mM EGTA	56.2 ± 4.2 #	59.1 ± 7.6

Values = mean (nM) ± SD; n = number of dogs tested; # = significantly different from resting, $p < 0.05$.

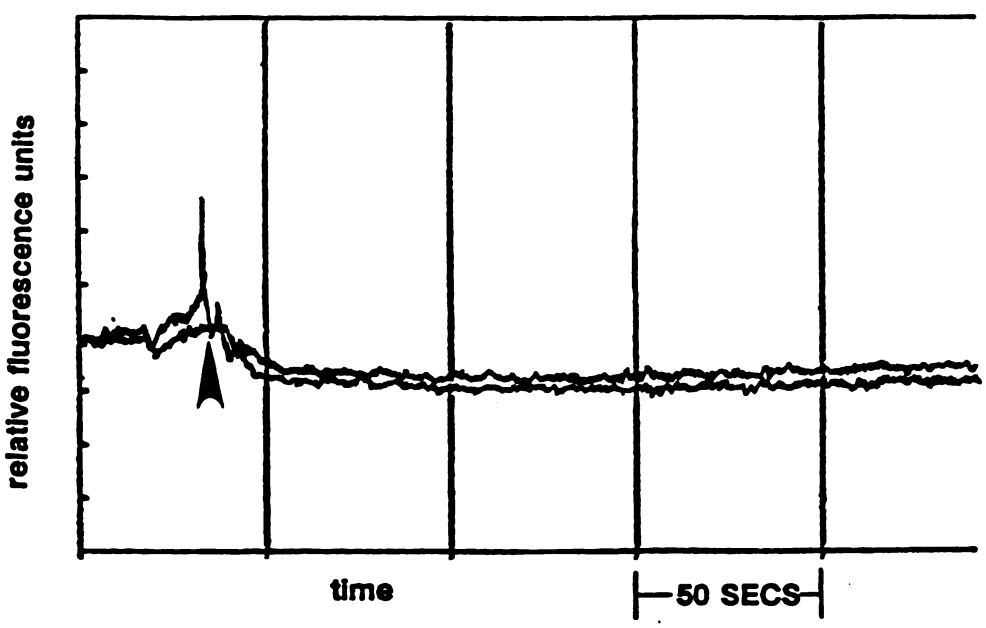


Figure 10. Representative fluorescence tracings of fura 2-loaded control and affected platelets in response to 3uM PMA in the presence of external Ca^{++}. Arrow = addition of agonist.

DISCUSSION

Changes in $[Ca^{++}]_i$ play a pivotal role in a number of important events or processes in platelet activation. First, increased $[Ca^{++}]_i$ stimulates MLCK activity which causes the subsequent phosphorylation of myosin light chain.[353] Phosphorylation of myosin light chain is correlated with granule centralization, the generation of a contractile force and shape change.[95,142] Secondly, elevated $[Ca^{++}]_i$ acts synergistically with PKC to promote granule secretion.[159,194,380] Thirdly, cytosolic Ca^{++} is essential for the proper functioning of a number of enzymes which have important roles in platelet activation. Increased $[Ca^{++}]_i$ is associated with activation of PLA_2[397] and the Ca^{++}-dependent proteases (calpains)[366] while resting to increased $[Ca^{++}]_i$ is required for PLC[23,25] and PKC[194] activity. Finally, cytosolic Ca^{++} is involved in the organization and composition of the platelet cytoskeleton.[377]

Resting and post-stimulation $[Ca^{++}]_i$ was measured in the platelets from dogs with Basset hound hereditary thrombopathy (BHT) and from control Basset hounds because previous findings suggest that the platelet disorder in BHT is due to a defect(s) in signal transduction or processing. In the current study, two different techniques were used to measure $[Ca^{++}]_i$: (1) the fluorescent dye fura 2; and (2) the luminescent photoprotein aequorin. It has been proposed that fura 2 and aequorin provide unique, but complimentary, information about changes in $[Ca^{++}]_i$.[190] Aequorin responds to regional elevations in $[Ca^{++}]_i$ with a corresponding exponential increase in the luminescent signal. While

aequorin effectively detects local zones of Ca^{++} change, it may not accurately reflect the average $[Ca^{++}]_i$.[97] Although aequorin is primarily located in the cytosol and not in the internal organelles, it has been proposed that bulk of the intracellular aequorin is closely associated with the plasma membrane and not distributed diffusely throughout the cytosol.[190] In contrast, fura 2 is distributed throughout the cytosol.[190] In some cells, fura 2 compartmentalizes in organelles; however, this has not been found to be a problem in platelets in suspension.[79,190] While fura 2 is not able to detect local Ca^{++} changes, it provides a better indication of average cytosolic Ca^{++} fluxes.[188,190]

The difference between the pool of Ca^{++} being measured using the two techniques is best illustrated by a comparison of the signals obtained using aequorin- and fura 2-loaded platelets. Aequorin gives a rapid luminescence peak which generally drops back to baseline levels within 60-90 seconds, irrespective of the presence or absence of external Ca^{++}. The rapid decrease in the aequorin luminescent signal cannot be explained by irreversible oxidation of the dye following reaction with Ca^{++} since the addition of a second agonist following the initial luminescence peak elicits another luminescent response.[190] It has been proposed that the shortness of the signal is due to rapid diffusion and/or sequestration of Ca^{++} from the region of dye location.[190] Fura 2 gives a rapid peak of fluorescence which tends to plateau before slowly decreasing toward baseline. This plateau is sustained much longer in the presence of external Ca^{++}.

A comparison of resting $[Ca^{++}]_i$ yielded no significant differences between the platelets of control and affected dogs using either aequorin-loaded or fura 2-loaded platelets, suggesting that affected platelets maintain normal resting Ca^{++} homeostasis. It is important to note the difference in the magnitude of the $[Ca^{++}]_i$ using the two techniques. The $[Ca^{++}]_i$ calculated using aequorin was 20 to 30 fold greater than the value calculated using fura 2. The values for resting $[Ca^{++}]_i$ in the current study were within the range of resting $[Ca^{++}]_i$ measured using aequorin[188] and fura 2[218,313,315] in human platelets.

The discrepancy between the $[Ca^{++}]_i$ calculated using fura 2 and aequorin can be partially explained by the effect of intracellular Mg^{++} on the aequorin signal. Mg^{++} competitively binds to aequorin without producing measurable luminescence and causes decreased sensitivity to Ca^{++}.[79,44] Though the $[Mg^{++}]$ in canine platelets is not known, the calibration curve used to calculate $[Ca^{++}]_i$ for the current study was constructed in a medium containing 1mM Mg^{++}. Recent studies in human platelets have shown that the cytosolic $[Mg^{++}]$ is closer to 0.3mM. In the reported studies, calculation of $[Ca^{++}]_i$ using an aequorin calibration curve constructed in a medium containing 0.25mM Mg^{++} yields resting platelet $[Ca^{++}]_i$ of approximately 300nM, much closer to the most frequently published $[Ca^{++}]_i$ of approximately 100nM in fura 2-loaded platelets.[440] The calculation of $[Ca^{++}]_i$ in fura 2-loaded platelets is also affected by the $[Mg^{++}]$, but to a much lesser extent than aequorin since fura 2 has a relatively high selectivity for Ca^{++} compared to Mg^{++}. The dissociation constant (K_d) for fura 2 used to calculate

$[Ca^{++}]_i$ in the present study also assumed an intracellular Mg^{++} concentration of 1mM.[79]

The absolute value for $[Ca^{++}]_i$ calculated using both dyes should be interpreted with caution. The fluorescence of fura 2 is dependent on the microviscosity of the medium surrounding the dye, therefore the fluorescence of fura 2 in the platelet cytosol differs from the fluorescence of the same amount of dye in the lysed platelet solution. Since the equation used to calculate $[Ca^{++}]_i$ is based on the comparison of dye fluorescence in the platelet cytosol to the fluorescence of dye in the lysed platelet suspension, the calculation of absolute $[Ca^{++}]_i$ has inherent error.[148,332] In aequorin-loaded platelets, the dye localizes in regions of high Ca^{++} concentrations, such as under the plasma membrane. Therefore, the $[Ca^{++}]_i$ measured does not reflect average $[Ca^{++}]_i$.[188,190] The absolute value for $[Ca^{++}]_i$ was not of primary interest in the current study since the study was designed to compare resting and post-stimulation $[Ca^{++}]_i$ in control and affected platelets using the same experimental techniques for each group.

A second interesting feature of the current study was the significant difference between $[Ca^{++}]_i$ in Ca^{++}- and EGTA-containing buffers in both aequorin- and fura 2-loaded platelets. It was most likely a feature of experimental design rather than a true physiologic difference since platelet resting $[Ca^{++}]_i$ is independent of large changes in external $[Ca^{++}]$.[51,331] One study that compared the extent and rate of decay of the aequorin signal in the presence and absence of external Ca^{++} has shown that the aequorin loaded into platelets is

partly accessible to external Ca^{++}. It was not determined whether this accessibility reflects the natural distribution of Ca^{++} in the cytoplasm and/or plasma membrane or is due to alterations in the platelet handling of Ca^{++} secondary to the permeabilization procedures used to load aequorin.[217] Other groups have proposed that the difference in resting $[Ca^{++}]_i$ in Ca^{++}- and EGTA-containing buffers reflects the effect of a few "leaky platelets" on the luminescence signal. Since the $[Ca^{++}]_i$ is measured in a platelet suspension, it is possible that a small number of injured cells have increased membrane permeability, thereby exposing their intracellular aequorin to the $[Ca^{++}]$ present in the external environment. In Ca^{++}-containing buffer, these cells would be exposed to a relatively high $[Ca^{++}]$. The kinetics of the aequorin reaction make the luminescence signal of platelets in suspension susceptible to dominance by a few damaged cells with large luminescence signals.[190,440]

The difference in resting $[Ca^{++}]_i$ between Ca^{++}- and EGTA-containing buffers was also noted in the fura 2 preparations. As with aequorin, the difference may be partly due to damage to cells during experimental manipulation and increased membrane permeability to Ca^{++}. Fura 2 is not as susceptible as aequorin to dominance of the fluorescence signal by a few "leaky platelets" in the platelet suspensions. Studies on quin2-loaded platelets have shown that the difference between resting $[Ca^{++}]_i$ in the presence and absence of external Ca^{++} is mainly due to buffering of internal Ca^{++} by quin2 itself. When external Ca^{++} is present, the buffering of quin2 is partly compensated for by the subsequent influx of external Ca^{++}.[190] This buffering effect is not as significant in fura 2-loaded platelets since

lower concentrations of fura 2 are loaded.[148,332] Another aspect for consideration is the effect of the fura 2 itself. Low concentrations of cytosolic fura 2 potentiate the aggregation and secretion responses of human platelets.[218] This finding was duplicated in the current study during pilot aggregation trials on fura 2-loaded canine platelets. The fura 2-loaded platelets had increased aggregation responses to all agonists tested when compared to non-loaded platelets. The loaded platelets also had a greater tendency, in the presence of external Ca^{++} and Mg^{++}, to undergo spontaneous shape change upon stirring, suggesting that $[Ca^{++}]_i$ is elevated. Finally, in the current study, the difference in the resting $[Ca^{++}]_i$ could be partially explained by leakage of fura 2 into the external medium. Due to the relatively low leakage rate, the calculation of $[Ca^{++}]_i$ did not correct for the presence of external dye. The fluorescence of the external dye was only mildly affected by the presence or absence of Ca^{++}, indicating that the form of dye in the supernatant consisted of both Ca^{++}-sensitive and Ca^{++}-insensitive forms. If leakage of dye was the main factor involved in the difference between the resting $[Ca^{++}]_i$ in the presence and absence of external Ca^{++}, it would be anticipated that resting $[Ca^{++}]_i$ would increase over the time period that the experiments were run. This was not found; instead, there was no significant difference in the resting $[Ca^{++}]_i$ calculated at the beginning and the end of each day's set of experiments.

ADP is a weak physiologic agonist which requires primary aggregation and the release of arachidonic acid metabolites for secretion and irreversible aggregation to occur.[377] It has been proposed that ADP activates platelets by stimulating Na^+-H^+ exchange and

causing a local, membrane-associated increase in pH_i. The localized alkalinization activates PLA_2 which mediates the release of small quantities of arachidonic acid. The arachidonic acid is metabolized via the cyclooxygenase pathway to yield endoperoxides and TxA_2 which amplify the response by binding to membrane receptors and activating PLC.[409,410,412] Inhibition of cyclooxygenase blocks the production of arachidonic acid metabolites in response to ADP but does not prevent primary aggregation[412] or Ca^{++} mobilization[92,229,290,394], suggesting these responses are independent of the TxA_2 pathway.

Kinetic studies on Ca^{++} mobilization in fura 2-loaded platelets have demonstrated that, in the presence of external Ca^{++}, ADP stimulation induces an almost instantaneous increase $[Ca^{++}]_i$.[351,352] This suggests that there is a direct association, independent of the production of diffusible second messengers, between the ADP receptor and the plasma membrane Ca^{++} channel. The nature of this association is not known; however, some evidence implicates the involvement of a G protein.[331,352] In the absence of external Ca^{++}, there is a lag period between ADP binding and Ca^{++} release, suggesting a requirement for the production of second messengers to mediate the release of Ca^{++} from internal stores.[351,352] The ADP-induced release of internal Ca^{++} is partially inhibited by agents which block Na^+/H^+ exchange.[394]

In the present study, a comparison of ADP-induced post-stimulation $[Ca^{++}]_i$ in EGTA-containing buffer yielded no significant differences between control and affected platelets using either fura 2 or aequorin, though there was a trend for values to be decreased in

affected platelets. These findings suggest that affected platelets have normal to slightly decreased ability to mobilize internal Ca^{++}. In the presence of external Ca^{++}, there was significantly increased post-stimulation $[Ca^{++}]_i$ in fura 2-loaded affected platelets relative to control platelets. These findings indicate that affected platelets have increased ability to stimulate Ca^{++} influx following stimulation with ADP when compared to control platelets. In contrast, in the presence of external Ca^{++}, affected aequorin-loaded platelets had decreased post-stimulation $[Ca^{++}]_i$ relative to control platelets. These findings suggest that affected platelets have normal to decreased influx of external Ca^{++} across the plasma membrane in response to ADP. It must be remembered that the Ca^{++} signal measured using aequorin is short lived and likely represents the flux in submembranous regions. The Ca^{++} signal measured by fura 2 is longer-lived and represents the overall cellular response.[188,190]

Caution must be used in the interpretation of changes in $[Ca^{++}]_i$ in the presence of external Ca^{++} due to the differing effects of aggregation on the signals measured using aequorin- or fura 2-loaded platelets. Control platelets are able to aggregate in response to ADP. Affected platelets do not undergo primary or secondary aggregation in response to ADP, though shape change and the rapid secretion of dense granule ATP occur.[254] The fura 2 fluorescence signal requires a homogeneous suspension for accurate measurement and is artificially suppressed by the formation of platelet aggregates in cell suspensions.[190] Although aggregation was not noted by gross examination of the control samples, the presence of microaggregates of the control

platelets cannot be ruled out. It has been shown in human platelets that ADP induces the formation of microaggregates in washed platelets in the absence of exogenous external fibrinogen.[190] The presence of microaggregates would artificially suppress the fluorescence signal in control platelets relative to affected platelets. The formation of microaggregates could also explain the difference in the appearance of the fluorescence curves between affected and control platelets. The more rapid decrease of the fluorescence signal in the control group could coincide with the formation of aggregates. In aequorin-loaded platelets, the luminescence signal itself is not affected by aggregation;[189] however, a recent study in aequorin-loaded platelets has shown that the stimulation of Ca^{++}-mobilization by weak agonists, such as ADP, requires aggregation-dependent amplification for full Ca^{++} responses to occur.[229] Lack of aggregation, therefore, would suppress the luminescence signal in the affected platelets relative to control platelets.

PAF is a physiologic agonist of intermediate strength. Secretion occurs independent of prior aggregation or the production of arachidonic acid metabolites; however, treatment of platelets with cyclooxygenase inhibitors or ADP scavengers reduces the extent of the aggregation and secretion responses.[377] PAF causes activation of PLC, hydrolysis of membrane phosphoinositides and the production of the second messengers (1,4,5)IP_3 and 1,2-DG.[106,283,375] Kinetic studies have demonstrated a lag period between agonist binding and the initiation of increased $[Ca^{++}]_i$, suggesting a requirement for the production of second messengers to mediate both Ca^{++} release from internal stores and the

influx of external Ca^{++}.[352] PAF-induced Ca^{++} mobilization is almost completely independent of TxA_2 production and cyclooxygenase inhibition.[155]

In the presence of external Ca^{++}, the post-stimulation $[Ca^{++}]_i$ in response to high concentrations of PAF was increased in fura 2-loaded affected platelets when compared to control platelets. There was no difference between the two groups using aequorin-loaded platelets. As discussed under ADP, part, if not all, of the difference between the two groups of fura 2-loaded platelets may be due to the formation of platelet aggregates in the control samples which would artificially lower the fluorescence signal. In affected platelets, PAF stimulation causes shape change but not primary or secondary aggregation.[254] Therefore, the fluorescence signal would not be affected by the formation of aggregates. The lack of difference using aequorin-loaded platelets is consistent with this hypothesis since the aequorin luminescent signal is not directly affected by aggregation;[190] however, the presence of aggregation in the control samples may reinforce or amplify the Ca^{++} response to PAF.[229]

In EGTA-containing buffer, post-stimulation $[Ca^{++}]_i$ was decreased in affected platelets following stimulation with either concentration of PAF, suggesting that affected platelets do not mobilize internal Ca^{++} stores as well as control platelets. In spite of lower post-stimulation $[Ca^{++}]_i$, affected platelets mobilize sufficient quantities of intracellular Ca^{++} to cause shape change[254] and the Ca^{++}-dependent phosphorylation of the 20kDa myosin light chain similar to control

platelets.[255] Phosphorylation of myosin light chain is associated with activation of MLCK,[177,353] a Ca^{++}-calmodulin dependent enzyme.[164]

Thrombin is a strong physiologic agonist that induces secretion and aggregation responses which are not affected by cyclooxygenase inhibition.[377] Thrombin stimulates platelet by causing activation of PLC.[90,262,263,346] Thrombin also initiates platelet aggregation and secretion independent of PLC activation and phosphoinositide activation.[57,226] Thrombin mobilizes intracellular and extracellular Ca^{++}, though the bulk of the increase in post-stimulation $[Ca^{++}]_i$ comes from the influx of external Ca^{++}.[192,243,438] Kinetic studies have shown a lag period between the addition of thrombin and the start of Ca^{++} mobilization, suggesting that it is the production of second messengers which actually mediate the mobilization of both internal and external Ca^{++}.[351,352,476]

In the presence of external Ca^{++}, the thrombin-induced post-stimulation $[Ca^{++}]_i$ was not significantly different between affected and control groups using either aequorin- or fura 2-loaded platelets. It is important to note that thrombin causes aggregation and dense granule secretion in both affected and control platelets, negating the selective effect of aggregation on the Ca^{++} response in control platelets.[254] In EGTA-containing buffer, the post-stimulation $[Ca^{++}]_i$ was significantly decreased in affected platelets loaded with fura 2. The same trend was seen using aequorin, though the difference was not statistically significant. This indicates that affected platelets mobilize Ca^{++} as well as control platelets only when Ca^{++} influx from the external

environment occurs while Ca^{++} mobilization from internal stores is impaired. The amount of internal Ca^{++} release is large enough to cause shape change and the Ca^{++}-dependent phosphorylation of the 20kDa protein.[255]

Ionomycin and calcium ionophore A23187 are non-physiologic agonists that transport Ca^{++} across cell membranes.[457] The ionophores cause sufficient Ca^{++} release from the DTS to initiate aggregation and secretion independent of the influx of external Ca^{++}; however, the presence of external Ca^{++} potentiates the responses.[121,457] The ionophore-induced elevation in $[Ca^{++}]_i$ activates a number of Ca^{++}-dependent enzymes, including PLA_2. Activation of PLA_2 mediates the release of the arachidonic acid metabolites[314] from membrane phospholipids and the subsequent activation of PLC, hydrolysis of membrane phosphoinositides and production of intracellular second messengers.[337,339,444] The ionophore-induced activation of PLC is blocked by cyclooxygenase inhibition while the increase in $[Ca^{++}]_i$ is not prevented.[337,339]

In the presence of external Ca^{++}, there was no significant difference between affected and control platelets in the post-stimulation $[Ca^{++}]_i$ in response to ionomycin using aequorin- or fura 2-loaded platelets. There was a tendency for the post-stimulation $[Ca^{++}]_i$ to be decreased following stimulation with high concentrations of ionomycin in fura 2-loaded affected platelets; however, the difference was not statistically significant due to the wide range of responses within the groups. Stimulation of affected platelets with calcium

ionophore A23187 causes shape change without significant aggregation or secretion of dense granule ATP.[254] Since, in the current study, the high concentration of ionomycin caused visible aggregation of control platelets but not affected platelets, it is possible that the formation of aggregates artificially lowered the fluorescence signal selectively in the control platelets. This suggests that post-stimulation $[Ca^{++}]_i$ in the control platelets could be higher than the calculated value and that the presence of aggregates negated any difference in the post-stimulation Ca^{++} response to ionomycin.

In EGTA-containing buffer, there was significantly decreased $[Ca^{++}]_i$ both aequorin- and fura 2-loaded affected platelets following stimulation with high concentrations of ionomycin, indicating decreased Ca^{++} release from internal stores. Calcium ionophores cause a non-receptor-mediated release of Ca^{++} from the DTS which is separate from the receptor-mediated release by the inositol phosphates.[11,313,377] In spite of a defect in the internal mobilization of Ca^{++} in response to ionomycin, the amount of Ca^{++} released by calcium ionophores is sufficient to cause shape change and phosphorylation of the 20kDa and 47kDa proteins.[255]

Phorbol myristate acetate is a non-physiologic agonist which causes platelet activation by direct stimulation of PKC by increasing the affinity of PKC for Ca^{++} and phospholipid.[71,72] It was originally reported that phorbol esters initiate platelet activation in a Ca^{++}-independent manner since the fluorescent dyes quin2 or fura 2 do not detect any increase in sample fluorescence in PMA-stimulated

platelets.[117,439] Studies using aequorin-loaded platelets have identified a post-stimulation Ca^{++} peak in response to phorbol esters.[117,439] The mechanism for this PMA-induced Ca^{++} flux is not known. The marked discrepancy between the $[Ca^{++}]_i$ measured using aequorin and that measured using the fluorescent chelator dyes is not easily resolved but illustrates the fact that the techniques are measuring different aspects of Ca^{++} metabolism. It has been suggested that aequorin is measuring local elevations in $[Ca^{++}]_i$, most likely submembranous in origin, which are not detectable by the use of fura 2 or quin2.[190]

In the presence of external Ca^{++}, there was increased post-stimulation $[Ca^{++}]_i$ in response to PMA using aequorin-loaded platelets similar to that reported in the literature.[437] This response was significantly decreased in the affected platelets when compared to control platelets. In the presence of external EGTA, there was no significant difference between the two groups of platelets or between the stimulated and resting $[Ca^{++}]_i$ within each group. This suggests two conclusions: (1) the primary component of the PMA-induced Ca^{++} is the influx of external Ca^{++} in both control and affected platelets; and (2) the influx of external Ca^{++} is decreased in affected platelets in response to PMA.

In the presence of external Ca^{++}, the post-stimulation $[Ca^{++}]_i$ in fura 2-loaded platelets was significantly decreased when compared to resting $[Ca^{++}]_i$. This decrease was of similar magnitude in both groups of platelets. This finding was expected because phorbol enhances Ca^{++}

resequestration into internal storage sites[315,471,472,473] and Ca^{++} efflux across the plasma membrane.[315,331] In addition, phorbol esters cause aggregation and secretion in both affected and control platelets,[254] therefore the fluorescence signal in both groups would be equally altered.

In summary, the measurement of post-stimulation $[Ca^{++}]_i$ in the absence of external Ca^{++} has shown that affected platelets do not mobilize internal stores of Ca^{++} as well as control platelets following stimulation with PAF, thrombin and high concentrations of the calcium ionophore ionomycin. The same trend was seen following stimulation with ADP; however, the difference was not statistically significant. The results are summarized in Tables 13 and 14.

Thrombin, PAF and ADP do not directly cause release of internal Ca^{++}; instead, these agonists require the production of intracellular second messengers[351,352] which initiate a receptor-mediated release of intracellular Ca^{++} from the DTS.[331] There is also a small quantity of Ca^{++} which may be released from the membrane phospholipids in response to agonist stimulation.[64] Possible explanations for the defective release of internal Ca^{++} in affected platelets include: (1) decreased production of second messengers or production of defective second messengers which normally mediate Ca^{++} release from the DTS (primarily 1,4,5-IP_3); (2) decreased ability of the DTS to respond to the second messengers; or (3) decreased storage of releasable Ca^{++} within the DTS. In contrast, calcium ionophores bypass the physiologic, receptor-mediated release of internal Ca^{++}.[350] It has been shown that the

Table 13. Summary of resting and post-stimulation $[Ca^{++}]_i$ results in control and affected platelets loaded with aequorin.

Agonist	Concentration	Control (n = 3)	Affected (n = 4)
1mM External Ca^{++}			
Resting	-	3.6 ± 0.4 (-)	3.2 ± 0.3 (-)
ADP	10 uM	6.0 ± 1.1 (+)	4.6 ± 0.4 (-)
PAF	1 uM	16.7 ± 2.9 (+)	16.6 ± 2.8 (-)
	0.01 uM	9.1 ± 0.7 (+/-)	7.4 ± 1.0 (-)
Thrombin	0.25U/ml	13.4 ± 3.3 (+)	12.2 ± 0.9 (+)
	0.01U/ml	6.1 ± 0.7 (+/-)	5.5 ± 1.0 (-)
Ionomycin	2 uM	10.1 ± 2.0 (+)	10.6 ± 2.1 (-)
	0.5 uM	5.6 ± 1.3 (+/-)	5.3 ± 0.8 (-)
PMA	3 uM	6.6 ± 1.1 (+)	* 4.7 ± 0.5 (+)
1mM External EGTA			
Resting	-	2.2 ± 0.3 (-)	2.0 ± 0.2 (-)
ADP	10 uM	2.5 ± 0.4 (-)	2.2 ± 0.4 (-)
PAF	1 uM	5.2 ± 0.8 (-)	4.2 ± 0.5 (-)
	0.01 uM	5.0 ± 0.9 (-)	4.0 ± 0.6 (-)
Thrombin	0.25U/ml	5.5 ± 1.4 (-)	3.9 ± 0.4 (-)
	0.01U/ml	5.1 ± 1.5 (-)	3.5 ± 0.3 (-)
Ionomycin	2 uM	3.2 ± 0.3 (-)	* 2.6 ± 0.3 (-)
	0.05 uM	2.7 ± 0.3 (-)	2.3 ± 0.3 (-)
PMA	3 uM	2.7 ± 0.4 (-)	2.3 ± 0.4 (-)

Values = mean (uM) ± S.D.; * = significantly different from control, $P < 0.05$; (+) = aggregation occurs; (+/-) = aggregation may or may not occur; (-) = aggregation does not occur.

Table 14. Summary of resting and post-stimulation $[Ca^{++}]_i$ in control and affected platelets loaded with fura 2.

Agonist	Concentration	Control (n = 4)	Affected (n = 5)
1mM External Ca^{++}			
Resting	-	130.6 ± 16.0 (-)	140.0 ± 9.8 (-)
ADP	10 uM	311.0 ± 19.1 (+/-)	* 399.5 ± 26.9 (-)
PAF	1 uM	676.8 ± 98.7 (+)	* 842.9 ± 47.8 (+/-)
	0.01 uM	485.9 ± 74.5 (+/-)	465.2 ± 71.1 (-)
Thrombin	0.25U/ml	744.4 ± 88.7 (+)	696.2 ± 53.2 (+)
	0.01U/ml	292.1 ± 42.6 (+/-)	299.4 ± 31.2 (-)
Ionomycin	5 uM	817.6 ± 252.2 (+)	609.4 ± 47.5 (-)
	2 uM	315.8 ± 37.8 (+/-)	318.0 ± 19.7 (-)
PMA	3 uM	101.4 ± 6.8 (+)	97.5 ± 15.6 (+)
1mM External EGTA			
Resting	-	75.7 ± 7.1 (-)	73.7 ± 9.1 (-)
ADP	10 uM	125.2 ± 15.4 (-)	114.0 ± 14.4 (-)
PAF	1 uM	248.6 ± 25.5 (-)	* 176.6 ± 9.0 (-)
	0.01 uM	218.1 ± 18.2 (-)	* 164.8 ± 17.9 (-)
Thrombin	0.25U/ml	235.3 ± 5.5 (-)	* 161.8 ± 15.3 (-)
	0.01U/ml	155.4 ± 27.9 (-)	124.3 ± 20.9 (-)
Ionomycin	5 uM	141.6 ± 1.3 (-)	* 119.3 ± 14.7 (-)
	2 uM	110.4 ± 9.0 (-)	96.9 ± 8.8 (-)
PMA	3 uM	56.2 ± 4.2 (-)	59.1 ± 7.6 (-)

Value = mean (nM) ± S.D.; * = significantly different from control, $p < 0.05$; (+) = aggregation occurs; (+/-) = aggregation may or may not occur; (-) = aggregation does not occur.

increase $[Ca^{++}]_i$ following stimulation with ionomycin is not inhibited by blockage of cyclooxygenase or by removal of external ADP, suggesting that the mobilization of Ca^{++} is independent of the secondary stimulation of PLC and the production of second messengers.[339] Therefore, the decreased post-stimulation $[Ca^{++}]_i$ in affected platelets following the addition of ionomycin suggests that there is a defect in the storage of releasable Ca^{++} in the DTS.

Affected platelets have increased basal intracellular concentrations of cAMP, possibly due to defective regulation of the cAMP phosphodiesterase activity.[48,49] Increased cAMP could partially explain the Ca^{++} defect in affected platelets since cAMP inhibits agonist-induced increases in $[Ca^{++}]_i$ by blocking both the influx of external Ca^{++} and the release of Ca^{++} from internal stores.[331,350,433] Cyclic AMP also stimulates resequestration of Ca^{++} into the DTS following agonist-induced release.[43,120,167] Increased cAMP does not affect the post-stimulation $[Ca^{++}]_i$ following stimulation with ionomycin in platelets treated with cyclooxygenase inhibitors.[350] This suggests that the decreased post-stimulation $[Ca^{++}]_i$ in response to ionomycin in the platelets from dogs with BHT is not due to increased cAMP; however, affected platelets were not treated with cyclooxygenase inhibitors to eliminate any secondary effects due to the generation of arachidonate metabolites.

The situation in Ca^{++}-containing buffer, where the post-stimulation $[Ca^{++}]_i$ represents a combination of internal release and influx of external Ca^{++}, is less clear. Using aequorin-loaded

platelets, there was a trend for post-stimulation $[Ca^{++}]_i$ to be similar to slightly decreased in affected platelets when compared to control platelets in response to all of the agonists tested; however, the difference was only statistically significant following the addition of PMA. These findings suggest that the ability of affected platelets to generate an agonist-induced influx of external Ca^{++} is similar to slightly decreased when compared to control platelets. Unfortunately, the absence of aggregation in affected platelets negates the aggregation-dependent reinforcement of platelet Ca^{++} flux which plays a role in the Ca^{++} response to weak agonists.[229]

In the presence of external Ca^{++}, the difference between affected and control platelets using fura 2 varied according to the agonist; however, the data overall suggests that Ca^{++} influx is normal to slightly increased in affected platelets. The results must be viewed cautiously because of the effect of aggregation, which artificially decreases the fluorescence signal. In general, stimulation with agonists which cause aggregation of control but not affected platelets was associated with significantly increased post-stimulation $[Ca^{++}]_i$ in affected platelets. Stimulation with agonists which aggregated both groups of platelets did not cause any significant difference in post-stimulation $[Ca^{++}]_i$ between affected and control platelets. One exception was the response to ionomycin when there was a trend, though not statistically significant, for post-stimulation $[Ca^{++}]_i$ to be lower in affected dogs. This was the only agonist tested that caused visible aggregation only in control platelets where the post-stimulation $[Ca^{++}]_i$ was higher in control than affected platelets. It would be expected

that aggregation would, if anything, cause decreased $[Ca^{++}]_i$.

It is interesting to speculate on the different Ca^{++} responses of fura-2 loaded control and affected platelets to agonist stimulation in the presence of external Ca^{++}. One explanation is the role ADP which is released from the dense granules and could positively feedback on the membrane Ca^{++} channel to enhance Ca^{++} influx. Thrombin, ADP and PAF, which cause normal to increased post-stimulation $[Ca^{++}]_i$ in affected platelets in spite of decreased release of internal Ca^{++}, also cause the release of dense granules.[254] The calcium ionophores, which cause normal to decreased post-stimulation $[Ca^{++}]_i$ in BHT affected platelets, do not cause significant dense granule secretion in affected platelets.[254] A second explanation is the role of released TxA_2, which acts as another amplification signal to enhance platelet activation. In response to all agonists which cause increased production of TxB_2 in affected platelets,[256] the post-stimulation $[Ca^{++}]_i$ was normal to slightly increased. Calcium ionophore A23187 was the only agonist tested which causes decreased production of TxB_2 in affected platelets when compared to control platelets. To definitely determine the effects of aggregation, ADP secretion and TxA_2 production on the Ca^{++} response in the presence of external Ca^{++} would require additional studies utilizing: (1) non-aggregating preparations which still contain external Ca^{++} (ie. monoclonal antibodies against GPIIb-IIIa[281]); (2) ADP-mobilizing enzymes; (3) and cyclooxygenase inhibitors respectively.

In conclusion, although affected platelets have some identifiable abnormalities in Ca^{++} mobilization, the defects cannot explain the other

pathologic findings in BHT. In spite of decreased mobilization of internal Ca^{++} in affected platelets, the quantity released is large enough to cause shape change[254] and similar phosphorylation of the 20kDa and 47kDa proteins in affected and control platelets in non-aggregating, EDTA-containing preparations.[256] Shape change, secretion and aggregation have different Ca^{++} thresholds, with shape change requiring the lowest $[Ca^{++}]_i$ and aggregation requiring the highest $[Ca^{++}]_i$.[331,335] Therefore, normal shape change and 20kDa protein phosphorylation is not contradictory to the decreased internal Ca^{++} mobilization response in affected platelets.

There is no apparent correlation between the post-stimulation Ca^{++} response and the platelet responses of aggregation and secretion, though direct comparison of results is not possible due to variation in the concentration of external Ca^{++} in the different investigations. In the presence of external Ca^{++}, affected platelets do not have any consistent abnormality in agonist-stimulated Ca^{++} mobilization; yet, affected platelets have marked defects in aggregation and secretion.[254] For example, in the presence of external Ca^{++}, PAF produced the largest Ca^{++} fluxes using both fura 2- and aequorin-loaded platelets. Yet, PAF does not induce aggregation in affected platelets. Ionomycin, which does not induce aggregation or secretion in affected platelets, produced Ca^{++} levels comparable to thrombin, which stimulates all of these responses. There is also no apparent correlation between Ca^{++} mobilization in the presence of external Ca^{++} and TxB_2 production in affected platelets.[256] Since PLA_2, the enzyme which regulates arachidonate release, is activated by increasing cytosolic $[Ca^{++}]_i$,[27,151,339,397] any alteration

in activity could be associated with changes in Ca^{++} metabolism. In spite of the lack of consistent differences in post-stimulation $[Ca^{++}]_i$ between control and affected platelets in the presence of external Ca^{++}, affected platelets have significantly increased TxB_2 production following stimulation with high concentrations of PAF, ADP and thrombin and significantly decreased TxB_2 following stimulation with low concentrations of PAF and calcium ionophore.[256]

CHAPTER 3: RESTING AND POST-STIMULATION PLATELET CYTOSOLIC pH

INTRODUCTION

In order to function and survive, cells must maintain cytosolic pH (pH_i) within the physiologic range. Although partially dependent on maintenance of a physiologic external pH, cells must also have an internal mechanism(s) for regulating pH_i irrespective of external pH. First, cells generate H^+ during metabolic processes (ie. ATP hydrolysis and glycolysis) which must be removed. Secondly, the typical cell has a large interior-negative membrane potential which promotes the passive cytosolic influx of H^+ and efflux of OH^- and/or HCO_3^-. If these two processes are not reversed, they lead to toxic cytosolic acidification. To maintain a physiologic pH_i, therefore, cells must have a mechanism(s) for actively removing H^+ or accumulating HCO_3^-.[146,246]

A variety of cells respond to stimulation by increasing pH_i above resting levels. In platelets, cytosolic alkalinization occurs following stimulation with thrombin,[4,98,99,145,387,391,402,474,475] PAF,[45] phorbol esters,[287,475] synthetic diacylglycerols,[387] the calcium ionophores A23187 or ionomycin,[287,475] arachidonic acid,[145,287] TxA_2 mimetic U46619[394] and ADP.[45] As with maintenance of resting pH_i, the production of post-stimulation alkalinization requires a mechanism(s) for actively extruding H^+ and/or accumulating HCO_3^-. The degree of alkalinization

depends on the amount of H^+ generated by energy-producing metabolic activity, the buffering capacity of the cytosol and the capacity of the platelet to extrude H^+.[475]

Cellular mechanisms for regulation of resting and post-stimulation pH_i include Na^+/H^+ exchange, Na^+-independent HCO_3^-/Cl^- exchange and Na^+-dependent HCO_3^-/Cl^- exchange. Relatively little has been published in platelets on the role of the latter two exchange mechanisms. One study has demonstrated that a Na^+-independent HCO_3^-/Cl^- exchange is active in thrombin-stimulated platelets maintained in a Na^+-deficient buffer; however, when external Na^+ is present, the alkalinization due to anion exchange is minimal relative to the alkalinization resulting from Na^+/H^+ exchange.[294] A different study has shown that anion exchange is not involved in the regulation of pH_i in resting or stimulated platelets. It was suggested that anion exchange plays a role in the restoration of resting pH_i following recovery from cytosolic alkalinization.[77]

The extrusion of H^+ in platelets is primarily mediated by Na^+/H^+ exchange.[77] The exchanger does not directly require energy; instead, it is coupled to the influx of Na^+ into the cell along its concentration gradient. The inwardly directed electrochemical Na^+ gradient is maintained by a separate plasma membrane Na^+-K^+-ATPase which exchanges intracellular Na^+ for extracellular K^+ in an energy-requiring manner.[246] Inhibition of the ATPase by ouabain causes an increase in the cytosolic concentration of Na^+ and a secondary inhibition of Na^+/H^+ exchange.[237] The Na^+/H^+ exchanger has a 1:1 stoichiometry.[246] It is specific for the cations Na^+ and Li^+ and does not transport significant amounts of K^+,

Rb^+, Cs^+, choline ions or N-methyl-D-glucamine.[237,386] Amiloride and its derivatives inhibit the exchanger in a competitive manner with Na^+, suggesting that the binding of amiloride and Na^+ are mutually exclusive.[237]

The Na^+/H^+ exchanger or antiport is relatively inactive at physiologic pH_i, although the addition of amiloride to resting platelets causes a slow decrease in pH_i. This suggests a role for Na^+/H^+ exchange in the extrusion of H^+ ions which are continuously generated during basal platelet metabolic processes.[475] When platelets are stimulated, they expend a large amount of energy.[169] The energy-generating reactions release H^+ and cause rapid cytosolic acidification. In thrombin-stimulated platelets, this cytosolic acidification is concentration-dependent and independent of Na^+/H^+ exchange or Ca^{++} mobilization, suggesting that the accumulation of cytosolic H^+ is one of the earliest, measurable responses to thrombin activation.[99]

In thrombin-stimulated platelets, cytosolic acidification is followed immediately by sustained alkalinization which is primarily mediated by Na^+/H^+ exchange.[99,475] The activity of the exchanger is greatly enhanced by cytosolic acidification,[237] suggesting that H^+ has a role in the regulation of the exchanger. It has been proposed that the exchanger has two internal binding sites for H^+, one that regulates antiport activity and one that exchanges internal H^+ for external Na^+. The H^+-dependent activation of Na^+/H^+ exchange provides a mechanism for the rapid extrusion of cytosolic H^+, protecting against deleterious accumulations of H^+. The exchange has an apparent setpoint or

threshold. When the cytosolic concentration of H^+ decreases below a certain level, the rate of Na^+/H^+ exchange becomes very slow. This protects the platelet against the generation of potentially harmful intracellular alkalosis.[246,386]

The acidification of the cytosol generated during agonist-stimulation of platelets is not large enough to explain the subsequent alkalinization phase where the pH_i increases above resting pH_i. There must be additional mechanisms by which agonist-stimulation leads to activation of Na^+/H^+ exchange. Some studies indicate that activation of PKC is involved in the agonist-mediated stimulation of Na^+/H^+ exchange.[387,391,475] PKC, or one of its phosphorylated products, alters the regulatory site on the exchanger, shifting the setpoint or threshold so that it is activated by lower cytosolic concentrations of H^+. Agonist-stimulated increases in $[Ca^{++}]_i$ may also play a role in activation of Na^+/H^+ exchange. Simultaneous measurement of pH_i and $[Ca^{++}]_i$ has shown that agonist-stimulated Ca^{++} mobilization precedes measurable cytosolic alkalinization.[99,358,398,474] In addition, stimulation of platelets with calcium ionophore A23187, which causes the release of internal stores of Ca^{++}, induces a Na^+ influx consistent with activation of Na^+/H^+ exchange.[390] It has been proposed that increased $[Ca^{++}]_i$ does not directly regulate the exchanger. Instead, Ca^{++} most likely plays a role in the activation of the Ca^{++}-dependent PKC.[246,386]

Finally, the Na^+/H^+ antiport is regulated by mechanisms other than H^+, PKC and Ca^{++}. Stimulation of platelets with weak agonists, such as epinephrine or ADP, is associated with activation of Na^+/H^+ exchange

independent of PKC activation. It has been proposed that binding of weak agonists to surface receptors activates the antiport in a fibrinogen-binding-dependent manner, producing a local region of alkalinization. The increase in pH_i, in combination with a local increase in $[Ca^{++}]_i$, stimulates PLA_2 which mediates the subsequent release of arachidonic acid products. These arachidonate metabolites amplify the platelet response by activating PLC, resulting in the production of the second messengers (1,4,5)IP_3 and 1,2-DG.[20,409,411,412]

Although most studies agree that Na^+/H^+ exchange and cytosolic alkalinization accompany platelet activation, there is considerable disagreement as to whether they are required for platelet activation. Artificial induction of cytosolic alkalinization by monovalent cation ionophores or by the addition of NH_4Cl does not, by itself, induce platelet activation as indicated by measurement of Ca^{++} mobilization, PA production, aggregation or protein phosphorylation.[385,387,398] Artificial alkalinization does enhance platelet responses to agonist-induced activation as indicated by aggregation and/or measurement of Ca^{++} mobilization.[384] Treatment of platelets with the cation ionophores monensin or nigericin induces shape change in a manner dependent on the external pH, suggesting that increasing pH_i has a direct effect on cytoskeletal assembly.[231]

Numerous studies have demonstrated that activation of the Na^+/H^+ exchange and cytosolic alkalinization play a vital role in the platelet activation response. Most of these studies have investigated the effect of <u>in vitro</u> inhibition of Na^+/H^+ exchange on some physiologic response

which occurs during platelet activation. Techniques used to inhibit Na^+/H^+ exchange include: (1) removal of external Na^+; (2) addition of amiloride or one of its derivatives; (3) elevation of extracellular H^+ concentration; or (4) inhibition of Na^+-K^+-ATPase to remove the inwardly directed Na^+ gradient. The addition of amiloride to platelet samples inhibits the rate of ADP shape change and spreading.[231] Inhibition of Na^+/H^+ exchange blocks ADP- and epinephrine-induced activation of PLA_2 and PLC.[409,411,412] Inhibition of the antiport also inhibits secretion,[86,413] Ca^{++} mobilization[45,145,384,387,395] and aggregation[388,390,423] in response to a variety of agonists including PAF, ADP, thrombin, calcium ionophores, the TxA_2 mimetic U46619 and vasopressin. The effect of Na^+/H^+ exchange and subsequent cytosolic alkalinization on Ca^{++} mobilization may be two-fold. First, the (1,4,5)IP_3-mediated release of Ca^{++} from internal stores is enhanced in alkaline pH.[55] Secondly, it has been proposed that an increase in pH_i enhances Ca^{++} influx, possibly by modulating the production of inositol phosphate metabolites.[386]

In contrast, other studies have shown that Na^+/H^+ exchange and cytosolic alkalinization are not essential events for Ca^{++} mobilization, shape change or aggregation in response to thrombin[4,99,176,358,474] or ADP.[135,398] It has been proposed that the inhibition of the platelet responses noted in the studies discussed in the previous paragraph are not due to inhibition of cytosolic alkalinization but are due to secondary effects of the experimental techniques used to block Na^+/H^+ exchange.[4,135,176] These latter studies do not dispute the proposal that pH_i plays a modulatory role in platelet activation.

Although the role of cytosolic alkalinization in platelet activation is still uncertain, resting and post-stimulation pH_i were measured in affected and control platelets using the fluorescent dye BCECF (2,7-biscarboxyethyl-5(6)-carboxyfluoroscein) for a variety of reasons. First, at this point in the project it was determined that affected platelets have aggregation and secretion responses which show increased sensitivity to cyclooxygenase inhibition, suggesting increased dependance on the production of arachidonic acid metabolites.[254] As mentioned above, stimulation of Na^+/H^+ exchange plays an important role in the activation of PLA_2 and subsequent production of arachidonate metabolites, particularly in response to weak agonists. Any alteration in pH_i could have secondary effects on PLA_2 activity. Secondly, as discussed in the previous chapter, affected platelets have decreased mobilization of internal Ca^{++} in response to stimulation with a variety of agonists. Since an alkaline pH_i potentiates the $(1,4,5)IP_3$-mediated release of Ca^{++} from internal stores,[55] a decrease in pH_i could potentially inhibit the release of internal Ca^{++}. Finally, the phosphorylation studies on affected platelets identified a 64-67kDa protein which is not phosphorylated normally.[255] The role and function of this protein has not been identified. The α_2-adrenergic receptor on human platelets is a 64kDa protein[327] which may be closely linked to, if not actually part of, the Na^+/H^+ exchange mechanism.[410] If the 64-67kDa protein which is abnormally phosphorylated in affected platelets is the α_2-adrenergic receptor, then any alteration in its structure or function would have a major effect on pH_i and subsequent platelet activation.

MATERIALS AND METHODS

Experimental Subjects

The affected group consisted of five dogs with BHT. There were four female and one male dogs. The control group consisted of four normal Basset hounds. There were one male and three female dogs. All dogs were housed in kennels in the Michigan State University Veterinary Clinical Center, fed a commercial dry dog chow (Hill's Pet Products, Topeka, KS) and exercised regularly. The dogs were free from signs of clinical disease other than those associated with BHT and were not receiving any medication during the study.

Reagents

BCECF-AM was purchased from Calbiochem Biochemicals (San Diego, CA). Stock solution (1mg in 227ul anhydrous DMSO) was aliquoted and stored at -20°C in a dessicator jar. Nigericin was purchased from Sigma Chemical Company (St. Louis, MO). Stock solution (5mM) was mixed in ethanol and stored at 4°C in a dessicator jar. U46619 was purchased from Sigma. Stock solution (5.2mM) was mixed in ethanol and stored at -20°C. Fibrinogen was purchased from Helena Laboratories (Beaumont, TX) and stored in the dessicated form at 4°C. Each day before use, 10mg fibrinogen was dissolved in 0.5ml HBSS. Epinephrine was obtained from Parke-Davis (Morris Plains, NJ). Stock solution (1mg/ml in sealed 1ml ampules) was stored at room temperature. Thrombin, PAF, ADP, digitonin

and PGE_1 were purchased, mixed and stored as described in the previous chapter.

Measurement of pH_i Using BCECF

pH_i was measured by modification of the technique described by Zavoico et al.[475] PRP was isolated as described in the previous chapter (pages 119-120). PGE_1 (1uM final concentration) was added to the PRP and the sample incubated at room temperature for 5 minutes prior to centrifugation at 800 x g for 15 minutes. The supernatant was discarded and the platelet pellet resuspended in 10ml Ca^{++}-Mg^{++}-free Tyrode's buffer (137mM NaCl, 2.7mM KCl, 5.5mM glucose, 0.35mM Na_2HPO_4, 10mM HEPES, pH 7.4) to which 1M citric acid was added at a ratio of 9ul acid to 1ml buffer. BCECF-AM (1.5uM final concentration) was added from stock solution and the suspension incubated at 37°C for 30 minutes. The suspension was centrifuged for 15 minutes at 800 x g. The supernatant was discarded and the platelet pellet resuspended in 1ml of Ca^{++}-Mg^{++}-free Tyrode's buffer containing 0.02% bovine serum albumin. The platelet suspension was passed over a 10ml Sepharose 4B column equilibrated with the same buffer. The gel-filtered platelets were manually counted using a Neubauer hemocytometer and diluted to a final concentration of 7.5 x 10^7 platelets/ml using the same buffer. $MgCl_2$ (0.8mM final concentration) was added and the suspension divided into 1.75ml aliquots and placed into polystyrene spectrofluorometer cuvettes covered with parafilm. The cuvettes were maintained at room temperature for the remainder of the experimental procedures. Five minutes prior to measuring sample fluorescence, a teflon-coated stir bar was added along

with either: (1) 1mM $CaCl_2$; (2) 1mM $CaCl_2$ and 0.2mg/ml fibrinogen; or (3) 1mM EGTA. The cuvettes were placed into a Perkin Elmer MPF 66 spectrofluorometer. The samples were stirred and a resting (baseline) fluorescence recorded using an excitation wavelength of 495nm and an emission wavelength of 530nm. Agonist was added (70ul) and the change in fluorescence recorded over a nine minute period. The pH of the samples was determined by comparing measured fluorescence to calibration curves generated each day by modification of the technique described by Thomas et al.[420] A small quantity of gel-filtered platelets were resuspended in K^+-HEPES buffer (140mM KCl, 5.5mM glucose, 10mM HEPES, pH 7.4) to a final concentration of 7.5×10^7 platelets/ml. The monovalent cation ionophore nigericin (2uM final concentration) was added to allow equalization of internal and external pH. The external pH was measured using a pH probe. The sample fluorescence was measured as the external pH was varied from 6.7 to 7.7 by the stepwise addition of KOH. The suspension was lysed by the addition of 50uM digitonin and the fluorescence measured as the pH was varied from 7.7 to 6.7 by the stepwise addition of HCl. The amount of dye leakage from the cells was calculated by centrifugation of the platelet samples in Ca^{++}-Mg^{++}-free Tyrode's buffer at 11,600 x g for 60 seconds at set time periods during the experimental runs. The fluorescence of the supernatant (indicative of the portion of sample fluorescence due to external dye) was compared to the total fluorescence in lysed platelet suspension (indicative of fluorescence due to both internal and external dye). The leakage of dye was calculated as % leakage/hr. The presence of external dye was not corrected for in the calculation of pH_i.

Statistical Analysis

The resting and post-stimulation pH_i were measured in response to each agonist on two separate occasions for each dog. The post-stimulation pH_i was recorded as the maximum change in pH_i from the corresponding resting value. The values for each dog were averaged to calculate a representative value which was used in the statistical comparisons. All data was analyzed using a computer software statistical/graphics program (Statgraphics, STSC, Rockville, MD). Control and affected values were compared using the student's t test for independent samples. Changes in pH_i following the addition of agonist and the addition of buffer only were compared using paired t tests.

RESULTS

As in the previous chapter, the results will be presented according to agonist. The agonists included those used in the Ca^{++} studies (PAF, ADP, thrombin and ionomycin) as well as additional agonists (TxA_2 mimetic U46619 and epinephrine). The concentration of agonists were chosen based on aggregation studies on platelets loaded with BCECF and treated in a similar manner to that used for the pH studies. The aggregation studies showed that both control and affected BCECF-loaded platelets had a marked tendency to undergo spontaneous shape change following the initiation of stirring in the aggregometer. When two concentrations of agonist were used, the high concentration was believed to stimulate platelet activation independent of TxA_2 production while the lower concentration was believed to be partially dependent on

TxA_2 production for full activation to occur. When only one concentration of agonist was tested, it was a concentration found to give maximal aggregation responses. The concentration of agonist listed in the tables is the final concentration in solution. The buffer used to dilute the agonists was also added to stirred platelet suspensions to assess its effects on resting pH_i.

The values for post-stimulation pH_i are listed as the change in pH_i from the corresponding, stirred resting value in the same sample. The figures are representative fluorescence tracings for both control and affected platelets. In the fluorescence tracings, it is important to note that BCECF increases its fluorescence as the pH_i of the solution increases. With the exception of thrombin, post-stimulation pH_i was measured with and without the addition of exogenous fibrinogen to assess the role of external fibrinogen in the alkalinization process. The aggregation studies indicated that the addition of fibrinogen enhanced the rate and extent of the platelet aggregation response in control platelets. The pH_i response was also measured in the presence and absence of external Ca^{++} to assess the role of external Ca^{++} in the regulation of Na^+/H^+ exchange. The addition of external EGTA (1mM final concentration) prevented aggregation in both groups of platelets.

Resting pH_i

Resting pH_i was measured in both stirred and non-stirred platelet suspensions. There was no difference between affected and control platelets in the resting pH_i of stirred or non-stirred suspensions. In

control platelets, the pH_i of the non-stirred suspensions was significantly increased relative to the pH_i of the same suspensions following the initiation of stirring. This same trend was also found in affected platelets; however, the difference between non-stirred and stirred pH_i was not statistically significant. The results are summarized in Table 15.

Table 15. Resting pH_i of control and affected platelets in non-stirred and stirred BCECF-loaded platelets.

Suspension	Control (n = 4)	Affected (n = 5)
Non-Stirred	7.23 ± 0.03#	7.20 ± 0.03
Stirred	7.18 ± 0.02	7.18 ± 0.02

Values = mean pH_i ± SD; n = number of dogs tested ; # = significantly different from stirred pH_i, $p < 0.05$.

Buffer

The addition of the buffer used to dilute the agonists was added to the platelet suspensions to serve as a negative control for comparison of post-stimulation pH_i in both groups of platelets. The addition of buffer caused an immediate decrease in sample fluorescence in affected and control platelets which was interpreted from the calibration curve

as a decrease in sample pH_i. The addition of 70ul buffer to 1.75ml of platelet suspension caused a 3.8% dilution by volume. A comparison of the average resting fluorescence of samples pre- and post-addition of buffer showed a 4.0 ± 1.6% decrease in fluorescence in the affected platelets and a 2.8 ± 1.2% decrease in fluorescence in control platelets.

The appearance of the fluorescent tracings were similar in both groups of platelets and were characterized by a sharp decrease in sample fluorescence which remained constant or gradually decreased further over the 9 minute time span during which sample fluorescence was recorded. See figure 11 for representative tracings. There was no significant difference between affected and control platelets in the pH_i response following the addition of buffer. See Table 16 for a summary of results.

Table 16. The change in pH_i from resting levels in control and affected BCECF-loaded platelets following the addition of buffer.

External buffer	Control (n = 4)	Affected (n = 5)
Ca^{++}/ fibrinogen	-0.04 ± 0.02	-0.06 ± 0.02

Values = mean ± SD; n = number of dogs tested ; external buffer contained 1mM Ca^{++} and 0.2 mg/ml fibrinogen.

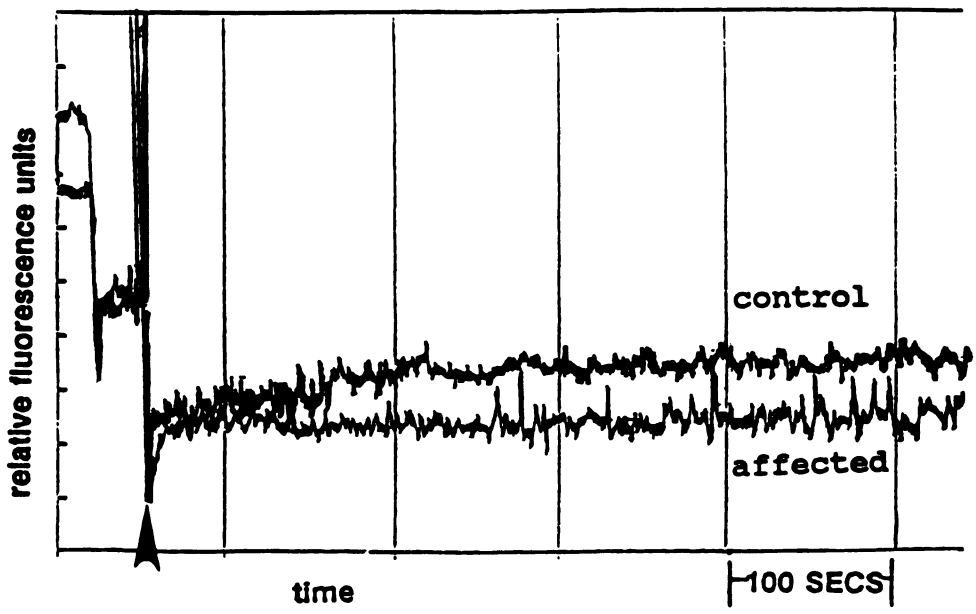


Figure 11. Representative fluorescence tracings of BCECF-loaded control and affected platelets in response to the addition of buffer in the presence of external Ca^{++} and fibrinogen. Arrow = agonist addition.

ADP

In an external buffer containing both fibrinogen and Ca^{++}, ADP stimulation caused significantly different pH_i changes in control and affected. The control platelets had an increase in pH_i following ADP stimulation. The change in pH_i was significantly different than that following the addition of buffer. The degree of alkalinization appeared to parallel the occurrence of aggregation. The average change in pH_i in those samples with grossly visible aggregation was 0.17 ± 0.06 while the average change in pH_i in those samples without visible aggregation was only 0.01 ± 0.05. The affected platelets had an immediate decrease in

sample fluorescence, interpreted as a decrease in pH_i, following agonist addition which was similar to the decrease following the addition of buffer. Though the decrease in pH_i following ADP was not as marked as that following buffer, the difference between the two was not statistically significant. Grossly visible aggregation was never noted in the affected samples. See Table 17 for a summary of the results.

Table 17. The change in pH_i relative to resting values in control and affected BCECF-loaded platelets following the addition of 10uM ADP in the presence of external Ca^{++} and fibrinogen.

Agonist	Control (n = 4)	Affected (n = 5)
10uM ADP	0.09 ± 0.06 #	-0.03 ± 0.03 *
Buffer	-0.04 ± 0.02	-0.06 ± 0.02

Values = mean ± SD; * = significantly different from control, $p < 0.05$; # = significantly different from buffer, $p < 0.05$.

The appearance of the fluorescence tracings were markedly different in affected and control dogs. In control dogs, there was an immediate decrease in fluorescence following ADP addition which was followed by a gradual increase to fluorescence levels above resting. In affected dogs, there was an immediate decrease in fluorescence following agonist addition similar to the decrease noted following the addition of buffer.

The fluorescence remained stable or increased slightly over the time period recorded. See Figure 12 for representative tracings.

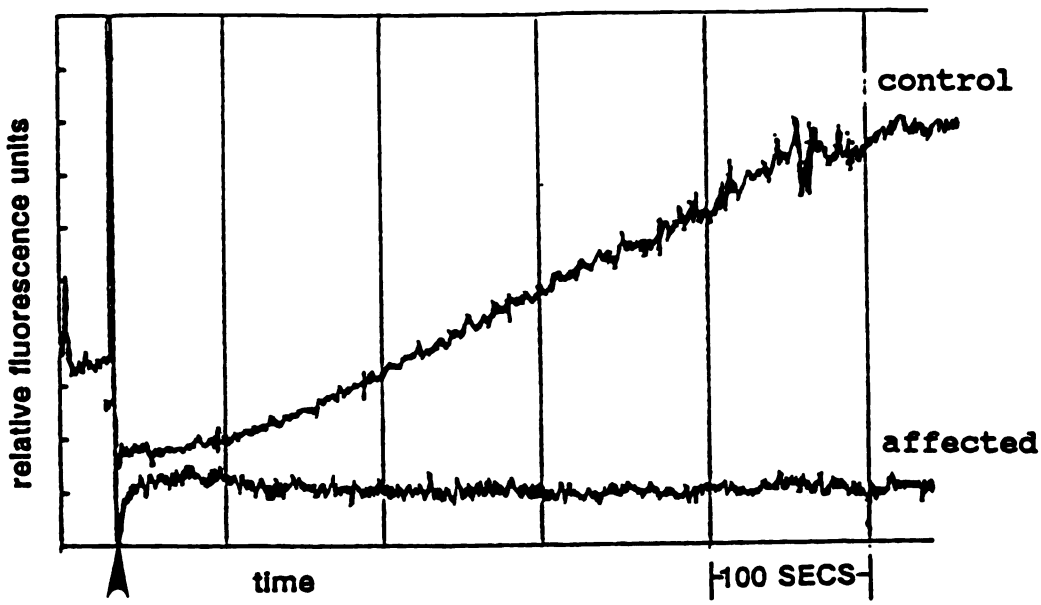


Figure 12. Representative fluorescence tracings of BCECF-loaded control and affected platelets following the addition of 10uM ADP in the presence of external Ca^{++} and fibrinogen. Arrow = agonist addition.

It has been proposed that the weak agonist epinephrine requires fibrinogen binding for measurable increases in pH_i.[20] Since ADP is also a weak agonist, it was assumed that fibrinogen binding was required to obtain measurable alkalinization following ADP stimulation. In platelet samples from two of the control dogs, the pH_i response to ADP was measured in the presence and absence of exogenously added external fibrinogen. The average change in pH_i from resting in the presence of external fibrinogen was 0.08 while the average change in pH_i in the

absence of external fibrinogen was -0.04. The latter value was not significantly different from the response following the addition of buffer alone. Therefore, the pH_i response to ADP was not measured in any of the other dogs in platelet suspensions either lacking fibrinogen or containing EGTA which inhibits fibrinogen binding and aggregation.

Platelet-Activating Factor (PAF)

The pH_i responses to a high concentration of PAF (1uM) were measured in platelet suspensions in the presence of external Ca^{++}, with and without added fibrinogen, and in the presence of external EGTA. When external Ca^{++} was present, the control platelets had much greater elevations in post-stimulation pH_i than affected platelets. The increase in pH_i was significantly larger in the presence of external fibrinogen, where all of the samples had grossly visible aggregation, than in the absence of added external fibrinogen, where only 1 out of 8 samples had grossly visible aggregation. The affected platelets had significantly less alkalinization in response to PAF stimulation than control platelets. The affected platelets had similar changes in pH_i irrespective of the presence or absence of fibrinogen. The pH_i changes in all groups were significantly different than those obtained following the addition of buffer only. Grossly visible aggregation was not noted in any of the affected samples. When external Ca^{++} was removed by the addition of EGTA, there was no significant difference in pH_i changes between control and affected platelets. In both groups, the post-stimulation alkalinization in the absence of external Ca^{++} was

significantly larger than the pH_i measured following the addition of buffer only.

The change in pH_i in response to low concentrations of PAF (0.01uM) was measured in the presence of external Ca^{++} and fibrinogen. The affected platelets had significantly decreased post-stimulation alkalinization when compared to control platelets. The pH_i changes in both groups of platelets were similar to those obtained following stimulation with high concentrations of PAF. Grossly visible aggregation was not noted in any of the platelet samples. See Table 18 of a summary of results.

Table 18. The change in pH_i relative to resting levels in control and affected BCECF-loaded platelets following stimulation with PAF.

Agonist	Conc	External Buffer	Control (n = 4)	Affected (n = 5)
Buffer		Ca^{++}/fibrinogen	-0.04 ± 0.2	-0.06 ± 0.02
PAF	1uM	Ca^{++}/ fibrinogen	0.25 ± 0.04 #@	0.12 ± 0.03 *#
		Ca^{++}	0.20 ± 0.02 #	0.12 ± 0.03 *#
		EGTA	0.05 ± 0.02 #	0.04 ± 0.02 #
PAF	0.01uM	Ca^{++}/ fibrinogen	0.23 ± 0.04 #	0.10 ± 0.04 *#

Values = mean ± SD; n = number of dogs tested; * = significantly different from control, $p < 0.05$; # = significantly different from buffer, $p < 0.05$; @ = significantly different from non-fibrinogen-containing buffer, $p < 0.05$.

The appearance of the fluorescence tracings were similar in control and affected. In Ca^{++}-containing buffer, there was an immediate decrease in fluorescence after the addition of PAF which was followed by a sharp increase to fluorescence levels greater than resting. In control platelets, there was a tendency for the fluorescence to gradually increase over the measured time period. In affected platelets, this fluorescence remained relatively constant through the measured time period. See figure 13 for representative tracings. In EGTA-containing buffer, both control and affected platelets had an immediate decrease in sample fluorescence after the addition of PAF which was followed by an increase in fluorescence to levels greater than resting. This level of sample fluorescence stayed constant or gradually decreased over the measured time period.

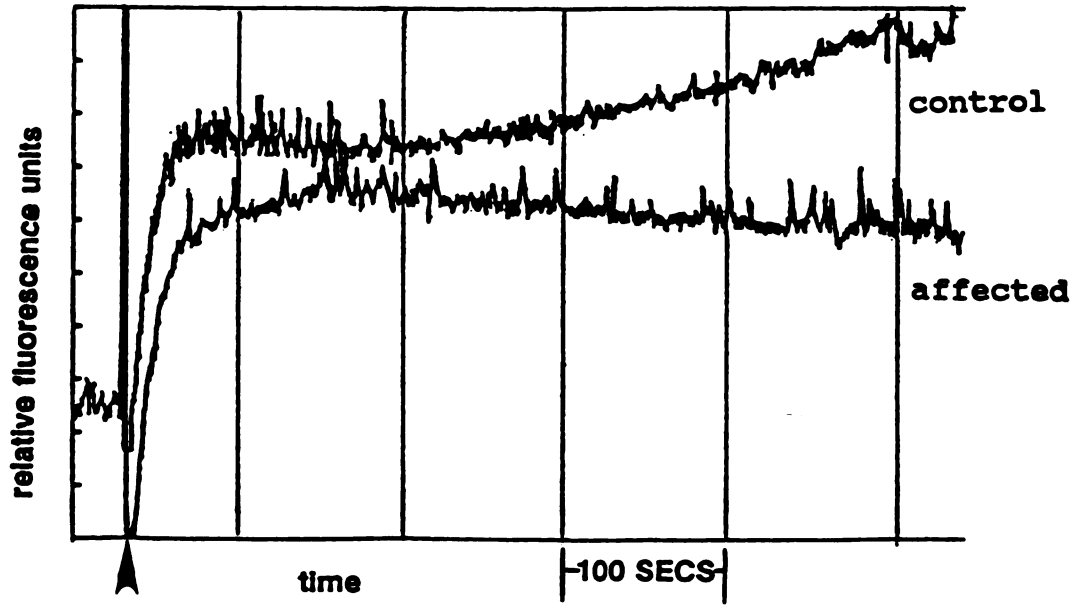


Figure 13. Representative fluorescence tracings of BCECF-loaded control and affected platelets in response to 1uM PAF in the presence of external Ca^{++}. Arrow = agonist addition.

Thrombin

The change in pH_i in response to high concentrations of thrombin (0.6U/ml) was measured in the presence and absence of external Ca^{++} while the change in pH_i in response to lower concentrations of thrombin (0.1U/ml) was measured only in the presence of external Ca^{++}. Although there was a trend for the agonist-induced alkalinization to be greater in control platelets, there was no significant difference between affected and control platelets. Interestingly, the alkalinization response following stimulation with the lower concentration of thrombin was greater than that found following stimulation with the high concentration of thrombin. This difference was similar in both groups of dogs. In all cases, the change in pH_i was significantly different than the change in pH_i following the addition of buffer alone. See Table 19 for a summary of the results.

The appearance of the fluorescence tracings was similar in both groups of platelets. In Ca^{++}-containing buffer, the addition of agonist was accompanied by an immediate decrease in fluorescence which was followed by an increase in sample fluorescence above resting levels. See Figure 14 for representative fluorescence tracings. In EGTA-containing buffer, the addition of agonist was accompanied by an immediate decrease in fluorescence which was followed by an increase in fluorescence to levels similar to resting values.

Table 19. The change in pH_i relative to resting levels in control and affected BCECF-loaded platelets following stimulation with thrombin.

Agonist	Conc.	External Buffer	Control (n = 4)	Affected (n = 5)
Buffer		Ca^{++}/fibrinogen	-0.04 ± 0.02	-0.06 ± 0.02
Thrombin	0.6U/ml	Ca^{++}	0.07 ± 0.03 #	0.04 ± 0.02 #
		EGTA	0.02 ± 0.02 #	0.01 ± 0.03 #
Thrombin	0.1U/ml	Ca^{++}	0.20 ± 0.03 #	0.16 ± 0.02 #

Value = mean ± SD; n = number of dogs tested ; # = significantly different from addition of buffer only, $p < 0.05$.

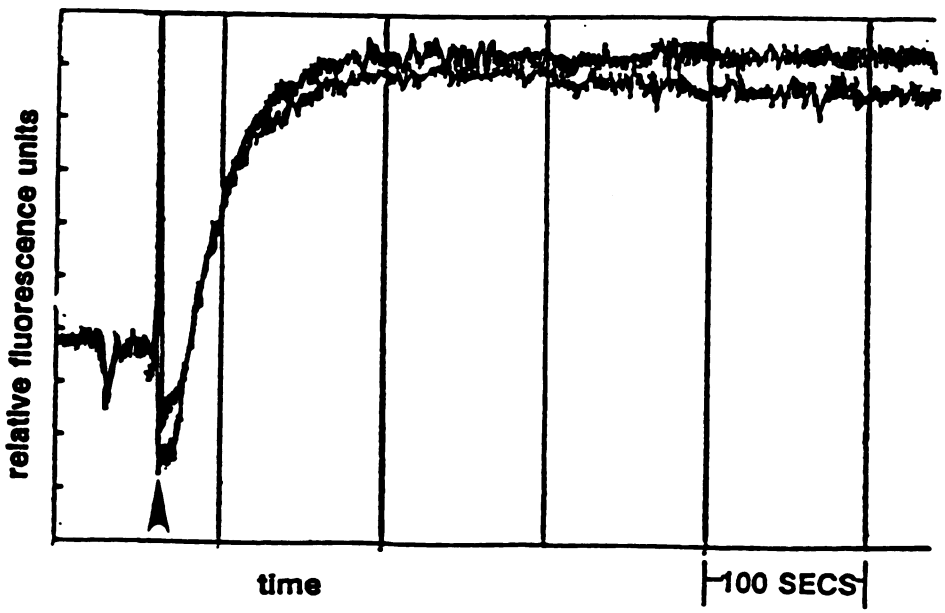


Figure 14. Representative fluorescence tracings of BCECF-loaded control and affected platelets in response to 0.1U/ml thrombin in the presence of external Ca^{++}. Arrow = agonist addition.

Ionomycin

The pH_i changes in response to ionomycin were measured in Ca^{++}-containing buffers in the presence and absence of fibrinogen and in EGTA-containing buffer. In the presence of external Ca^{++}, the agonist-induced alkalinization was significantly lower in affected platelets when compared to control platelets. Within each group, there was no significant difference between the pH_i changes obtained in the presence or absence of fibrinogen. All of the control dog platelet samples had visible aggregation in the presence of exogenous fibrinogen and no visible aggregation in the absence of fibrinogen. Visible aggregation was not noted in any of the affected samples. In the absence of external Ca^{++}, there was no significant difference between control and affected platelets in the pH_i response to ionomycin. There was no statistical difference between the pH_i change in response to ionomycin in the presence of EGTA and the pH_i change in response to the addition of buffer, indicating that there was no significant pH_i response to ionomycin stimulation in the absence of external Ca^{++}. See Table 20 for a summary of the results.

The appearance of the fluorescence curves in the Ca^{++}-containing buffers differed slightly between control and affected platelets. In both groups, the addition of ionomycin was accompanied by an immediate decrease in fluorescence followed by a relative slow increase in fluorescence to levels greater than resting. In affected platelets, the fluorescence tended to level off and maintain a constant intensity while in control platelets, the fluorescence tended to increase over the

entire time period measured. See Figure 15 for representative tracings. The appearance of the fluorescence curves in EGTA-containing buffers was similar in both groups of platelets and was characterized by a sharp decrease in fluorescence which remained constant or slightly increased.

Table 20. The change in pH_i relative to resting levels in control and affected BCECF-loaded platelets stimulated with luM ionomycin.

Agonist	External Buffer	Control (n = 4)	Affected (n = 5)
Buffer	Ca^{++}/ fibrinogen	-0.04 ± 0.02	-0.06 ± 0.02
Ionomycin	Ca^{++}/ fibrinogen	0.40 ± 0.10 #	0.17 ± 0.11 *#
	Ca^{++}	0.38 ± 0.06 #	0.16 ± 0.14 *#
	EGTA	-0.03 ± 0.02	-0.03 ± 0.03

Values = mean ± SD; n = number of dogs tested; * = significantly different from control, $p < 0.05$; # = significantly different from addition of buffer, $p < 0.05$.

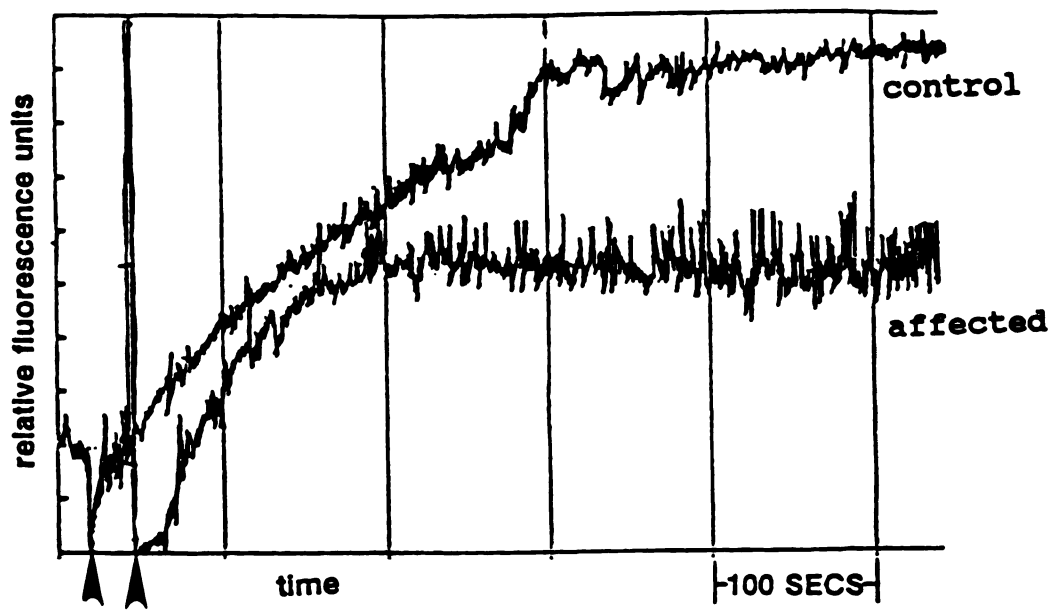


Figure 15. Representative fluorescence tracings of BCECF-loaded control and affected BCECF-loaded platelets in response to 1uM ionomycin in the presence of external Ca^{++}. Arrow = agonist addition.

U46619/Epinephrine

The pH_i changes in response to 1uM U46619 were measured in Ca^{++}/fibrinogen-containing buffers in the presence and absence of 1uM epinephrine. Without the addition of subthreshold concentrations of epinephrine, there was no significant difference between the two groups of platelets in the pH_i response following the addition of U46619. The response was similar to that recorded when buffer alone was added, suggesting no significant effect of U46619, by itself, on pH_i. In the presence of external epinephrine and fibrinogen, there was a significantly decreased alkalinization response to U46619 in the

affected platelets relative to the alkalinization response in control platelets. When compared to pH_i changes following the addition of buffer only, both control and affected platelets had a significant alkalinization response. In the control platelets, it is important to note that when gross aggregation was visible, the mean pH_i change was 0.21 ± 0.07. When gross aggregation was not visible, the mean pH_i change was only 0.08 ± 0.04. Gross aggregation was never noted in affected platelets. In the presence of external epinephrine without added fibrinogen, there was no significant difference in the pH_i response in the affected platelets when compared to the control platelets. The response in affected platelets was similar irrespective of the presence or absence of external fibrinogen. In the control platelets, there was a tendency, though not statistically significant, for increased alkalinization in the presence of external fibrinogen relative to the absence of fibrinogen. In the presence of EGTA, there was no significant difference between control and affected platelets in the pH_i response. The response in both groups was not significantly different than the response following the addition of buffer only.

The pH_i response to low concentrations of U46619 (0.05uM) was measured in the presence of external fibrinogen and epinephrine. There was a significantly decreased alkalinization response in the affected platelets when compared to the control platelets. In the control platelets, the post-stimulation pH_i was significantly more alkaline than the pH_i following the addition of buffer. In the affected platelets, the post-stimulation pH_i was actually lower than the resting pH_i;

however, it was more alkaline than the pH_i following the addition of buffer alone. See Table 21 for a summary of the results.

Table 21. The pH_i change relative to resting levels in control and affected BCECF-loaded platelets following stimulation with U46619 and epinephrine.

Agonist (Conc.)	External Buffer	Control (n = 4)	Affected (n = 5)
Buffer	Ca^{++}/fibrinogen	-0.04 ± 0.02	-0.06 ± 0.02
U46619 (1uM)	Ca^{++}/fibrinogen/epi	0.13 ± 0.08 #	-0.01 ± 0.03 *#
	Ca^{++}/epi	0.01 ± 0.02	-0.01 ± 0.02
	Ca^{++}/fibrinogen	-0.06 ± 0.03	-0.07 ± 0.03
	EGTA	-0.05 ± 0.02	-0.07 ± 0.03
U46619 0.5uM)	Ca^{++}/fibrinogen/epi	0.07 ± 0.04 #	-0.02 ± 0.02
10.5uM Epi	Ca^{++}/fibrinogen	-0.05 ± 0.01	-0.05 ± 0.02

Values = mean ± SD; n = number of dogs tested; * = significantly different from control, $p < 0.05$; # = significantly different from addition of buffer, $p < 0.05$; concentration of epinephrine in external buffer = 1uM.

The appearance of the fluorescence tracing were different in control and affected platelets. In buffer containing both Ca^{++} and epinephrine, the control platelets showed a decrease in fluorescence followed by a gradual increase to levels similar to or greater than resting values. The increase in control platelets was greatly potentiated by the addition of exogenous fibrinogen. In a similar

buffer, the affected platelets had a rapid decrease in sample fluorescence followed by a gradual increase toward the resting levels. See Figure 16 for representative tracings. In EGTA-containing buffer or in Ca^{++}-containing buffer without external epinephrine, there was no difference in the appearance of the fluorescence curves between affected and control platelets. In all cases, the curves were characterized by an immediate decrease in fluorescence which remained relatively constant or slowly increased toward resting values.

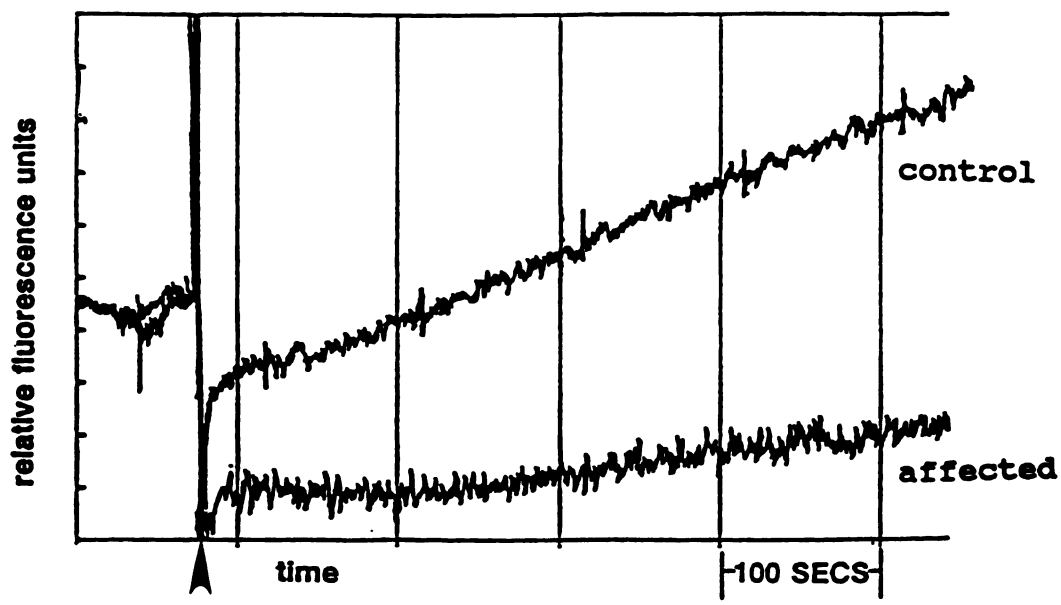


Figure 16. Representative fluorescence tracings of BCECF-loaded control and affected platelets in response to 1uM U46619/1uM epinephrine in the presence of external Ca^{++} and fibrinogen. Arrow = agonist addition.

In the presence of external Ca^{++} and fibrinogen, there was no significant difference between control and affected platelets in the pH_i

response to 10.5uM epinephrine. In both cases, there was an immediate decrease in fluorescence which was similar to that found following the addition of buffer alone, indicating no specific effect of epinephrine on pH_i. See Table 21 for a summary of the results.

Dye Leakage Rates

There was no significant difference between affected and control platelets in the rate of leakage of BCECF from the cytosol. In the affected platelets, the leakage rate was 9.7 ± 0.9% /hr. while in the control platelets the rate was 8.7 ± 0.9% /hr..

DISCUSSION

Stimulation of platelets is usually accompanied by cytosolic alkalinization which is primarily mediated by a Na^+/H^+ exchange mechanism.[386,475] The importance of the role of cytosolic alkalinization in platelet activation is not clear. Early studies were hampered by a lack of readily available techniques to measure pH_i, since platelets, as well as other blood cells, are too small for microinjection of indicators or impalement by pH-sensitive microelectrodes.[336] The pH_i is conveniently measured using fluorescent probes and spectroscopic techniques. One of the first fluorescent probes developed to measure pH_i was 6-carboxyfluorescein. 6-Carboxyfluorescein is taken up by the cell and subsequently hydrolyzed in the cytoplasm to a less permeable fluorescent form. Unfortunately, this dye has a low sensitivity at pH > 7.2 and suffers from severe

leakage problems.[98,336] This lead to the development of a newer fluorescent derivative, 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF). BCECF is available in a non-fluorescent ester form which is lipophilic and readily penetrates across the platelet membrane. Once in the cytosol, the ester group is removed by non-specific esterases to yield a relatively impermeable fluorescent probe. Relative to 6-carboxyfluorescein, BCECF has the advantages of pH sensitivity to 7.8 or 7.9 and decreased permeability.[336] The intensity of the fluorescence of BCECF-loaded platelets is directly related to the pH_i, with the intensity of fluorescence increasing as the pH_i becomes more alkaline.[146] BCECF is diffusely distributed throughout the cytosol and, therefore, measures average pH_i.

Using BCECF as the pH_i indicator, resting pH_i was measured in control and affected platelets. When the platelet suspensions were not stirred, the pH_i of the affected platelets tended to be more acidic than the pH_i of the control platelets. Following initiation of stirring, the pH_i of the control platelets became significantly more acidic while the pH_i of the affected platelets stayed relatively the same. There was no significant difference in the stirred, resting pH_i between the two groups.

Initial activation of platelets is accompanied by a transient acidification stage.[99] It has been proposed that acidification results from the generation of protons due to increased metabolic activity and the production of energy.[398,475] Others have proposed that the acidification results from platelet shape change which changes the light

transmitting properties of the platelet suspension, thereby decreasing sample fluorescence.[393] The data on resting pH_i suggest that the affected platelets were already in a state of activation before stirring and were not affected by stirring while the control platelets were activated in some manner by stirring. Previous studies in the laboratory have indicated that affected platelets readily undergo partial activation with handling. The aggregation studies performed on BCECF-loaded control platelets showed the platelets were susceptible to spontaneous shape change upon the initiation of stirring in the aggregometer. It is likely that the same effect occurred upon initiation of stirring in the spectrofluorometer.

The buffer used to dilute agonists was added to stirred platelet suspensions to determine what effect, if any, it had on resting pH_i. Unexpectedly, the addition of buffer caused an immediate and sustained decrease in measured pH_i which was of similar magnitude in both affected and control platelets. This decrease may be partially due to a dilutional effect. Comparison of the percent dilution of the platelet suspension to the percent decrease in the sample fluorescence following the addition of buffer showed similar values in both groups of platelets. Since the spectrofluorometer measures the fluorescence produced by the excitation of the sample by a discrete beam of light, decreasing the concentration of dye-loaded platelets within the beam concomitantly decreases the emission fluorescence measured. Another possible explanation for the decrease in pH_i is that the buffer itself was causing some degree of platelet activation; however, this is unlikely since the buffer used was a physiologic solution. Regardless

of the cause of the decrease in measured pH_i, the magnitude of the change was similar in both control and affected platelets. Therefore, the most accurate assessment of agonist-induced alterations in pH_i was to compare the change in pH_i from the corresponding stirred, resting value following the addition of agonist to the change in pH_i obtained following the addition of buffer without added agonist.

ADP initiates platelet activation by mobilizing a small pool of arachidonic acid in a manner dependent on Na^+/H^+ exchange, the production of a localized zone of alkalinization and PLA_2 activation. The mechanism by which ADP activates the exchanger is not known.[409,411,412] Inhibition of Na^+/H^+ exchange prevents PLA_2 activation following ADP stimulation while artificial alkalinization of the platelet cytosol reverses the inhibitory effects of the prevention of Na^+/H^+ exchange.[411] Shape change and primary aggregation are not prevented by blockage of Na^+/H^+ exchange, suggesting that these responses are mediated directly by ADP binding and not by the production of cytosolic alkalinization.[412] Studies have shown that ADP-induced irreversible aggregation[388] and Ca^{++} mobilization[394] are fully or partially inhibited by experimental manipulations that block Na^+/H^+ exchange and cytosolic alkalinization. In converse, other studies have demonstrated that cytosolic alkalinization is not essential for ADP-induced aggregation[135] and Ca^{++} mobilization.[398] These studies do not dispute that Na^+/H^+ exchange is important in the release of arachidonic acid and the secondary enhancement of platelet activation in response to ADP; however, it has been suggested that the concentration of amiloride

used in the previous studies was higher than necessary to block Na^+/H^+ exchange and was having secondary effects on activation.[135]

There were marked differences between control and affected platelets in the pH_i response to addition of ADP. In the control platelets hand, addition of ADP caused immediate acidification which was followed by alkalinization to post-stimulation pH_i levels higher than resting. This indicates that ADP stimulation of control platelets causes a significant pH_i response. In contrast, the affected platelets had an immediate and sustained decrease in sample fluorescence which was not significantly different from the response following the addition of buffer alone. This indicates that ADP stimulation of affected platelets is not associated with any significant acidification or alkalinization of the cytosol.

Inhibition of fibrinogen binding in BCECF-loaded platelets blocks alkalinization following stimulation with the weak agonist epinephrine, indicating a requirement for fibrinogen binding in the production of measurable pH_i changes.[20,410] The lack of measurable alkalinization in control platelets stimulated with ADP in the absence of exogenously added fibrinogen suggests that fibrinogen binding is also essential for a pH_i response to ADP. The data presented in the results section further indicate that the production of measurable post-stimulation changes in pH_i depends not only on fibrinogen binding, but also on the close platelet-platelet interactions that occur during aggregation or on some other post-fibrinogen binding event. This conclusion is based on the fact that, in control platelets, the degree of post-stimulation

alkalinization was significantly greater in those cases where aggregation was grossly visible when compared to those cases where aggregation was not visible. Since the gross identification of aggregation is a very crude technique, the lack of visible aggregates does not preclude the presence of microaggregates which could account for the small degree of alkalinization measured. In spite of normal ADP-induced fibrinogen binding,[199,298] affected platelets do not support primary or secondary aggregation,[254] therefore the pH_i measured in the current study was not affected by platelet aggregation.

Comparison of the alkalinization response to ADP stimulation and some of the other measurable responses in BHT affected platelets suggests that there must be a primary defect in some post-fibrinogen binding event(s) other than Na^+/H^+ exchange. Affected platelets produce significantly greater quantities of TxB_2 in response to ADP stimulation than control platelets.[256] It is impossible to make direct correlations between the pH_i data and the TxB_2 data since the pH_i measurements used different experimental conditions (ie. different concentrations of external Ca^{++}). Based on the proposed mechanism of ADP activation presented above, comparison of the pH_i and the TxB_2 results suggest that the addition of ADP to affected platelets does cause activation of Na^+/H^+ exchange and the production of a small, localized zone of alkalinization that BCECF is not able to detect. Aggregation or some other post-fibrinogen binding event may be necessary to produce measurable alkalinization. Alternatively, it is possible that the PLA_2 present in affected platelets is independent of regulation by pH_i. In addition, primary aggregation induced by ADP is not affected by

inhibition of Na^+/H^+ exchange, suggesting that the binding of ADP to surface receptors, the exposure of the fibrinogen binding site and subsequent fibrinogen binding of adjacent platelets is not inhibited by pH_i.[412] Primary aggregation, which is absent in affected platelets,[254] should not be affected by pH_i changes. Finally, in spite of no measurable pH_i response to ADP, affected platelets mobilize Ca^{++} as well as control platelets under similar experimental conditions as discussed in the previous chapter.

PAF causes receptor-mediated activation of PLC[106,221,375] and subsequent stimulation of Na^+/H^+ exchange by at least two separate routes. One mechanism is mediated by PKC[45,386] while the second route is independent of PKC.[45] Blockage of Na^+/H^+ exchange inhibits the release of arachidonic acid metabolites following PAF stimulation, suggesting that Na^+/H^+ exchange is involved in the regulation of arachidonic acid release and/or metabolism.[200,410,413] Inhibition of Na^+/H^+ exchange or artificial acidification of the platelet cytosol also partially inhibits PAF-induced dense granule secretion,[410,413] aggregation[45,388] and Ca^{++} mobilization,[45] suggesting a significant role for the exchanger in platelet activation. Removal of external Na^+ inhibits TxB_2 production and serotonin dense granule secretion following stimulation with PAF in buffers containing low (ie. non-physiologic) concentrations of Ca^{++} but not in buffers containing physiologic (2mM) concentrations of Ca^{++}.[200] The removal of extracellular Na^+ does not affect Ca^{++} mobilization in platelets treated with cyclooxygenase inhibitors,[349] indicating that the inhibition of Ca^{++} mobilization by

blockade of Na^+/H^+ exchange reported above is due to an effect on arachidonic acid release and not a direct effect on Ca^{++} fluxes.

Unlike ADP, the addition of PAF to control and affected platelets was associated with significantly increased post-stimulation pH_i in the presence and absence of external Ca^{++}. In the presence of external Ca^{++}, the degree of cytosolic alkalinization was significantly greater in the control platelets than in the affected platelets. Within the control group, but not the affected group, the degree of alkalinization was significantly greater in those samples containing fibrinogen, where aggregation was grossly visible, than in the samples without fibrinogen, where aggregation was not always visible. This suggests that the production of measurable pH_i is partially dependent on aggregation. Unlike ADP, PAF also causes activation of the exchanger independent of aggregation as indicated by the post-stimulation cytosolic alkalinization in the affected platelets in spite of an inability to undergo either primary or secondary aggregation in response to PAF.[254] When external Ca^{++} was removed by EGTA, the alkalinization response was significantly decreased when compared to the response in the presence of external Ca^{++}, but there was no significant difference between control and affected platelets in the post-stimulation pH_i. These findings demonstrate that PAF initiates Na^+/H^+ exchange irrespective of external Ca^{++}, fibrinogen binding and aggregation. Affected and control platelets show similar phosphorylation of the 47kDa protein in the presence of external EDTA, indicating similar activation of PKC.[255] Therefore, it is likely that PKC mediates the stimulation of Na^+/H^+ exchange when external Ca^{++} is removed.

As discussed for the ADP data, the difference between control and affected platelets in the pH_i responses to PAF cannot explain the platelet abnormalities previously identified in BHT. First, it has been shown that inhibition of Na^+/H^+ exchange does not inhibit primary platelet aggregation in response to PAF;[413] yet, affected platelets do not aggregate in response to PAF stimulation.[254] In spite of an inability to aggregate, PAF-induced cytosolic alkalinization of affected platelets occurs, though the post-stimulation pH_i was decreased when compared to control platelets. Secondly, one of the primary roles proposed for Na^+/H^+ exchange is regulation of arachidonic acid mobilization.[413] Yet, the alkalinization response was much greater in control platelets in response to high concentrations of PAF while TxB_2 production was significantly less than that of control platelets. In addition, PAF stimulation of affected platelets causes an increased production of TxB_2 in response to high concentrations of PAF but decreased production of TxB_2 in response to low concentrations of PAF when compared to control platelets.[256] In contrast, the degree of post-stimulation cytosolic alkalinization was similar following stimulation with both high and low concentrations of PAF in affected platelets. Unfortunately, it is impossible to directly correlate these results since the measurement of TxA_2 production was performed in plasma containing 0.32% trisodium citrate (a low Ca^{++} concentration medium) while the pH_i changes were assessed in a buffer containing higher concentrations of Ca^{++} (1mM). In platelet suspensions maintained in the presence of physiologic concentrations of external Ca^{++} (2mM), PAF-induced production of arachidonate metabolites (TxA_2) is significantly inhibited when compared to the same measurement in platelets maintained

in suspensions containing low concentrations of external Ca^{++}. The mechanism for this Ca^{++}-dependent inhibition of TxA_2 production is not known.[200] Finally, it was proposed that decreased post-stimulation pH_i in affected platelets could partially explain the decrease release of intracellular Ca^{++} as discussed in the previous chapter; however, this was not supported by the current study. In the presence of external Ca^{++}, there was no significant decrease in Ca^{++} mobilization in affected platelets relative to control platelets. It was only in the presence of external Ca^{++} that there was a difference in post-stimulation cytosolic alkalinization between affected and control platelets. The affected platelets had significantly less internal Ca^{++} released in the presence of external EGTA; yet, there was no significant difference between control and affected platelets in the pH_i response to PAF in buffers containing EGTA.

Similar to PAF, thrombin causes the activation of PLC, hydrolysis of membrane phosphoinositides and the production of the second messengers (1,4,5)IP_3 and 1,2-DG.[21,34,90,262,346] 1,2-DG activates PKC which stimulates Na^+/H^+ exchange, causing cytosolic alkalinization.[386,391] Thrombin also stimulates Na^+/H^+ exchange by some undefined mechanism that is independent of PKC activation.[475] The role of Na^+/H^+ exchange and subsequent cytosolic alkalinization in thrombin-stimulated platelet activation is under intense debate. Na^+/H^+ exchange is most important in the platelet responses to low concentrations of thrombin and one study concluded that thrombin concentrations of >0.2U/ml were relatively independent of pH_i effects.[381] Inhibition of Na^+/H^+ exchange by amiloride attenuates platelet aggregation in response

to thrombin[388] while inhibition of Na^+/H^+ exchange by removal of external Na^+ decreases aggregation and secretion in response to thrombin.[86] Inhibition of Na^+/H^+ exchange blocks the release of arachidonic acid following stimulation with low concentrations of thrombin.[412] Blockade of Na^+/H^+ exchange by the removal of external Na^+ or by use of amiloride derivatives also partially blocks the influx of external Ca^{++} and the release of Ca^{++} from internal stores.[145,211,384,387] This inhibitory action on Ca^{++} mobilization is bypassed by artificial alkalinization of the platelet cytosol.[395] These effects on thrombin stimulation are not mediated by inhibition of PLC since manipulations which alter Na^+/H^+ exchange have no effect on thrombin-induced hydrolysis of membrane phosphoinositides.[395,409]

Other studies have demonstrated that Ca^{++} mobilization and platelet activation in response to thrombin occur independent of cytosolic alkalinization or activation of Na^+/H^+ exchange. These studies have shown that: (1) the increase in $[Ca^{++}]_i$ precedes cytosolic alkalinization;[99,398] (2) artificial acidification of the platelet cytosol and inactivation of Na^+/H^+ exchange have minimal effects on the Ca^{++} mobilization in response to high or low concentrations of thrombin;[474] and (3) removal of external Na^+ does not affect Ca^{++} mobilization in aspirin-treated platelets in response to thrombin stimulation.[349] A recent study has demonstrated that removal of external Na^+ enhances thrombin-induced serotonin secretion due to inhibition of cytosolic alkalinization and other undefined effects of Na^+ itself.[211] Inhibition of PKC activity blocks thrombin-induced cytoplasmic alkalinization without affecting Ca^{++} mobilization,[358]

suggesting that Ca^{++} mobilization occurs independently of pH_i effects. It has been proposed that amiloride derivatives inhibit thrombin-induced platelet aggregation and Ca^{++} mobilization at levels 100-fold greater than those required to inhibit Na^+/H^+ exchange,[176] indicating that the inhibitory effects demonstrated in the previous studies are secondary to other effects of amiloride and not to inhibition of Na^+/H^+ exchange.

Thrombin was the only agonist tested which showed a similar degree of cytosolic alkalinization in control and affected platelets in both the presence and absence of external Ca^{++}. Thrombin was also the only agonist tested which causes aggregation of platelets from both groups of dogs.[254] In spite of similar pH_i responses, previous studies have demonstrated that affected platelets produce significantly increased quantities of TxB_2.[256] This suggests that, though Na^+/H^+ exchange and pH_i may modulate PLA_2 activity,[27,412] they are not the only factors regulating its activity.

The smaller increases in pH_i measured in the presence of external EGTA in both control and affected platelets indicate a requirement for external Ca^{++}, fibrinogen binding and aggregation for full activation of Na^+/H^+ exchange following stimulation with thrombin. In the previous chapter, it was shown that thrombin-stimulation of affected platelets in EGTA-containing buffers is associated with decreased release of internal stores of Ca^{++} relative to control platelets. Since there was no difference in cytosolic alkalinization in response to thrombin, alterations in pH_i responses cannot explain the defective Ca^{++} mobilization. It should be noted that 0.25U/ml thrombin was used in the

Ca^{++} studies while 0.6U/ml was used in the pH_i studies. Both of these are considered concentrations at which the effects of thrombin have been shown to be independent of pH_i.[387]

Ionomycin is a calcium ionophore that activates platelets by increasing membrane permeability to Ca^{++} [121,313,377,457] and releasing internal stores of Ca^{++} in a non-receptor-mediated manner.[11,313,377] Relatively little has been published regarding the effects of calcium ionophores on Na^+/H^+ exchange and cytosolic alkalinization. Blockade of Na^+/H^+ exchange by amiloride derivatives inhibits aggregation in response to low concentrations of A23187[390] while platelets activated with calcium ionophore A23187 extrude H^+ in a manner compatible with Na^+/H^+ exchange.[391] Whereas perturbation of Na^+/H^+ exchange inhibits PLA_2 activation in response to ADP and epinephrine, it does not have any significant effect on PLA_2 activation by A23187.[411] Studies using fluorescent dyes to measure pH_i have demonstrated that the addition of ionomycin causes an initial acidification of the platelet suspension followed by minimal to no cytosolic alkalinization.[358,398,475]

Contrary to previously published results, the current pH_i studies on control and affected platelets have demonstrated significant alkalinization in response to ionomycin. This difference may be due to a variety of reasons. First, the concentration of ionomycin used in the current study was approximately 10-fold higher than that used in the published studies. The concentration of agonist chosen was based on aggregation studies in BCECF-loaded control canine platelets showing that 1uM ionomycin was required to cause strong aggregation responses

while 0.1uM ionomycin (the concentration used in the published studies) did not reliably induce aggregation. It is important to note that there was a narrow range between ionomycin concentrations that cause no aggregation, aggregation and visible cell lysis. Since concurrent enzyme leakage studies were not performed, it cannot be ruled out that some of the alkalinization measured in the platelet samples represents the leakage of dye from injured or damaged platelets. Since the external pH was more alkaline than the pH_i, the fluorescence of external dye would artificially elevate the measured signal. If this were the case, it would be anticipated that levels would be similar in both control and affected platelets. Another explanation for the difference between the current study and the published results was that the published studies used cyclooxygenase inhibitors to eliminate the amplification effects of TxA_2 production while the current study did not.

In a Ca^{++}-containing buffer, the addition of ionomycin caused significantly less cytosolic alkalinization in affected platelets when compared to control platelets. The absence or presence of exogenously added external fibrinogen had minimal effect on the post-stimulation pH_i in either group. As with the other agonists tested, visible aggregation occurred in response to ionomycin in control platelets while affected platelets have been previously demonstrated to not aggregate in response to the calcium ionophore A23187.[254] Therefore, part, if not all, of the decrease in cytosolic alkalinization in affected platelets may be due to the absence of aggregation or some other post-fibrinogen binding event.

Although there were significant differences between affected and control platelets in the degree of cytosolic alkalinization in response to ionomycin stimulation, the results do not correlate with the other abnormalities detected in BHT. Previous studies have demonstrated that affected platelets release significantly less TxB_2 in response to calcium ionophore A23187 than control platelets.[256] Decreased post-stimulation pH_i could, theoretically, be associated with decreased PLA_2 activity and decreased release of arachidonic acid since PLA_2 activity is enhanced in alkaline pH;[27] however, studies have shown that inhibition of Na^+/H^+ exchange has no effect on calcium ionophore A23187-induced activation of PLA_2.[411] In addition, in the presence of external Ca^{++}, the level of ionomycin-induced cytosolic alkalinization in affected platelets was as large as the alkalinization following stimulation with PAF or thrombin which cause increased production of TxB_2 in affected platelets.[256] This indicates that alkalinization alone does not mediate the release of arachidonate metabolites.

The lack of post-stimulation pH_i response in the presence of external EGTA in both affected and control platelets was not expected and indicates that measurable alterations in pH_i following stimulation with ionomycin are dependent on the presence of external Ca^{++}. External Ca^{++} could be required for: (1) fibrinogen binding and aggregation; or (2) potentiation of the Ca^{++} flux into the platelet cytosol. Since neither control nor affected platelets showed significant alkalinization in response to ionomycin in the absence of external Ca^{++}, the decreased release of internal stores of Ca^{++} in response to ionomycin in affected platelets does not appear to be secondary to a defect in Na^+/H^+

exchange. This was expected since ionomycin causes non-receptor-mediated Ca^{++} release[11,313,377] and increased pH has been shown to potentiate (1,4,5)IP_3-mediated release of internal Ca^{++}.[55]

U46619 is a synthetic PGH_2/TxA_2 mimetic which causes platelet activation by receptor-mediated stimulation of PLC.[337,374] In dogs, full platelet activation in response to U46619 requires the addition of subthreshold doses of epinephrine.[66] This finding was supported by the present study in which the addition of U46619 alone caused no significant change in pH_i in either control or affected platelets when compared to the addition of buffer only. This was also supported by the results of the aggregation studies used to establish concentrations of agonists for the pH_i measurements. In all control dogs tested, the addition of U46619 by itself caused only shape change while the prior addition of a subthreshold concentration of epinephrine (1uM) was required to initiate aggregation or secretion.

U46619 stimulates Na^+/H^+ exchange primarily by the PLC-mediated hydrolysis of membrane PI and the production of 1,2-DG.[394] U46619-induced hydrolysis of membrane phosphoinositides is not inhibited by <u>in vitro</u> manipulations that block Na^+/H^+ exchange, suggesting that U46619-induced activation of PLC is independent of Na^+/H^+ exchange and subsequent alkalinization.[409] U46619-induced aggregation and secretion is also not inhibited by removal of external Na^+, indicating that these responses occur independent of Na^+/H^+ exchange.[86] Recent studies have demonstrated that blockage of Na^+/H^+ exchange partially inhibits U46619-induced Ca^{++} mobilization. Ca^{++} mobilization is restored by artificial

alkalinization of the platelet cytosol, suggesting a dependence on increased pH_i at the level of (1,4,5)IP_3-mediated release of Ca^{++} from internal stores.[394]

The significance of the U46619-stimulated pH_i changes to the defect in BHT is not clear since U46619 has not been used in any of the previous studies. It was used in the current study due to the suggestions that: (1) affected platelets are more susceptible to cyclooxygenase inhibition[254]; and (2) the pathway leading to the generation of TxA_2 is not under proper regulatory control in affected platelets.[256] In the presence of external Ca^{++} and epinephrine, affected platelets had weak to no alkalinization responses to U46619. These responses were markedly depressed compared to those in control platelets. It is likely that part, if not all, of the difference was due to the absence of aggregation in affected platelets. This conclusion is supported by the pH_i response in control platelets. In the presence of external fibrinogen, the control platelets had greater post-stimulation pH_i relative to the situation in the absence of fibrinogen where the post-stimulation pH_i was similar to that in affected platelets. The aggregation trials performed in control platelets showed that external fibrinogen was not needed to maintain measurable aggregation; however, the rate of aggregation was slower and the extent of aggregation less in the absence of external fibrinogen.

Incubation with EGTA inhibited any significant pH_i response in either control or affected platelets stimulated by U46619/epinephrine. These findings were not expected since a recent published study has

shown that BCECF-loaded platelets in the presence of external EGTA have an initial decrease in sample fluorescence after addition of the agonist, due to initial shape change, followed by a return to initial resting levels.[394] Possible explanations for the lack of measurable pH_i responses following the removal of external Ca^{++} include requirements for: (1) the Ca^{++}-dependent membrane GPIIb-IIIa complex, fibrinogen binding and aggregation; (2) the influx of external Ca^{++} to potentiate and sustain the intracellular Ca^{++} release involved in the regulation of Ca^{++}-dependent processes; or (3) Ca^{++} to mediate U46619 receptor binding. Platelets have been demonstrated to be less responsive to TxA_2 or TxA_2 mimetics in low Ca^{++} media.[200]

Epinephrine is proposed to have a mechanism of action similar to ADP whereby the binding of epinephrine to its receptor initiates local alkalinization by activation of Na^+/H^+ exchange. This causes the subsequent release of a localized, very small quantity of arachidonic acid.[409,411,412] This response is dependent on the exposure of the fibrinogen binding site and subsequent fibrinogen binding.[20] The released arachidonic acid metabolites then amplify the original stimulatory response by activating PLC.

In both control and affected platelets, epinephrine did not cause any significant pH_i response when compared to the addition of buffer only. Even though measurable cytosolic alkalinization was not identified in response to epinephrine using BCECF, it cannot be ruled out that both control and affected platelets do produce local, membrane-associated activation of Na^+/H^+ exchange. The lack of measurable

alkalinization does not indicate that epinephrine does not play a potentiating role. Examination of U46619 data shows that the prior addition of subthreshold doses of epinephrine was needed to cause full platelet activation. Other studies have shown that inhibition of cytosolic alkalinization by prevention of Na^+/H^+ exchange does not affect the epinephrine potentiation of thrombin-induced PLC activation,[402] suggesting that the mechanism of epinephrine potentiation of platelet activation is independent of pH effects.

In conclusion, the pH_i studies yielded significant differences between control and affected platelets as summarized in Table 22. The findings suggest that these alterations are secondary to failure of aggregation and do not explain the primary platelet defect in BHT based on current knowledge of platelet function. Since many events in platelet activation are occurring within a very short time period, it is often difficult to determine which events are primary and which are secondary. In the current study, it is difficult to separate those events which result from activation of Na^+/H^+ exchange and those events that initiate Na^+/H^+ exchange. There is no apparent correlation between pH_i and the platelet responses of dense granule ATP secretion, Ca^{++} mobilization and aggregation in affected platelets. ATP secretion occurs in affected platelets in response to ADP and high concentrations of PAF;[254] yet, post-stimulation pH_i was significantly decreased when compared to control platelets. ATP secretion is absent in affected platelets following stimulation with low concentrations of PAF; yet, in affected platelets there was no significant difference between the pH_i response to high or low concentrations of PAF. Calcium ionophores do

Table 22. Summary of the change in pH_i from resting levels in control and affected BCECF-loaded platelets.

Agonist (Conc.)	External Buffer	Control (n = 4)	Affected (n = 5)
Buffer	Ca^{++}/fibrinogen	-0.04 ±0.02 (-)	-0.06 ±0.02 (-)
ADP (10uM)	Ca^{++}/fibrinogen	0.09 ±0.06 #(+/-) 0.17 ±0.06 #(+) 0.01 ±0.01 #(-)	-0.03 ±0.03 * (-)
PAF (1uM)	Ca^{++}/fibrinogen	0.25 ±0.04 #(+)	0.12 ±0.03 *#(-)
	Ca^{++}	0.20 ±0.02 #(+/-)	0.12 ±0.03 *#(-)
	EGTA	0.05 ±0.02 #(-)	0.05 ±0.02 # (-)
PAF (0.01uM)	Ca^{++}/fibrinogen	0.23 ±0.04 #(+)	0.10 ±0.04 *#(-)
Thrombin (0.6U/ml)	Ca^{++}	0.07 ±0.03 #(-)	0.04 ±0.02 # (-)
	EGTA	0.02 ±0.03 #(-)	0.01 ±0.03 # (-)
Thrombin (0.1U/ml)	Ca^{++}	0.20 ±0.03 #(-)	0.16 ±0.02 # (-)
Ionomycin (1uM)	Ca^{++}/fibrinogen	0.40 ±0.10 #(+/-)	0.17 ±0.11 *#(-)
	Ca^{++}	0.38 ±0.06 #(-)	0.16 ±0.14 *#(-)
	EGTA	-0.03 ±0.02 (-)	-0.03 ±0.03 (-)
U46619 (1uM)	Ca^{++}/fibrinogen/ 1uM epinephrine	0.13 ±0.08 #(+/-) 0.21 ±0.07 #(+) 0.08 ±0.04 #(-)	-0.01 ±0.03 *#(-)
	Ca^{++}/epinephrine	0.01 ±0.02 (-)	-0.01 ±0.02 (-)
	EGTA	-0.05 ±0.02 (-)	-0.07 ±0.03 (-)
Epinephrine (10.5uM)	Ca^{++}/fibrinogen	-0.05 ±0.01 (-)	-0.06 ±0.02 (-)

Values = mean change in pH_i ± S.D.; n = number of dogs tested; * = significantly different from control, $p < 0.05$; # = significantly different from addition of buffer, $p < 0.05$; (+) = visible aggregation occurs; (+/-) = visible aggregation may or may not occur; (-) = visible aggregation does not occur.

not cause secretion in affected platelets.[254] While the calcium ionophore ionomycin-induced post-stimulation pH_i was decreased in affected platelets relative to control platelets, the absolute level was similar to that obtained following stimulation with other agonists which do induce secretion in affected platelets. In terms of Ca^{++} mobilization, affected platelets had a decreased pH_i response in the presence of external Ca^{++}; yet, measurement of post-stimulation $[Ca^{++}]_i$ in similar experimental conditions shows normal to increased values. In the presence of external EGTA, affected platelets have normal to decreased post-stimulation $[Ca^{++}]_i$ relative to control platelets; yet, there was no significant difference in post-stimulation pH_i in similar experimental conditions.

Enhancement of platelet activation by Na^+/H^+ exchange and subsequent cytosolic alkalinization is most important in response to weak agonists.[386] Activation of Na^+/H^+ exchange appears to be vital in the activation of PLA_2, release of arachidonate metabolites and subsequent activation of PLC in response to weak agonists such as ADP, epinephrine and low concentrations of thrombin.[410] In the current study, activation of affected platelets with ADP was associated with a significantly decreased alkalinization response when compared to control platelets. In spite of this finding, it has been shown that affected platelets produce significantly greater quantities of TxB_2 than control platelets.[256] This suggests that weak agonists do initiate local activation of Na^+/H^+ exchange which is not detected using BCECF. Alternately, it is possible that affected platelets contain a PLA_2 system that is activated independent of changes in pH_i.

The cause of the decreased alkalinization response in affected platelets was not determined in the current study. Since it was seen in response to all agonists but thrombin, the defect is not likely secondary to abnormal agonist binding or receptor coupling to effector systems. While ADP and epinephrine cause direct receptor-mediated activation of Na^+/H^+ exchange,[409,411,412] other agonists activate Na^+/H^+ exchange in a manner primarily mediated by PLC hydrolysis of phosphoinositides, DG production and PKC activation. Therefore, it cannot be ruled out that there is decreased activation of PKC or decreased PKC activation of Na^+/H^+ exchange in affected platelets. This theory is not substantiated by similar patterns of phosphorylation of the 47kDa protein in response to agonist stimulation in control and affected platelets.[255]

Alternately, the decreased post-stimulation alkalinization response in the affected platelets may be due to the lack of the potentiating effects of aggregation. This theory is supported by a number of findings in the current study. First, there were significant differences in the post-stimulation pH_i of control samples in the presence and absence of added fibrinogen which appeared to correlate to the presence or absence of visible aggregation. Secondly, thrombin, the only agonist which induced similar pH_i responses in control and affected platelets, was also the only agonist tested that causes aggregation of both groups of platelets.[254] Finally, there was no significant difference between affected and control platelets in the pH_i response to any of the agonists tested in platelet suspensions incubated with

external EGTA, a situation which inhibits aggregation and fibrinogen binding in both groups of platelets.

CHAPTER 4: 1,2-DIACYLGLYCEROL PRODUCTION

INTRODUCTION

Platelet activation is associated with the hydrolysis of membrane phosphoinositides due to either: (1) direct stimulation of PLC by an activated receptor-agonist complex; or (2) stimulation of Ca^{++}-dependent PLA_2, release of arachidonic acid metabolites and secondary activation of PLC by PGH_2/TxA_2.[337] Initially, PLC preferentially hydrolyses PIP_2 to release (1,4,5)IP_3 and 1,2-DG.[17,25,248,323,343] IP_3 production is detectable within seconds of agonist stimulation and the quantity produced is sufficient to initiate internal Ca^{++} release.[340] The elevation in $[Ca^{++}]_i$ stimulates further activation of PLC and the hydrolysis of PI in addition to PIP and PIP_2, leading to the release of the corresponding inositol phosphate and additional quantities of 1,2-DG.[435,463] The majority of the membrane phosphoinositides are present as PI rather than PIP or PIP_2,[337] therefore the bulk of 1,2-DG production comes from the hydrolysis of PI.[342]

Diacylglycerol is generated within 5 seconds of the addition of agonist and production generally peaks by 30 seconds,[318,342] at which time the concentration of 1,2-DG is elevated 2-3 fold over resting levels.[318] Studies in other cells have shown that approximately one-half of the post-stimulation 1,2-DG comes from the breakdown of non-

phosphoinositide lipids.[323] In platelets, potential sources for the formation of 1,2-DG include triglyceride, PA, monoglyceride, PI and phospholipids other than PI including phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine. It has been shown that the level of [^{3}H]arachidonic acid in platelet triglyceride, monoglyceride and phospholipids other than PI does not change following agonist stimulation during the period in which 1,2-DG production is peaking. In platelets, the levels of [^{3}H]arachidonic acid in PA increase during the same time period,[342] indicating that the primary source for the activation-associated production of 1,2-DG is PI. It has recently been demonstrated that radiolabelled phosphatidylcholine is not metabolized during agonist-induced platelet activation, supporting the primary role of PI in the production of 1,2-DG.[39]

The concentration of 1,2-DG in resting platelets is actively maintained at low levels[318,342] by two different biochemical pathways: (1) phosphorylation to PA by diacylglycerol kinase; and (2) hydrolysis by diglyceride lipase.[33] Diacylglycerol kinase catalyses the reaction: 1,2-DG + ATP → PA + ADP.[422] Studies using diacylglycerol kinase inhibitors or measurement of the breakdown of exogenous diacylglycerols suggest that the diacylglycerol kinase pathway is the primary route by which platelets metabolize 1,2-DG.[41,422] The PA formed is then recycled back into the phosphoinositide pathway. PA also plays a role in platelet activation as a second messenger which amplifies the stimulatory signal.[214] Diglyceride lipase catalyzes the deacylation of the sn-1 fatty acid from 1,2-DG and the subsequent action of monoglyceride lipase catalyzes the hydrolysis of the sn-2 fatty acid,

releasing the glycerol backbone which is recycled back into the membrane lipids.[76] The rate-limiting enzyme in this pathway is diglyceride lipase. Since 71% of the total platelet phosphoinositol is present as the 1-stearoyl, 2-arachidonyl form,[244] the primary form of 1,2-DG is also the 1-stearoyl, 2-arachidonyl form. Both lipases show some specificity for arachidonyl-containing substrates, therefore this pathway plays a role in the release of arachidonic acid.[76] A relatively minor pathway for the removal of 1,2-DG is acylation to triacylglycerol.[41]

1,2-DG is a neutral lipid that remains within the membrane. It activates PKC[377] by increasing the affinity of PKC for Ca^{++} and permitting enzyme activation at $[Ca^{++}]_i$ present in the resting platelet.[193,414] This reaction is dependent on the presence of membrane phosphatidylserine, which binds Ca^{++} and provides a surface for PKC binding,[138] and the presence of diacylglycerols of the sn-1,2 configuration.[224,377] The effects of endogenously produced 1,2-DG can be mimicked <u>in vitro</u> by the use of synthetic diacylglycerols or the tumor promoting phorbol esters which intercalate into the plasma membrane and bind to PKC to induce activation.[72,194,224]

Based on current knowledge of platelet function, no single defect can explain the platelet abnormalities identified in BHT; however, it was determined that measurement of PLC activity in intact platelets was essential since the enzyme plays a central role in platelet activation. Agonists which stimulate platelet activation independent of PLC activation and the associated hydrolysis of membrane phosphoinositides

(such as phorbol esters[337] or high concentrations of thrombin[57,226]) cause aggregation and secretion of both control and affected platelets. In contrast, those agonists that rely on either direct or secondary activation of PLC for full activation (such as PAF, ADP and calcium ionophores) do not induce aggregation of affected platelets.[254] PLC activity can be assessed *in vitro* by the measurement of the production of the metabolites of phosphoinositide hydrolysis such as the inositol phosphates, 1,2-DG or PA. Due to the compatibility of experimental techniques with equipment available in the laboratory, 1,2-DG production was measured following stimulation with the same range of agonists used in previous studies. It was anticipated that there would be decreased production of 1,2-DG in response to those agonists dependent on PLC activation for the full range of platelet activation responses.

MATERIALS AND METHODS

Experimental Subjects

The affected group consisted of three dogs with BHT. There were one male and two female dogs. The control group consisted of three normal Basset hounds. There were one male and two female dogs. All dogs were housed in kennels at the Michigan State University Veterinary Clinical Center, fed a commercial dry dog chow (Hill's Pet Products, Topeka, KS) and exercised regularly. The dogs were free from signs of clinical disease other than those associated with BHT and were not receiving any medication during the study.

Reagents

[^{3}H]Arachidonic acid was purchased from Amersham Corporation (Arlington Hghts, IL, 250uCi/250ul) and from Dupont/ NEN Products (Boston, MA, 250uCi/2.5ml). The labeled arachidonic acid was stored in its original container at -20°C. Silica 60A (250uM) glass-backed thin layer chromatography (TLC) plates with 19 channels and preabsorbent strips were purchased from Whatman LabSales (Hillsboro, OR). Lipid standards were purchased from Sigma Chemical Company (St. Louis, MO) and included the following: (1) diolein containing 85% 1,3-diolein and 15% 1,2-diolein, (2) 1,3-diolein, (3) phosphatidylcholine, (4) free fatty acid (oleate), (5) monoglyceride, (6) cholesterol oleate, (7) triolein and (8) cholesterol. Each standard was dissolved in 1ml chloroform and stored in a dessicator jar at -20°C. Scintillation fluid was purchased from Beckman (Arlington Hghts, IL) and from Research Products International Corp.(Mount Prospect, IL). Organic solvents (chloroform, methanol, benzene, diethyl ether and NH_4OH) were of HPLC grade and were purchased from Aldrich Chemical Company (Milwaukee, WI). Thrombin, PAF, ADP, calcium ionophore A23187, U46619 and epinephrine were purchased and stored as discussed in the previous chapters (pages 119-120, 175).

Preparation of [^{3}H]Arachidonic Acid Labelled Platelets

1,2-DG was measured by modification of a technique described by Brass et al.[57] Platelets were collected and PRP isolated as described in the previous chapter (page 119-120). PGE_1 (1uM final concentration) was added to the PRP and the suspension incubated for 5 minutes at room

temperature prior to centrifugation at 800 x g for 15 minutes. The supernatant was discarded and the pellet resuspended in 1.5ml autologous PPP acidified by the addition of 1M citric acid to yield a final pH 6.5 as measured using a pH meter. A total of 6uCi [^{3}H]arachidonic acid was added and the suspension incubated at 37°C for 1 hour. The suspension was passed over a 15ml column of Sepharose 4B equilibrated with Ca^{++}-Mg^{++}-free Tyrode's buffer (137mM NaCl, 2.7mM KCl, 5.5mM glucose, 0.35mM Na_2HPO_4, 10mM HEPES, pH 7.4) to remove the excess radiolabel. The platelets were manually counted using the Unopette system and a Neubauer hemocytometer. The eluate was diluted to a final concentration of 3 x 10^8 platelets/ml using the same buffer containing 1mM EDTA. Aliquots (0.5ml) were placed in polypropylene centrifuge tubes, covered and maintained at room temperature for 15 minutes. Agonist (20ul) was added and the samples briefly swirled to gently mix the suspension. The reaction was stopped at 5, 15, 30, 60 and 120 seconds post-stimulation by the addition of 1.8ml ice cold chloroform/ methanol solution (1:2 v/v) which was freshly mixed each day. The buffer used to dilute the agonists was added alone to additional samples and the procedure repeated to establish baseline samples for comparison of results following the addition of agonists. Resting (0 seconds) 1,2-DG levels were established by adding stopper solution to an aliquot of platelet suspension which was not agitated and which did not have anything added to it. Following the addition of the stopper buffer, the suspensions were vigorously vortexed and maintained at room temperature for 30 minutes. The lipids were extracted by the addition of 0.9ml 2M KCl and 0.9ml chloroform to each aliquot. The suspensions were thoroughly vortexed and the layers were separated by centrifugation at 800 x g for

10 minutes. The lower layer was removed and maintained at room temperature. An additional 1.8ml chloroform was added to the upper layer of each sample. The suspensions were vortexed followed by centrifugation at 800 x g for 10 minutes. The lower layer was removed and combined with the lower phase previously collected. The samples were placed in a water bath at 37°C and dried under N_2 for storage at -20°C. For each day's collection, a protein assay (BCA Protein Assay, Pierce, Rockford, IL) was performed according to the directions provided with the kit to determine the protein content per sample.

Separation of Lipids by Thin Layer Chromatography

The Silica plates were dried by either baking in an oven at 150°F for one hour or drying under a stream of hot air from a hair dryer for 15 minutes. Once dried, the plates were kept in an air tight container with Drierite to prevent moisture from accumulating on the plates. Each dried platelet sample was redissolved in 65ul chloroform/methanol solution (2:1 v/v) and 3ul of the stock diolein standard (containing 85% 1,3-diolein and 15% 1,2-diolein) was added. The suspension was vigorously vortexed, spotted onto the preabsorbent strip on a TLC plate and allowed to dry at room temperature in a dessicator container. The plates were placed into a pre-equilibrated developing tank containing benzene/diethyl ether/NH_4OH (100:80:0.2 v/v/v) which was mixed fresh every day. The plates were removed from the tank when the solvent front reached 2cm from the upper edge of the plate. The samples were dried well in a dessicator container before being placed in an iodine tank to allow visualization of the lipid bands. The bands were identified by

comparison with the migration of the lipid standards listed in the reagent section. The R_f value for each lipid was calculated by measuring the distance that the lipid band traveled from the edge of the preabsorbent strip and dividing by the distance that the solvent front traveled from the edge of the preabsorbent strip.

The band which co-migrated with the 1,2-diolein standard was scraped off the plate and placed into a scintillation vial containing 1ml methanol. The suspension was vortexed and allowed to sit at room temperature for 30 minutes. 9ml of scintillation fluid was added and the samples vortexed. The radioactivity was determined by liquid scintillation counting using a Beckman LS9000 liquid scintillation counter. Each sample was counted twice, the counts per minute (CPM) recorded and the average value recorded. The background CPM of the scintillation fluid alone was measured and subtracted from the sample CPM.

Statistical Analysis

The resting and post-stimulation CPM of 1,2-DG was measured in response to each agonist on two separate occasions for each dog. The corresponding values were averaged to obtain representative values which were used in the statistical comparisons. All data was analyzed using a computer software statistical/graphics program (Statgraphics, STSC, Rockville, MD). Affected and control data were compared using the Student's t test for independent samples. The comparison of post-stimulation data to resting values within affected and control groups

was performed using a one way analysis of variance (Tukey's test) for multiple comparisons.

RESULTS

As in the previous chapters, the results will be presented according to agonist. The agonists used included PAF, ADP, thrombin, calcium ionophore A23187, PMA and U46619/epinephrine. In addition, buffer used to dilute the agonists was added by itself to use as a baseline for comparison with the post-stimulation values. The amount of radioactivity recovered following the extraction and TLC separation procedures is indicative of the amount of 1,2-DG present in the platelets samples. The resting values will be presented as CPM/sample. The post-stimulation results will be presented as a ratio of post-stimulation CPM : resting (0 time) CPM for the agonists PAF, thrombin, ADP and PMA. The post-stimulation results will be presented as a ratio of post-stimulation CPM : time-matched CPM following the addition of buffer only for the agonists U46619/epinephrine and calcium ionophore A23187 since it was observed that the 1,2-DG produced in response to these agonists was relatively small and that the increase above resting levels alone was not representative. Each sample contained 0.5ml of the platelet suspension with a total of 1.5 x 10^8 platelets. The mg protein/sample was calculated each day to assure that similar quantities of platelets were present in the samples.

Lipid TLC Results

The platelet lipid samples were compared to known lipid standards. The region containing the 1,2-DG radioactivity was determined by co-migration with 1,2-diolein standards. See Figure 17 for a representative TLC plate. The R_f values were calculated over the course of the experiments and the mean values ± SD are as follows: (1) 1,2-diolein = 0.72 ± 0.06; (2) 1,3-diolein = 0.83 ± 0.04; (3) triolein = 0.92 ± 0.03; (4) monoglyceride = 0.08 ± 0.02; (5) phosphatidylcholine = 0.51 ± 0.04; (6) cholesterol oleate = 0.90 ± 0.05; and (7) cholesterol = 0.45 ± 0.06. It was found during the course of the experiments that the R_f values and resolution of the lipid bands were adversely affected by increasing ambient humidity and the subsequent accumulation of moisture within the plates. Care had to be taken to assure the TLC plates were adequately dried and maintained in a low humidity environment.

Resting Values

There was no significant difference between affected and control platelets in the resting 1,2-DG CPM in extracted lipid samples which had been separated on TLC plates. The total radioactivity of all of the platelet lipids recovered following the extraction procedure was also measured each day on a representative sample prior to lipid separation on TLC plates. There was no significant difference between the two groups. The mg protein/sample was measured each day and affected and control platelets contained similar amounts of protein. The ratio of the resting 1,2-DG CPM/sample : mg protein/sample and the ratio of the

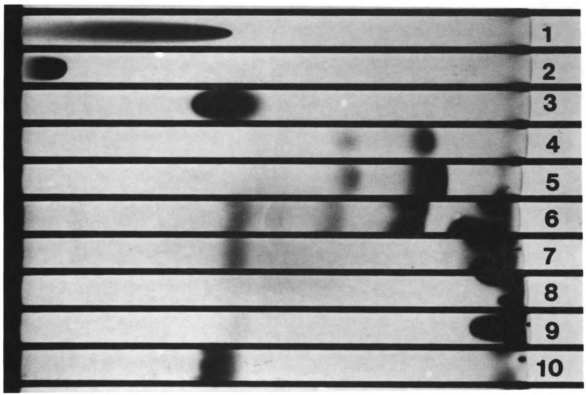


Figure 17. Representative TLC plate. Lane 1 = oleate; lane 2= monoglyceride; lane 3 = cholesterol; lane 4 = 1,3-diolein; lane 5 = 85% 1,3-diolein, 15% 1,2-diolein; lane 6 = platelet sample + diolein mix; lane 7 = platelet sample without added standard; lane 8 = triolein; lane 9 = cholesterol oleate; lane 10 = phosphatidylcholine.

resting 1,2-DG CPM/sample : CPM for total extracted lipids/sample were also calculated. Though there was a tendency for the ratios to be lower in affected platelets, the differences between the two groups of platelets were not statistically significant. See Table 23 for a summary of the results.

Table 23. Comparison of values from the resting platelet samples of control and affected ^{3}H-AA-labelled platelets.

Value	Affected (n = 3)	Control (n = 3)
1,2-DG (CPM)	144.5 ± 17.4	129.0 ± 37.2
total extracted lipids (CPM)	72738.8 ± 12882.5	77651.2 ± 8867.5
mg protein/sample	0.41 ± 0.04	0.46 ± 0.05
1,2-DG CPM/mg protein	322.8 ± 35.1	299.2 ± 97.8
1,2-DG CPM/CPM of total extracted lipids	0.00206 ± 0.00029	0.00177 ± 0.00030

Values = mean ± SD; n = number of dogs tested.

Buffer

The addition of buffer without added agonist to the platelet samples of both affected and control dogs caused a decrease in sample CPM which was measurable 5 seconds after the addition of buffer and remained at a relatively constant level throughout all of the time periods tested. The CPM of the post-stimulation samples was not statistically different from resting values and was of similar magnitude in both groups. See Table 24 and Figure 18 for summaries of the results.

Table 24. Measurement of baseline 1,2-DG following the addition of buffer alone to control and affected ^{3}H-AA-labelled platelets.

Post-stimulation time	Control (n = 3)	Affected (n = 3)
0 Seconds (Resting)	155.8 ± 27.9	152.2 ± 39.9
5 Seconds	123.0 ± 16.0	109.2 ± 27.1
15 Seconds	119.5 ± 11.9	109.7 ± 30.0
30 Seconds	128.1 ± 18.9	112.5 ± 27.7
60 Seconds	121.1 ± 17.0	106.4 ± 24.1
120 Seconds	124.6 ± 5.2	110.6 ± 26.3

Values = mean CPM ± SD; n = number of dogs tested.

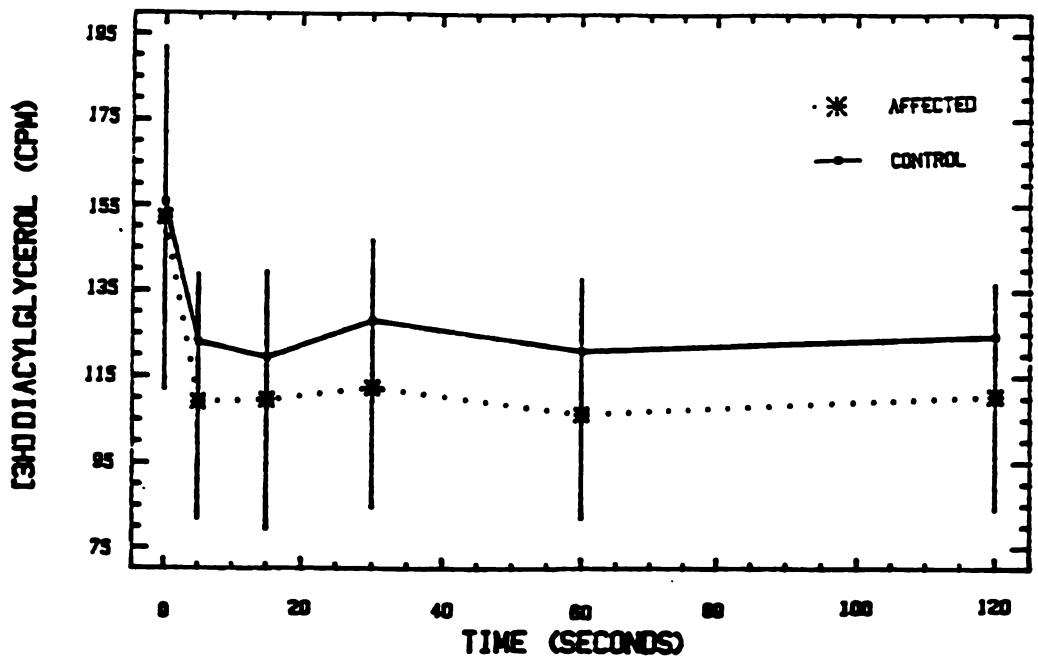


Figure 18. Comparison of the baseline 1,2-DG (CPM) following the addition of buffer in control and affected ^{3}H-AA-labelled platelet samples. Values represent mean ± SD.

Adenosine Diphosphate (ADP)

The ADP results are presented as the ratio of post-stimulation 1,2-DG CPM : resting 1,2-DG CPM. In both affected and control platelets, the addition of 10uM ADP caused a decrease in sample 1,2-DG CPM which was reflected as a decreased ratio. The decrease in CPM tended to be greater in the affected platelets; however, the difference between control and affected platelets was not significant. Resting and post-stimulation values were compared in affected and control platelets. The difference was statistically significant only in the affected platelets. See Table 25 and Figure 19 for summaries of the results.

Table 25. Comparison of 1,2-DG production in control and affected ^{3}H-AA-labelled platelets following the addition of 10uM ADP. Ratio of post-stimulation CPM : resting CPM.

Post-stimulation Time	Control (n = 3)	Affected (n = 3)
5 Seconds	0.80 ± 0.10	0.73 ± 0.08 @
15 Seconds	0.83 ± 0.14	0.71 ± 0.11 @
30 Seconds	0.82 ± 0.09	0.75 ± 0.09 @
60 Seconds	0.81 ± 0.10	0.74 ± 0.10 @
120 Seconds	0.79 ± 0.04	0.76 ± 0.07 @

Value = mean ± SD; n = number of dogs tested; @ = significantly different from resting value, $p < 0.05$.

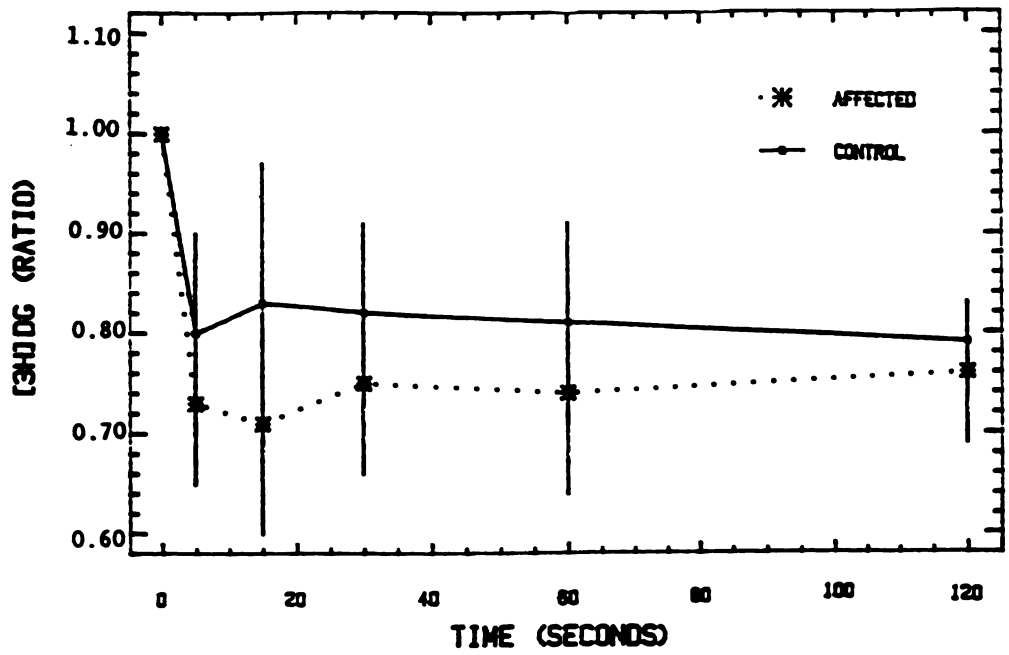


Figure 19. Comparison of 1,2-DG production in response to 10uM ADP in control and affected ^{3}H-AA-labelled platelet samples. Ratio of post-stimulation CPM : resting CPM. Values represent mean ± SD.

Comparison of ADP-induced 1,2-DG production (as indicated by the ratio of post-stimulation CPM : resting CPM following the addition of 10uM ADP) to the buffer induced 1,2-DG production (as indicated by the ratio of post-stimulation CPM : resting CPM) yielded no significant differences in either control or affected platelets. See Figures 20 and 21 for a summary of the results.

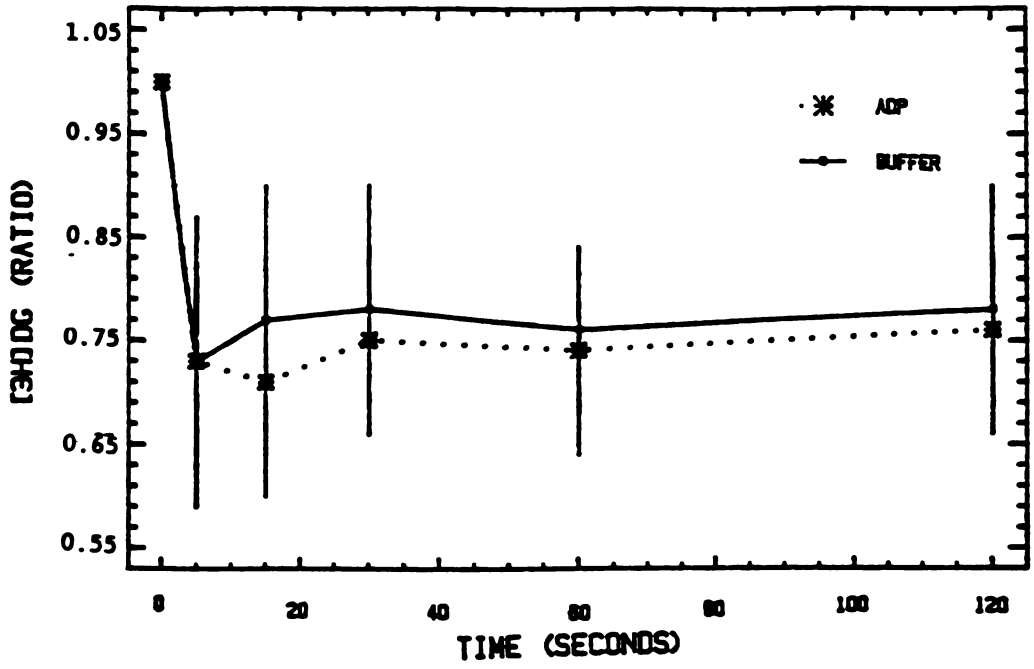


Figure 20. 1,2-DG production in response to 10uM ADP and buffer in $_3$H-AA-labelled affected platelet. Values represent mean ± SD.

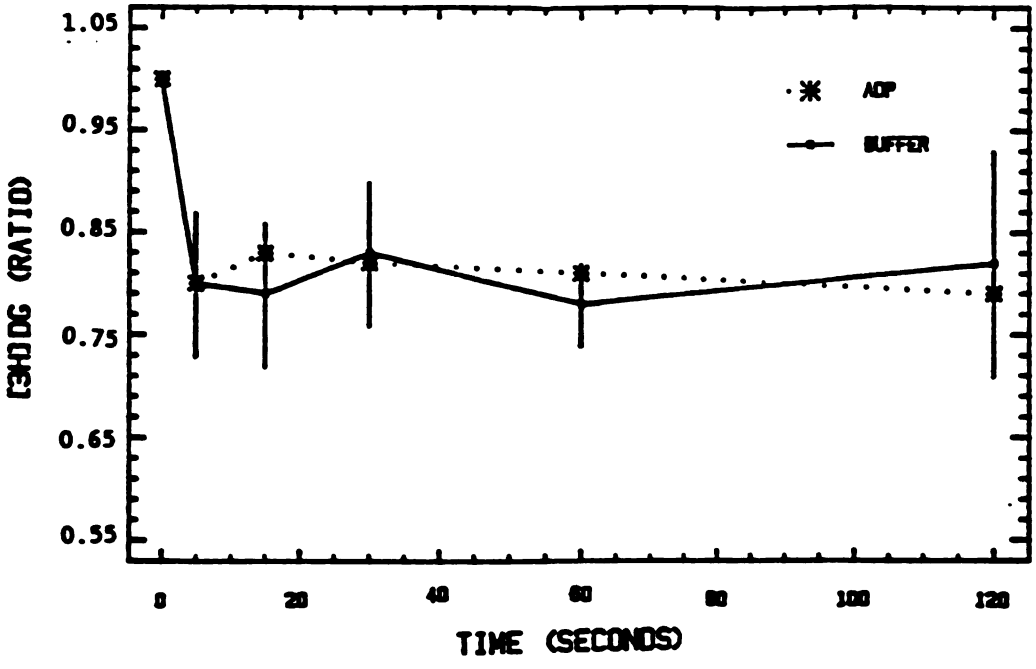


Figure 21. 1,2-DG production in response to 10uM ADP and buffer in ^{3}H-AA-labelled control platelets. Values represent mean ± SD.

Platelet-Activating Factor (PAF)

The PAF results are presented as the ratio of post-stimulation 1,2-DG CPM : resting 1,2-DG CPM. In response to stimulation with high concentrations of PAF (1uM), there was a rapid peak of 1,2-DG production at 5 seconds which was of similar magnitude in both control and affected platelets. By 15 seconds 1,2-DG production was decreasing and by 30 seconds values were similar to resting levels in both groups. At 60 seconds, the ratio was significantly decreased in affected platelets when compared to control samples. Though the difference was not significant, the same trend was seen at 120 seconds. See Table 26 and Figure 22 for a summary of the results.

Table 26. Comparison of 1,2-DG production in control and affected ^{3}H-AA-labelled platelets following stimulation with 1uM PAF. Ratio of post-stimulation CPM: resting CPM.

Post-Stimulation Time	Control (n = 3)	Affected (n = 3)
5 Seconds	2.30 ± 0.13 @	2.49 ± 0.74 @
15 Seconds	1.67 ± 0.20 @	1.64 ± 0.37
30 Seconds	1.19 ± 0.24	1.12 ± 0.12
60 Seconds	1.08 ± 0.09	0.92 ± 0.06 *
120 Seconds	1.10 ± 0.15	0.94 ± 0.08

Value = mean ± SD; n = number of dogs tested; * = significantly different from control, $p < 0.05$; @ = significantly different from resting, $p < 0.05$.

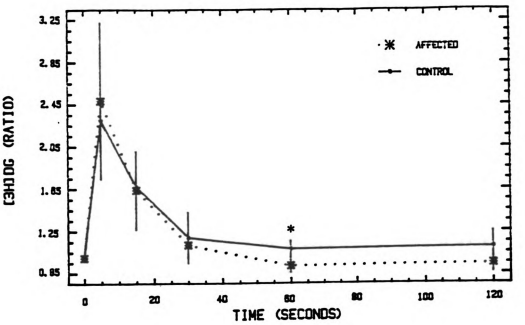


Figure 22. Comparison of 1,2-DG production in response to 1uM PAF in ^{3}H-AA-labelled control and affected platelets. Values represent mean ± SD. * = significantly different from control, $p < 0.05$.

Following stimulation with low concentrations of PAF (0.01uM), the response at 5 and 15 seconds was similar in control and affected platelets, although the peak post-stimulation ratio was not as large as that found following stimulation with 1uM PAF. By 30 and 60 seconds, the amount of 1,2-DG was similar to resting values and the ratio was significantly decreased in the affected platelets relative to the control samples. See Table 27 and Figure 23 for summaries of the results.

Table 27. Comparison of 1,2-DG production in control and affected ^{3}H-AA-labelled platelets following stimulation with 0.01uM PAF. Ratio of post-stimulation CPM: resting CPM.

Post-Stimulation Time	Control (n = 3)	Affected (n = 3)
5 Seconds	1.54 ± 0.06 @	1.61 ± 0.28 @
15 Seconds	1.27 ± 0.11 @	1.19 ± 0.13
30 Seconds	1.18 ± 0.11	0.92 ± 0.07 *
60 Seconds	1.02 ± 0.02	0.85 ± 0.09 *
120 Seconds	0.92 ± 0.10	0.84 ± 0.11

Values = mean ± SD; n = number of dogs tested; * = significantly different from control, $p < 0.05$; @ = significantly different from resting value, $p < 0.05$.

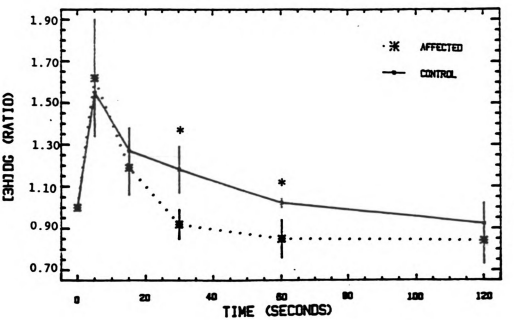


Figure 23. Comparison of 1,2-DG production in response to 0.01uM PAF in ^{3}H-AA-labelled control and affected platelets. Values represent mean ± SD. * = significantly different from control, $p < 0.05$.

THROMBIN

The thrombin results are presented as the ratio of post-stimulation 1,2-DG CPM : resting 1,2-DG CPM. Following the addition of 0.3 U/ml thrombin, there was a relatively slow increase in 1,2-DG production which reached its peak by 30 seconds in both control and affected platelets. At 60 and 120 seconds the 1,2-DG production was gradually decreasing toward resting levels in both groups, though there was a tendency for the decrease to be greater in affected platelets. The ratio was significantly decreased in affected platelets relative to control platelets at 120 seconds. See Table 28 and Figure 24 for summaries of the results.

Table 28. Comparison of 1,2-DG production in control and affected ^{3}H-AA-labelled platelets following stimulation with 0.3U/ml thrombin. Ratio of stimulated CPM: resting CPM.

Post-Stimulation Time	Control (n = 3)	Affected (n = 3)
5 Seconds	1.01 ± 0.09	0.92 ± 0.08
15 Seconds	1.31 ± 0.06 @	1.21 ± 0.15
30 Seconds	1.48 ± 0.17 @	1.52 ± 0.44
60 Seconds	1.46 ± 0.10 @	1.50 ± 0.03 @
120 Seconds	1.30 ± 0.06 @	1.15 ± 0.06 *

Values = mean ± SD; n = number of dogs tested; * = significantly different from control, $p < 0.05$; @ = significantly different from resting, $p < 0.05$.

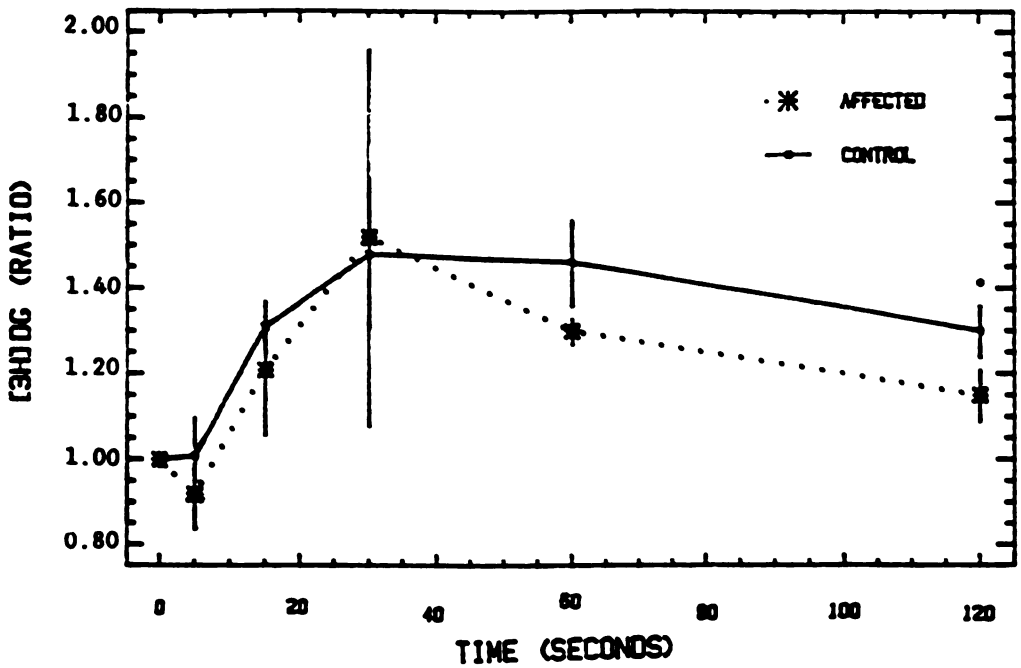


Figure 24. Comparison of 1,2-DG production in response to 0.3U/ml thrombin in ^{3}H-AA-labelled control and affected platelets. Values represent mean ± SD. * = significantly different from control, $p < 0.05$.

Calcium Ionophore A23187

The A23187 results are presented as the ratio of 1,2-DG CPM following stimulation with 1uM A23187 : 1,2-DG CPM following the baseline addition of buffer at matched time periods. Increased production of 1,2-DG was detectable by 5 seconds in both control and affected samples. The increase peaked by 30 to 60 seconds and was gradually decreasing by 120 seconds. There was no significant difference between control and affected platelets at any of the measured time periods. See Table 29 and Figure 24 for summaries of the results.

Table 29. Comparison of 1,2-DG production in control and affected ^{3}H-AA-labelled platelets following stimulation with 1uM calcium ionophore A23187. Ratio of 1,2-DG CPM following agonist stimulation : 1,2-DG CPM following addition of buffer only.

Post-Stimulation Time	Control (n = 3)	Affected (n = 3)
5 Seconds	1.08 ± 0.05	1.11 ± 0.04
15 Seconds	1.14 ± 0.06	1.12 ± 0.03
30 Seconds	1.22 ± 0.06 @	1.15 ± 0.04
60 Seconds	1.22 ± 0.02 @	1.22 ± 0.07 @
120 Seconds	1.15 ± 0.09	1.12 ± 0.10

Value = mean ± SD; n = number of dogs tested; @ = significantly different from baseline, $p < 0.05$.

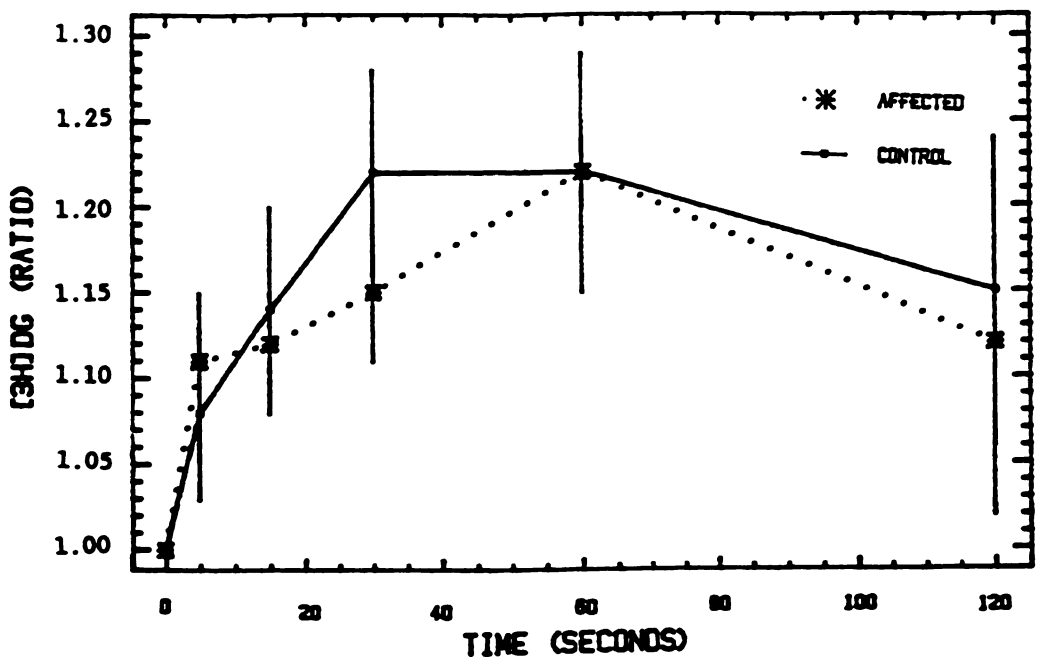


Figure 25. Comparison of 1,2-DG production in response to 1uM A23187 in ^{3}H-arachidonic acid labelled control and affected platelets. Values represent mean ± SD.

U46619/Epinephrine

The U46619/epinephrine results are presented as the ratio of 1,2-DG CPM following the addition of 2.5uM U46619/1uM epinephrine : baseline 1,2-DG CPM following the addition of buffer at matched time periods. The amount of 1,2-DG in the control platelets peaked at approximately 15 to 30 seconds post-stimulation after which it remained relatively constant over the time period measured. There was an initial sharp increase in 1,2-DG production in affected platelets that was significantly greater than the increase in control platelets. At 15 seconds, the amount of 1,2-DG had decreased, but was still greater than that of the control samples, though the difference was not significant. The amount of 1,2-DG stayed relatively constant at times 30 and 60 seconds. 1,2-DG production peaked again at 120 seconds in the affected platelets to a level that was significantly elevated over values present in control platelets. See Table 30 and Figure 26 for summaries of the results.

Table 30. Comparison of 1,2-DG production in control and affected ^{3}H-AA-labelled platelets following stimulation with 2.5uM U46619/1uM epinephrine. Ratio 1,2-DG CPM following agonist-stimulation: baseline 1,2-DG CPM following addition of buffer.

Post-Stimulation Time	Control (n = 3)	Affected (n = 3)
5 Seconds	1.18 ± 0.16	1.49 ± 0.05 *@
15 Seconds	1.20 ± 0.07	1.34 ± 0.06 @
30 Seconds	1.23 ± 0.21	1.28 ± 0.14 @
60 Seconds	1.22 ± 0.06	1.28 ± 0.10 @
120 Seconds	1.21 ± 0.06	1.37 ± 0.06 *@

Value = mean ± SD; n = number of dogs tested; * = significantly different from control, $p < 0.05$; @ = significantly different from baseline, $p < 0.05$.

Phorbol Myristate Acetate (PMA)

The PMA results are presented as the ratio of 1,2-DG CPM following the addition of 3uM PMA : resting 1,2-DG CPM. In both control and affected platelets, there was a rapid decrease in 1,2-DG levels which was of similar magnitude. The 1,2-DG values stayed relatively constant throughout the test period. See Table 31 and Figure 27 for summaries of the results. The values in both control and affected platelets were not statistically different than the 1,2-DG response following the addition of buffer without agonist. See Figures 28 and 29 for summaries of the results.

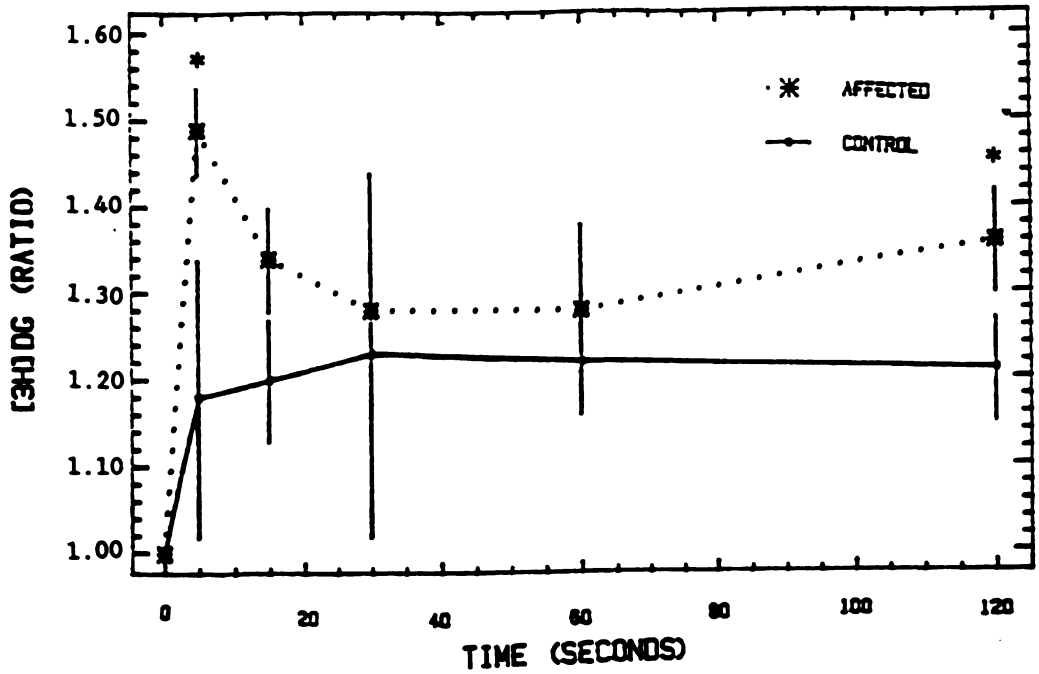


Figure 26. Comparison of 1,2-DG production in response to U46619/epinephrine in ^{3}H-AA-labelled control and affected platelets. Values = mean ± SD. * = significantly different from control, $p < 0.05$.

Table 31. Comparison of 1,2-DG production in control and affected ^{3}H-AA-labelled platelets following the addition of 3uM PMA. Ratio of post-stimulation 1,2-DG CPM : resting 1,2-DG CPM.

Post-Stimulation Time	Control (n = 3)	Affected (n = 3)
5 Seconds	0.73 ± 0.17	0.72 ± 0.10
15 Seconds	0.72 ± 0.09	0.79 ± 0.14
30 Seconds	0.76 ± 0.14	0.75 ± 0.13
60 Seconds	0.74 ± 0.11	0.74 ± 0.12
120 Seconds	0.72 ± 0.09	0.80 ± 0.12

Value = mean ± SD; n = number of dogs tested.

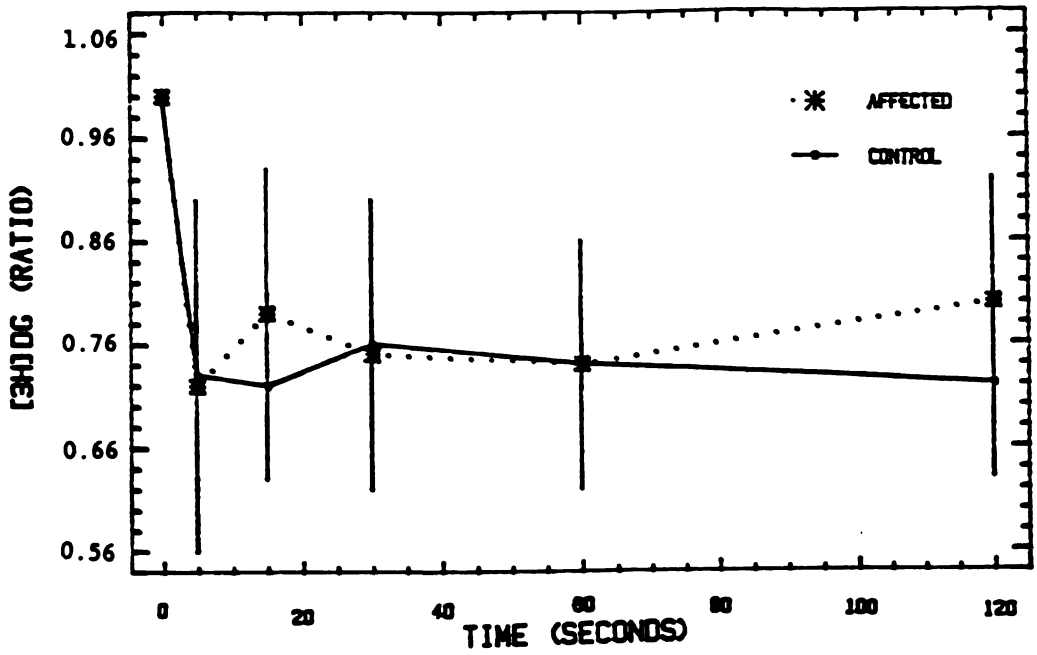


Figure 27. Comparison of 1,2-DG production in response to 3uM PMA in ^{3}H-AA-labelled control and affected platelets. Values = mean ± SD.

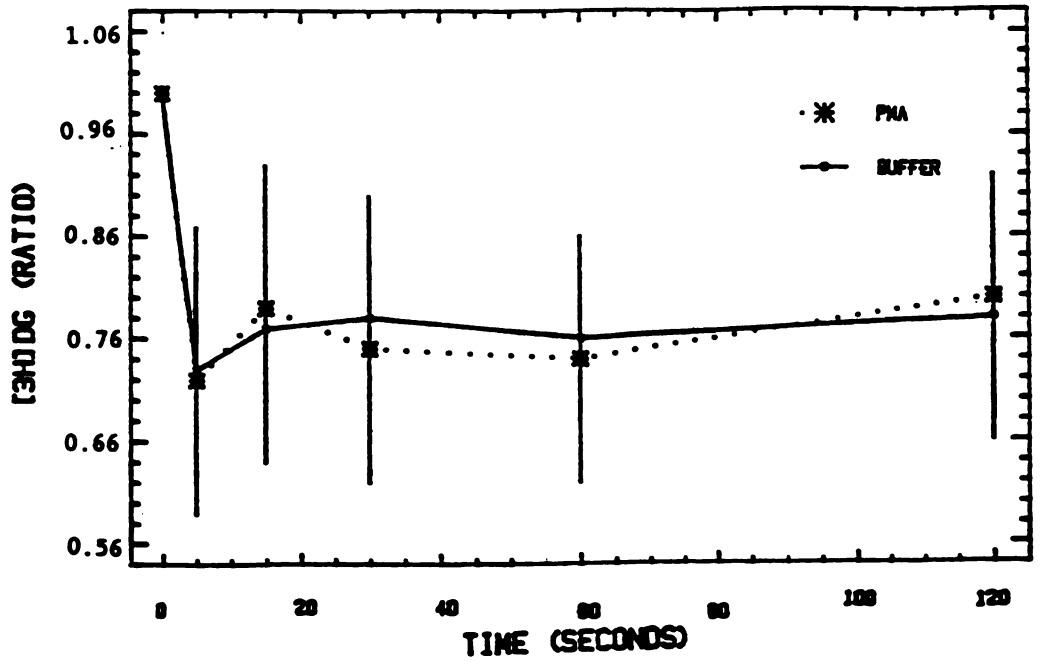


Figure 28. Comparison of 1,2-DG production in response to 3uM PMA and buffer in affected ^{3}H-AA-labelled platelets. Values = mean ± SD.

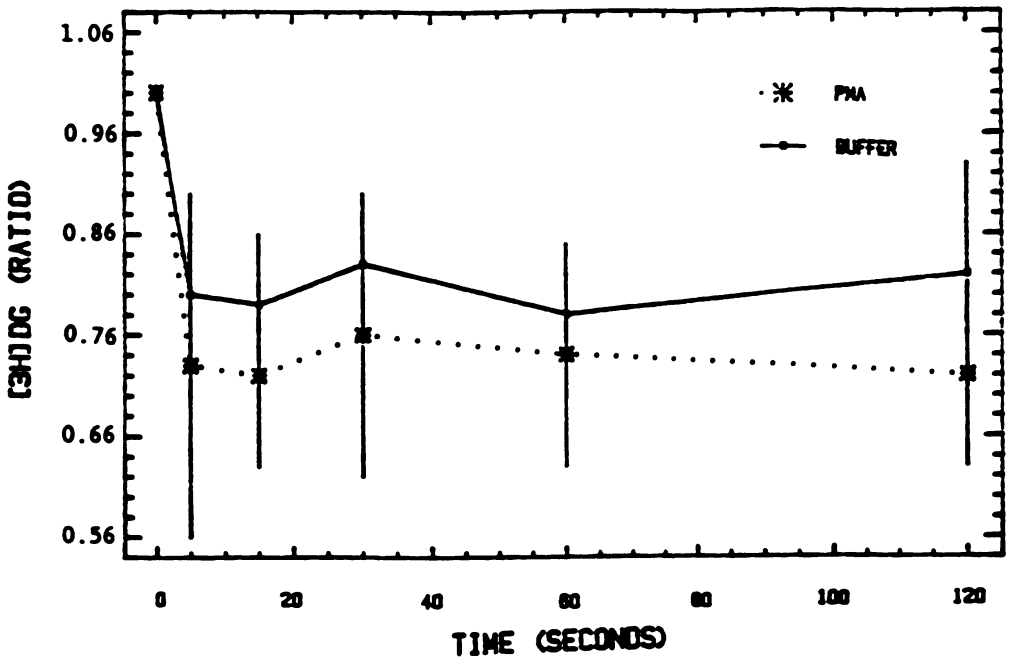


Figure 29. Comparison of 1,2-DG production in response to 3uM PMA and buffer in ^{3}H-AA-labelled control platelets. Values = mean ± SD.

DISCUSSION

Activation of phosphoinositide-specific PLC is a central effector pathway in platelet signal transduction.[377] Agonists activate PLC either directly by a receptor-mediated process or indirectly via the production of arachidonic acid metabolites which secondarily initiate receptor-mediated activation of PLC.[377] PLC stimulates the hydrolysis of membrane phosphoinositides to produce the intracellular second messengers (1,4,5)IP_3 and 1,2-DG. Therefore, the production of either of these products can be used to indirectly monitor PLC activity in intact platelets.

1,2-DG production was measured in control and affected platelets. Non-aggregating platelet preparations (ie. non-stirred, no exogenous fibrinogen, 1mM EDTA) were used to allow direct comparisons with the protein phosphorylation data previously reported[255] and to eliminate alterations in 1,2-DG production which occur secondary to the amplification processes resulting from aggregation itself. There was no significant difference between control and affected samples in the mg protein per sample, indicating that the samples being compared contained similar concentrations of platelets.

Resting 1,2-DG levels were measured and reported as CPM per sample. There was no significant difference between affected and control platelets in resting 1,2-DG concentrations using this parameter. There was marked day to day variation in the CPM values in both control and affected platelets. The range of values for each dog also changed on separate days. This was reflected in marked day to day variations in the radioactivity for the total quantity of extracted lipids/platelet sample. These findings suggest that there were unavoidable differences in loading of [^{3}H]arachidonic acid into platelet samples which occurred irrespective of uniform experimental loading techniques. Therefore, a better assessment of resting 1,2-DG was the comparison of the ratio of the radioactivity of 1,2-DG recovered from the TLC plates to the radioactivity of the total amount of lipids recovered from the extracted sample. The latter value was calculated in a representative platelet sample prior to separation of the lipids on TLC plates. Since there is only approximately 33% recovery of lipids from the TLC plates (personal communication M. Laposata), the ratio calculated cannot be used to

estimate the absolute percentage of total lipids in the resting platelet present as 1,2-DG. The ratio can be used to estimate differences between control and affected platelets in the relative amount of the total resting platelet lipids which is present as 1,2-DG. The data showed a trend for the ratio to be decreased in affected platelets, although the difference between affected and control was not statistically significant. This suggests that affected platelets have a tendency for a decreased percentage of the total lipids to be present as 1,2-DG in resting platelets. It may reflect decreased basal formation of 1,2-DG or increased rate of removal of 1,2-DG.

The quantity of 1,2-DG in resting platelets is maintained at relatively low levels[318,342] by two separate enzyme systems that actively remove 1,2-DG. The addition of exogenous diacylglycerols is rapidly followed by conversion to PA by diacylglycerol kinase or to glycerol by the sequential action of diglyceride and monoglyceride lipases. Studies have shown that 80% of exogenous [^{3}H]-labelled diacylglycerols are metabolized within 2.5 minutes, with the majority being removed by diglyceride kinase.[41] Inhibition of diglyceride kinase is associated with prolongation of the 1,2-DG signal while inhibition of diglyceride lipase, the rate limiting enzyme in the conversion of 1,2-DG to glycerol,[76] is not associated with prolongation of the 1,2-DG signal. Instead, when diglyceride lipase is inhibited, a greater percentage of the exogenously added 1,2-DG is metabolized by diglyceride kinase.[41]

The amount of 1,2-DG in platelet samples was measured following the addition of 20ul buffer without agonist in order to act as a time-matched baseline for comparison to the amount of 1,2-DG produced following the addition of agonist in the same group of platelet samples. Unexpectedly, there was a decrease in 1,2-DG following the addition of buffer which was evident by five seconds and persisted throughout all time points tested. It seemed unlikely that the response was due to the addition of buffer itself since the buffer was a physiologic solution. It was felt that a more likely source for the decrease was the gentle swirling applied briefly to the platelet samples to mix the suspensions after the addition of buffer or agonist. This hypothesis was examined in a few experiments where platelets samples were swirled but nothing was added. It was found that the shaking alone did cause a decrease in measurable 1,2-DG; however, the experiments were not repeated sufficient times to do statistical comparisons with the change in 1,2-DG following the addition of buffer.

The mechanism for the decrease in 1,2-DG is not known and has not, to my knowledge, been previously reported. Possible explanations include decreased basal formation of 1,2-DG or increased activity of diglyceride kinase and/or diglyceride lipase. As discussed above, the post-stimulation production of 1,2-DG is primarily regulated by PLC-mediated hydrolysis of membrane PI.[39,342] PLC is a Ca^{++}-dependent enzyme whose activity is modulated by a G protein.[53,377] In platelets, a number of forms of PLC have been identified which differ in molecular weight, substrate specificity, pH optima, Ca^{++} requirements and immunoreactivity.[53] Agents that inhibit PLC activity include of a group

of positively charged substances which interact with the negatively charged phosphate group on the phosphoinositides. These agents include neomycin, polyamines, phenothiazines and local anesthetics.[377] cAMP inhibits an agonist-induced increase in 1,2-DG but does not affect basal 1,2-DG levels.[318] Therefore the increased basal cAMP present in BHT affected platelets[48,49] cannot adequately explain the decrease in 1,2-DG following the addition of buffer. Relatively little has been published on diglyceride kinase activity in platelets; however, it is strongly dependent on membrane phospholipids for normal activity[318] and can be inhibited by a number of synthetic inhibitors.[41] Diglyceride lipase activity is optima at acidic pH, is not affected by Ca^{++} and/or Mg^{++} concentrations and is sensitive to sulfhydryl inhibitors.[76]

In the current study, the 1,2-DG response to buffer was not felt to be significant since the response was similar in control and affected platelets. In hindsight, it would have been desirable to compare all of the post-stimulation 1,2-DG CPM values to the baseline, time-matched values following the addition of buffer. Unfortunately, time-matched baseline values were only available for A23187 and U46619/epinephrine. The contribution of the different pathways of 1,2-DG removal to the decrease in 1,2-DG could be determined by measuring the concurrent production of the metabolites produced. If the decrease is due to increased activity of diglyceride kinase, a concurrent elevation in [^{3}H]PA would be expected. If the decrease is due to increased activity of diglyceride lipase, a concurrent elevation in [^{3}H]glycerol or 2-[^{3}H]oleoylmonoacylglycerol would be expected. Finally, if the decrease

is due to decreased activity of PLC, none of these metabolites would be increased.

ADP is a weak agonist which requires both cyclooxygenase activity and primary aggregation to induce secretion and irreversible aggregation.[377] Previous studies have shown that activation of PLC following stimulation with weak agonists, such as ADP, is dependent on the production of arachidonic acid metabolites.[409] ADP stimulation does not cause any measurable phosphorylation of the 20kDa and 47kDa proteins in platelet suspensions when granule secretion and the production of arachidonic acid metabolites is prevented by non-aggregating experimental conditions,[127,161] suggesting that ADP does not cause activation of PLC under non-aggregating conditions. In the presence of EDTA to inhibit aggregation, control and affected platelets do not have significantly increased phosphorylation of the 20kDa and 47kDa proteins following ADP stimulation.[255] These findings suggest that there is no significant Ca^{++} mobilization and PKC activation respectively, indirectly indicating that PLC activation does not occur.

The current study used a non-aggregating/non-fibrinogen binding preparation (ie non-stirred, no exogenous fibrinogen, 1mM EDTA). It was anticipated that ADP stimulation would not cause any measurable increase in 1,2-DG production relative to resting levels. Unexpectedly, it was found that the addition of ADP caused a decrease in sample 1,2-DG which was of similar magnitude in both affected and control platelets. Comparison of the 1,2-DG responses following the addition of ADP and following the addition of buffer alone showed no significant difference

in either group. These findings indicate that the decrease in 1,2-DG in response to ADP is not due to a specific effect of ADP but results from the same factors that cause a decrease in measurable 1,2-DG following the addition of buffer alone.

PAF is a physiologic agonist that induces receptor-mediated activation of PLC, hydrolysis of membrane phosphoinositides and the production of the second messengers (1,4,5)IP_3 and 1,2-DG.[106,221,375,383] The PAF-induced activation of PLC is similar both in the presence of external Ca^{++}/Mg^{++} and in the presence of external EDTA.[383] PAF-induced activation of PLC occurs independent of the release of arachidonic acid metabolites.[383]

In the presence of external EDTA, the phosphorylation pattern of the 20kDa and 47kDa proteins following addition of PAF is similar in control and affected platelets. Phosphorylation of the 20kDa protein peaks within 30 seconds of agonist addition while phosphorylation of the 47kDa protein peaks within 60 seconds.[255] These findings suggest that Ca^{++} mobilization and PKC activation is similar in control and affected platelets. In the current study, production of 1,2-DG in response to PAF stimulation was very rapid in both control and affected platelets. Production peaked by 5 seconds and gradually decreased over the 2 minute test period. The maximum level of 1,2-DG production was similar in both groups of platelets; however, the amount of 1,2-DG was significantly decreased in affected platelets relative to control platelets by 30 (1uM PAF) and 60 seconds (1uM and 0.01uM PAF) after agonist addition.

Decreased concentrations of 1,2-DG may have resulted from either decreased production by PLC or increased rate of removal of 1,2-DG. Since PAF stimulation of platelets is associated with an agonist-induced uncoupling of the membrane receptor from the PLC effector system,[272] it is possible that affected platelets have an enhanced rate of receptor uncoupling. Alternatively, affected platelets may have an increased rate of 1,2-DG metabolism due to enhanced activity of either diglyceride kinase or diglyceride lipase.

Thrombin is a physiologic agonist that causes receptor-mediated activation of PLC.[21,34,90,262,346] The thrombin-induced activation of PLC and subsequent production of 1,2-DG occurs in either the presence or absence of external Ca^{++}/Mg^{++} and is independent of the release of arachidonic acid metabolites.[383] Previous studies on the thrombin-induced phosphorylation of the 20kDa and 47kDa proteins have shown similar phosphorylation patterns in control and affected platelets, although peak phosphorylation of the 47kDa protein in affected platelets is not as extensive as in control platelets.[255] Measurement of 1,2-DG production in control and affected platelets showed no significant differences over the first 60 seconds following the addition of thrombin, though there was much greater variation in the values from the affected platelets. At 120 seconds post-stimulation, the 1,2-DG level was significantly decreased in the affected platelets relative to control platelets. As discussed with PAF, this may result from enhanced uncoupling of the PLC effector system from the membrane receptor or from increased removal of 1,2-DG by diglyceride kinase or diglyceride lipase.

The calcium ionophores A23187 and ionomycin are non-physiologic agonists that cause activation of PLC as indicated by the loss of membrane phosphoinositides and the concurrent formation of 1,2-DG and PA.[34,314,339] Inhibition of cyclooxygenase and removal of ADP completely block these responses, indicating that the activation of PLC is secondary to Ca^{++}-induced activation of PLA_2 and the release of ADP.[337,339] Examination of control and affected platelets have yielded similar phosphorylation patterns for both the 20kDa and 47kDa proteins in the presence of external EDTA,[255] indirectly indicating similar PLC activity. Phosphorylation of both of these proteins can occur independent of PLC activation due to Ca^{++}-mediated activation of PKC and MLCK.[225,361] Since affected platelets have decreased production of TxB_2[256] and no significant ATP dense granule secretion[254] following stimulation with A23187, it was anticipated that there would be decreased activation of PLC in affected platelets. However, measurement of 1,2-DG production showed a similar response in both control and affected platelets. The degree of activation of PLC, as indicated by relative 1,2-DG production, was much less following stimulation with A23187 than following stimulation with PAF or thrombin.

U46619 is a PGH_2/TxA_2 mimetic that causes direct receptor-mediated activation of PLC which is independent of arachidonic acid metabolism and the presence of external Ca^{++}.[337,379] In the present study, subthreshold concentrations of epinephrine were added prior to U46619 because canine platelets do not aggregate in response to U46619 by itself.[66] Epinephrine does not directly activate PLC; instead,

activation of PLC is dependent on the release of arachidonic acid metabolites and ADP secretion.[383,409]

When compared to baseline 1,2-DG following the addition of buffer, control platelets had relatively constant 1,2-DG concentrations over the entire time period tested following the addition of U46619/epinephrine. In contrast, affected platelets had significantly increased production of 1,2-DG following stimulation with U46619/epinephrine. By five seconds post-stimulation, affected platelets had a peak of 1,2-DG production not found in control platelets. 1,2-DG levels then decreased until 120 seconds post-stimulation at which time there was another, although smaller, peak of production. These findings suggest that affected platelets have increased sensitivity to U46619 and/or epinephrine which is specific for one, or both, of these agonists. Increased sensitivity to either agonist may result from (1) increased receptor number or affinity; or (2) decreased receptor downregulation and desensitization in affected platelets. Pretreatment of platelets with U46619 causes decreased responsiveness to addition of subsequent doses of U46619,[233] indicating that receptor downregulation and desensitization normally occur.

Phorbol esters (ie PMA) are non-physiologic agonists that intercalate into the membrane and directly activate PKC.[71,72,377] The addition of phorbol esters to platelets has no measurable effect on the levels of inositol phosphates[442] or the production of 1,2-DG,[39] indicating no direct effect on PLC activity and phosphoinositide metabolism. PKC activation causes decreased production of PA, 1,2-DG

and/or inositol phosphates in response to stimulation with ADP, PAF, U46069, thrombin or collagen.[39,380,421,442] This indicates that PKC inhibits agonist-induced PLC activation and the hydrolysis of the membrane phosphoinositides. One mechanism proposed for this inhibition is a negative effect of PKC on the G protein coupled to PLC.[5]

In the presence of external EDTA, affected and control platelets have similar phosphorylation of the 20kDa and 47kDa proteins following stimulation with PMA. There is rapid phosphorylation of the 47kDa protein and a slower phosphorylation of the 20kDa protein.[255] Unlike the other agonists discussed above, phosphorylation of the 20kDa and 47kDa proteins in response to PMA does not necessarily result from PLC activation. The 47kDa protein is a major substrate for phosphorylation by PKC.[362] PKC also phosphorylates the 20kDa protein at a distinct site from that phosphorylated by MLCK.[275]

There was no significant difference between control and affected platelets in 1,2-DG production following stimulation with PMA. In both groups, the addition of PMA was followed by a decrease in sample CPM which stayed relatively constant over the time periods measured. This response was similar to that found following the addition of buffer without agonist, indicating that it was not due to a specific effect of PMA. These findings suggest that the phosphorylation of the 20kDa and 47kDa proteins demonstrated in the previous study is due to PKC, not PLC, activation.

In conclusion, affected and control platelets produced similar quantities of 1,2-DG in response to all agonists tested with the exception of the PGH_2/TxA_2 mimetic U46619 and/or epinephrine. The combination of U46619/epinephrine caused affected platelets to produce significantly greater quantities of 1,2-DG. It was not possible to make direct comparisons between the current investigation and previous investigations on BHT because of the different experimental conditions; however, it appears that the platelet defects previously identified do not result from lack of PLC activation.

A direct comparison between the production of 1,2-DG and the phosphorylation of the 47kDa and 20kDa proteins[255] was possible since the studies had been performed under similar experimental conditions. Both investigations, which indirectly assess PLC activity in intact platelets, have demonstrated similar findings. The affected platelets have similar patterns of agonist-induced phosphorylation of the 20kDa and 47kDa proteins; however, there was a general tendency for affected platelets to phosphorylate the proteins less extensively or the dephosphorylate the proteins more rapidly.[255] This finding correlates well with the tendency for the production of 1,2-DG to decrease more rapidly in affected platelets when compared to control platelets. This decrease in 1,2-DG could result from either: (1) increased uncoupling of the agonist receptors or the modulatory G protein from PLC; or (2) enhanced metabolism of 1,2-DG by diglyceride kinase and/or diglyceride lipase. Unfortunately, the phosphorylation of platelet proteins in response to U46619/epinephrine has not been done in affected or control platelets to offer a comparison. Based on the measurement of 1,2-DG

production, it would be anticipated that affected platelets would show enhanced phosphorylation of the 47kDa and 20kDa proteins.

Increased production of 1,2-DG in response to U46619/epinephrine in affected platelets suggests increased sensitivity of PLC to stimulation with these specific agonists. As mentioned earlier, increased platelet sensitivity to U46619 could result from: (1) increased number or affinity of PGH_2/TxA_2 receptors; or (2) decreased receptor downregulation or desensitization. This finding is significant in view of the increased production of TxB_2 in affected platelets following stimulation with thrombin, ADP and high concentrations of PAF and decreased production of TxB_2 in response to stimulation with A23187 and low concentrations of PAF.[256] Although activation of PLC and the associated production of 1,2-DG is independent of the formation of arachidonate metabolites, the release of arachidonate metabolites does enhance the formation of PA.[383] It would be anticipated that increased production of TxB_2 in combination with enhanced sensitivity to TxA_2 mimetics would lead to increased PLC activity in affected platelets. However, there appeared to be no correlation between TxB_2 production and PLC activation, as indicated by 1,2-DG production.

It is not possible to make direct comparisons between TxB_2 production and 1,2-DG formation since the measurement of TxB_2 was performed in aggregating preparations in the presence of low concentrations of external Ca^{++} [256] while the measurement of 1,2-DG was performed in non-aggregating conditions in the absence of external Ca^{++} (1mM EDTA). It would be interesting to measure TxB_2 production in the

presence of EDTA to remove external cations and inhibit aggregation. If under these conditions, affected platelets do not produce increased concentrations of TxB_2, then it is possible that decreased activation of PLA_2 would be associated with decreased mobilization of internal Ca^{++} as discussed in the previous chapter. PLA_2 is a Ca^{++}-dependent enzyme[27,397] which is activated by increasing $[Ca^{++}]_i$;[122,151] however, PLA_2 activity is also influenced by other factors including cytosolic pH,[27] PKC activity[122,152,150] and G protein modulation.[6,137,280,396] Alternatively, if affected platelets still produce significantly increased quantities of TxB_2 in the presence of external divalent cation chelators, then the lack of significantly increased effect of the released arachidonic acid metabolites on PLC activation would be due to other factors. It has been demonstrated that affected platelets have increased basal cAMP concentrations.[48,49] Increased cAMP inhibits PLC-mediated hydrolysis of membrane phosphoinositides.[205,344,430,472] Therefore, the increased cAMP could offset the increased activation of PLC by the endogenously produced arachidonate metabolites.

Alternatively, increased 1,2-DG production in response to U46619/epinephrine may have resulted from increased sensitivity of affected platelets to epinephrine. The exact mechanism for epinephrine-mediated potentiation of platelet activation is not well defined. By itself, epinephrine does not stimulate PLC activity[383]. Stimulation of PLC by epinephrine is dependent on Na^+/H^+ exchange and the local production of arachidonic acid metabolites.[20,409] Epinephrine does potentiate Ca^{++} influx, Ca^{++} release from internal stores, protein phosphorylation of the 20kDa and 47kDa proteins, Na^+/H^+ exchange and PA

formation.[75,219,290,402] The potentiation of platelet responses can occur independent of PLC,[290] Na^+/H^+ exchange,[402] fibrinogen binding[317] and TxA_2 production.[317] To definitively determine whether the response indicates increased sensitivity to epinephrine would require the addition of subthreshold concentrations of epinephrine to platelets suspensions followed by the measurement of 1,2-DG production in response to the other agonists which have been shown to cause similar 1,2-DG production in affected and control platelets.

CHAPTER 5: SUMMARY AND CONCLUSIONS

Previous studies on BHT have shown that affected platelets have normal quantities of membrane GPIIb-IIIa[299] and bind fibrinogen following ADP stimulation.[119,298] Affected platelets do not aggregate in response to those agonists which are fully or partially dependent on arachidonic acid metabolism and PLC activation (ie. ADP, PAF and calcium ionophore A23187).[254] They do aggregate when phosphoinositide hydrolysis is bypassed (ie. phorbol ester and high concentrations of thrombin). In spite of an inability to aggregate, ATP dense granule secretion occurs in affected platelets in response to all agonists tested except for U46619/epinephrine and calcium ionophore A23187.[254] The activation responses in affected platelets are more sensitive to cyclooxygenase inhibition than those in control platelets. Affected platelets also produce significantly greater quantities of TxB_2 in response to all those agonists capable of stimulating secretion.[256] Phosphorylation of the two major proteins (20 and 47kDa) is similar in control and affected platelets following agonist stimulation. Unexpectedly, affected platelets have decreased phosphorylation of a 64-67kDa protein of unknown identity or functional significance.[255] Finally, basal concentrations of cAMP is increased in affected platelets.[48,49]

In the research for the current dissertation, resting and post-stimulation $[Ca^{++}]_i$ was measured. In the presence of external Ca^{++}, affected aequorin-loaded platelets have normal (ie.thrombin, ionomycin, ADP and PAF) to decreased (ie. PMA) Ca^{++} mobilization relative to control platelets while affected fura 2-loaded platelets have normal (ie. thrombin, ionomycin and PMA) to increased (ie. ADP and PAF) Ca^{++} mobilization. When external Ca^{++} is removed by EGTA, affected aequorin-loaded platelets have normal (ie. ADP, PAF, thrombin and PMA) to decreased (ie. ionomycin) post-stimulation $[Ca^{++}]_i$ relative to control platelets while affected fura 2-loaded platelets have normal (ie. ADP and phorbol) to decreased (ie. PAF, thrombin and ionomycin) post-stimulation $[Ca^{++}]_i$. There was no difference between control and affected platelets in resting $[Ca^{++}]_i$. These findings suggest that affected platelets have normal Ca^{++} homeostasis in the resting state. They appear to be able to mobilize Ca^{++} as well as control platelets when the influx of external Ca^{++} can occur. When external Ca^{++} is removed, the internal release of Ca^{++} is impaired. The previously-identified defects in affected platelets do not appear to be associated with defective Ca^{++} metabolism since the aggregation, secretion and TxB_2 production responses are abnormal in the presence of external Ca^{++}, a situation where the Ca^{++} fluxes appear to be normal.

The cause of the defective mobilization of internal Ca^{++} was not determined in the current study. Possible explanations include: (1) decreased production of the second messengers that mediate Ca^{++} release; (2) production of defective or non-functional second messengers; (3) decreased responsiveness of the DTS to the second messengers; or (4)

decreased stores of releasable Ca^{++} within the DTS. The first three could explain the decrease in response to the physiologic agonists. Increased cAMP, which has previously been identified in affected platelets,[48,49] decreases Ca^{++} mobilization in response to the physiologic agonists.[331,350,433] However, cAMP does not affect Ca^{++} release in response to the non-physiologic calcium ionophores.[350] The decrease post-stimulation $[Ca^{++}]_i$ in response to ionomycin suggests that there is also a defect in the releasable stores of Ca^{++} within the DTS.

The second portion of the dissertation research measured resting and post-stimulation pH_i in affected and control platelets. There was no significant difference between control and platelets in resting pH_i in stirred samples. In the presence of EGTA, a situation which inhibits fibrinogen binding and prevents aggregation, there was no significant difference between affected and control platelets in post-stimulation pH_i. In the presence of external Ca^{++}, there was significantly decreased post-stimulation pH_i in affected platelets in response to those agonists (ie. ADP, PAF, ionomycin, U46619/epinephrine) which cause aggregation in control, but not affected, platelets. In response to thrombin, the only agonist tested which causes aggregation in both affected and control platelets, there was no difference between the two groups.

These results demonstrate that affected platelets do have a functional Na^+/H^+ antiport as indicated by the similar pH_i responses in control and affected platelets in the presence of external EGTA. The results in the presence of external Ca^{++} suggest that the decreased

post-stimulation pH_i in the affected platelets is partially, if not fully, due to the lack of aggregation. In response to the weak agonist ADP, affected platelets do not show any significant post-stimulation increase in pH_i; yet, they have increased production of TxB_2. It has been proposed that the weak agonists initiate platelet activation by producing a local, membrane associated increase in pH_i. This local alkalinization then causes activation of PLA_2, the production of arachidonic acid metabolites and subsequent activation of PLC.[409,410,411,412] Since affected platelets produce increased quantities of TxA_2 in response to ADP,[256] it is likely that a local cytosolic alkalinization is occurring but is not detectable using the fluorescent pH indicator BCECF in platelet suspensions.

The final phase of the dissertation research assessed PLC activation in intact platelets by measurement of the production of the second messenger 1,2-DG in a non-aggregating preparation. When compared to control platelets, affected platelets produce similar quantities of 1,2-DG relative to control platelets in response to all agonists tested with the exception of TxA_2 mimetic U46619/epinephrine. U46619/epinephrine causes increased production of 1,2-DG in affected platelets. These findings suggest that the defect in BHT does not result from lack of activation of PLC. The increased responsiveness to U46619/epinephrine suggests increased responsiveness to U46619 and/or epinephrine.

The results described in the three portions of the dissertation research do not explain the platelet defect in BHT; however, they do

help to further localize the defect. Unfortunately, may events occur simultaneously during platelet activation, making it difficult to determine which events are primary and which events are secondary. In addition, the different biochemical pathways involved in the activation sequence are not isolated; instead, there are numerous checks and balances. Figure 30 presents a schematic model of platelet activation. Based on this model, the following conclusions on affected platelets can be drawn from previous studies as well as those reported in this dissertation:

1. Membrane GPIIb-IIIa complex is present in normal amounts[299] and is able to bind fibrinogen following ADP-stimulation.[119,298]
2. Na^+/H^+ exchange is functional and produces sufficient alkalinization to support PLA_2 activity. Whether it is under normal control mechanisms needs to be determined using a more sensitive technique than BCECF in platelet suspensions.
3. The pathway leading to the TxA_2 production is intact but not under proper regulatory control. Affected platelets are much more dependent on this pathway for activation than control.[254]
4. Based on measurement of 1,2-DG production, PLC activation is similar in control and affected platelets in response to most agonists. This suggests that the primary defect in BHT must lie in some pathway other than PLC. There is increased activation of PLC by U46619 and/or epinephrine.
5. When PKC is activated directly, full platelet activation occurs.[254] The mechanism by which PKC mediates aggregation is not known.

6. The influx of external Ca^{++} across the plasma membrane is normal in affected platelets while the release from internal stores is impaired. The amount released is sufficient to support the Ca^{++}-dependent processes of shape change, phosphorylation of the 20kDa protein and PLA_2 activity.
7. Phosphorylation of the major cytoskeletal proteins (20kDa and 47kDa) is normal in non-aggregating preparations.[255] Phosphorylation of the 20kDa protein is associated with shape change. The role of the 47kDa protein in platelet activation is under debate. It is not clear how these proteins mediate secretion and aggregation.
8. There is decreased phosphorylation of a 64-67kDa protein of unknown function or identity.[255]

In conclusion, these findings do not fit any described platelet thrombopathy and cannot be explained by current understanding of platelet function. The results do suggest areas for further investigation including:

1. Closer examination of the thromboxane pathway including measurement of PLA_2 activity, arachidonic acid release and PGH_2/TxA_2 receptor number and affinity.
2. Further investigation of the 64-67kDa protein which is not phosphorylated as well in affected platelets. This would include protein isolation, production of a monoclonal antibody and protein sequencing.

3. Further examination of the process of aggregation. None of the findings thus far can explain the lack of primary aggregation in affected platelets. Inherently, it seems logical that if platelets can bind fibrinogen, they can also aggregate. Therefore, further examination of surface charge and membrane fluidity is warranted.

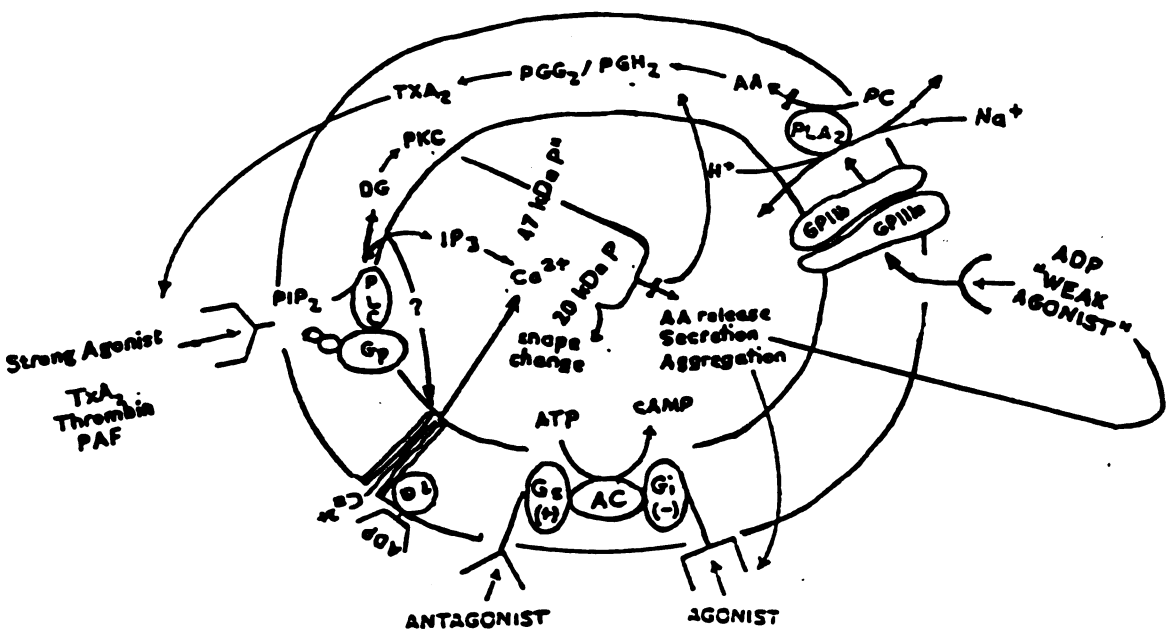


Figure 30. Schematic model for platelet activation. Modified from Kroll et al.[212]

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