BIOENERGETICS OF PLATELET AND ALVEOLAR MACROPHAGE FUNCTION

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ABSTRACT

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Part 1: Platelet bioenergetics

In modern day life, risk factors associated with life style such as high calorie diet, unhealthy eating habits, mental stress, and lack of exercise substantially increase the risk of cardiovascular diseases and diabetes. An important feature of platelets in both of these conditions is their enhanced sensitivity to the activation stimuli that can results in life threatening conditions such as heart attacks or strokes. Although platelet activating factors account for the larger fraction of this systemic thrombus formation, they do not explain the entire spectrum of platelet hyperactivity. We observed a correlation between; red blood cell (RBC) derived ATP release and platelet hyperactivity in the circulation. Therefore, it was predicted that platelet hyperactivity may be related to the physiological behavior of RBCs and their ATP release. Furthermore, the potential of ATP and its primary receptor on platelets, P2X1 have been underestimated as a result of the lack of available analytical tools and methodologies.

The work shown here demonstrates the biphasic nature of ATP and P2X1 receptor on platelet inhibition and aggregation in a concentration-dependent manner. We found that incremental increases of ATP concentration initially reduce the platelet aggregation, with a gradual increase in platelet $Ca^{2+}i$ and NO production. However, with further increase in ATP, platelets began to aggregate. Therefore our data explains the behavior of extracellular ATP on platelets: an antagonist at low concentrations and

an agonist at high concentrations. We also found that the most commonly used ATP analogue to study P2X1 function, α,β–methylene ATP, fails to induce the actual P2X1 receptor potential, due to its chemical structure and the behavior which abolishes the P2X1 function. In summary, investigations in chapter 2 and 3 of this dissertation demonstrate the biphasic nature of extracellular ATP as a platelet antagonist and an agonist. Also we were the first to demonstrate the actual potential of the P2X1 receptor and bioavailable ATP in platelet activation and aggregation which may be useful in future antiplatelet therapy.

Part 2: Bioenergetics of alveolar macrophages in cystic fibrosis pathogenesis

Cystic fibrosis (CF) is a genetic disorder associated with mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene and its protein product, CFTR. Although, the fatal CF lung disease is characterized by chronic pulmonary infections and inflammations, the available molecular level understanding cannot explain the failures in the lung defense against the pathogen invasion, which results in lung infections. Despite the fact that alveolar macrophages are the first defense in the lungs against particulate matter and pathogen invasion, there have never been correlations described between defective CFTR on macrophage function and defective macrophage function on CF lung defense failure.

Our investigations reveal the correlation between CFTR dysfunction and macrophage glucose uptake which eventually regulates the alveolar macrophages phagocytosis. We also found that Zn^{2+} activated C-peptide, a known glucose uptake sensitizer for RBCs, completely reversed the reduced glucose uptake and phagocytosis due to the CFTR inhibition.

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CHAPTER 1

1.1 INTRODUCTION

The average adult has about five liters of blood circulating in the vascular system. While traversing through vessels, blood is acting as a multifunctional tissue which delivers essential elements such as nutrients and oxygen and removes harmful wastes such as CO_2 and urea. Blood is the growth medium for the body's cells and by transporting nutrients from the digestive system, delivering regulatory hormones and distributing disease fighting substances, blood circulation maintains the regular dynamics of the body. Blood is considered as the largest tissue of the body containing multiple cell types and substances.

Blood plasma is a yellow liquid in which the blood cells are suspended. Plasma contributes 55% of the total blood volume. Although 90% of plasma is water by volume, it contains proteins (8%), glucose, electrolytes, clotting factors, hormones and carbon dioxide.

Cells are 45% of the total volume of the blood. The cellular component of the blood is mainly red blood cells (RBCs), although other cell types are important. For an example, white blood cells protect the body from infections and destroy unwanted cells and particulate matter. There are two types of white blood cells: granulocytes (neutrophils, basophils and eosinophils) and agranulocytes (monocytes, lymphocytes and macrophages). Granulocytes differentiate in to mature cells that are responsible for

specific immune responses (T lymphocytes) and phagocytosis (macrophages). Monocytes leave the circulation and become tissue macrophages. Macrophages are the front line defense in the lungs against pathogen and other harmful materials. Platelets are smaller anucleated cells that mediate the blood clotting mechanism, thereby preventing blood loss during vascular injury. Although white blood cells and platelets are smaller in percentage, they are equally as important as RBCs for a proper integrated body function.

Either one or several components of blood interact with any given cell in the body at all the times. Therefore any defect in function/structure of blood components can influence one or many tissues. On the other hand, an abnormal behavior or function of tissue or organ mostly manifests itself in blood. This is why blood is such an important component of medical diagnostics.

Many diverse disease conditions, where entirely dissimilar abnormalities occur, different cell types, tissues or an organ, give rise to common pathological conditions. For example; in some disease states, platelets are hyperactive and unusually susceptible for aggregation. Hyperactive platelets are prominent in diseases such as diabetes, multiple sclerosis and cystic fibrosis and cause an imminent threat of thrombus formation by blocking blood vessels. When analyzing the behavior of other cell types interacting with hyperactive platelets, our group and others have observed that bioavailable ATP released from RBCs are abnormal in these diseases due to either decreased deformability (in diabetes) or increased cell lysis (multiple sclerosis).[1-4]

Investigations in recent years by the Spence group and others suggest the potential involvement of RBCs in the regulation of energy in terms of glucose consumption and ATP production/release. Being the most exposed cell type to the abundant glucose absorbed from the digestive system, RBC glucose uptake may play a major role in glucose homeostasis. Also, the significance of bioavailable ATP (mostly derived from RBCs) in the circulatory system serves as a key regulator for vasodilatation and platelet inhibition through stimulating production nitric oxide (NO) in the cell types.

Although ATP was considered primarily as a simple energy molecule two decades ago, its importance as a secondary messenger and a ligand in purine nucleotide binding receptor signaling (purinergic signaling) has recently made it an important molecule in biological research. Despite the fact that ADP has long been recognized as one of the main platelet function regulators and ADP associated signal transduction pathway currently being the main target for treating hyperactive platelets (antiplatelet therapy), investigations described in this dissertation will demonstrate the significance of bioavailable ATP as a potent platelet function regulator. This would also provide another key signal transduction pathway for novel antiplatelet therapy that may eventually help in preventing and managing cardiovascular diseases. Here, we also investigated the ATP mediated regulation of macrophage glucose uptake and their phagocytic ability which is very important in preventing lung infections in especially in diseases like cystic fibrosis (CF). We believe that, molecular level picture of macrophages function described in this dissertation will be able to enhance the strategies in fighting against cystic fibrosis.

1.2 PLATELET STRUCTURE, FUNCTION AND REGULATION

1.2.1 Platelet structure

Platelets, or thrombocytes, are anucleated irregular shaped cellular bodies that are 2-4 μm in diameter. Platelets are derived from megakaryocytes during hematopoisis. Although the basic function of platelets is hemostasis, the prevention of bleeding during vascular injuries, they are also critical in numerous and diverse pathological processes including thrombus formation in cardiovascular disease, hemorrhage, inflammation-immune disorders and tumor metastasis.[5] Platelets are of great interest in the bio-medical research community as they play a significant role in coronary artery disease and other common diseases including stroke, peripheral vascular disease and diabetes.[5] Although platelets have a short lifespan of 10 days, they are the source of many cellular growth factors and cellular messengers. There are about 150,000 to 400,000 platelets per micro liter of blood in a healthy person.[5,6] Platelets in large excess (thrombocytosis) or platelet hyperactivity could possibly lead to unnecessary thrombus formation leading to fatal conditions. Similarly, platelets in low numbers (thrombocytopenia) or decreased platelet activity can cause severe and fatal hemorrhagic situations.[7]

Platelet structure can be divided in to three key components: membrane structure, cytoskeleton, and the secretory organelles system (Figure 1.1). The outermost part of the platelet membrane is mainly comprised of glycoprotein and is collectively called glycocalyx. Transmembrane and sub membrane structures mediate responses to

platelet stimulation, immune responses and express specific antigenic characteristics. The platelet cytoskeleton is a detergent-insoluble, intricate three-dimensional network of filamentous proteins. The filaments are composed of three types of polymeric proteins called microfilaments, intermediate filaments and microtubules.[8,9] Changes in actin and myosin filaments associated with increased platelet cytosolic calcium ($Ca^{2+}i$) concentrations lead to platelet shape change and aggregation.[10,11]

Platelet alpha granules, dense granules and lysosomes are the main secretory organelles.[8] Alpha granules consist of a large spectrum of components that regulate a diverse range of functions.[12] These components include coagulation proteins (fibrinogen, factor V-proaccelerin), soluble adhesion molecules (von Willebrand factor-vWF, vitronectin), growth factors (PDGF-platelet derived growth factor, epidermal growth factor), protease inhibitors (plasminogen activator inhibitor-1, α_2-antiplasmin), and membrane adhesion molecules (P-selectin, GPIIb/IIIa). Small molecules (ADP/ATP-adenosine diphosphate/adenosine triphosphate) and important ions (calcium and magnesium) that mediate platelets activation are stored in platelet dense granules.[13-16] Secretory organelles are responsible for control of platelets adhesion, cell growth, and cellular signaling cascades.[17]

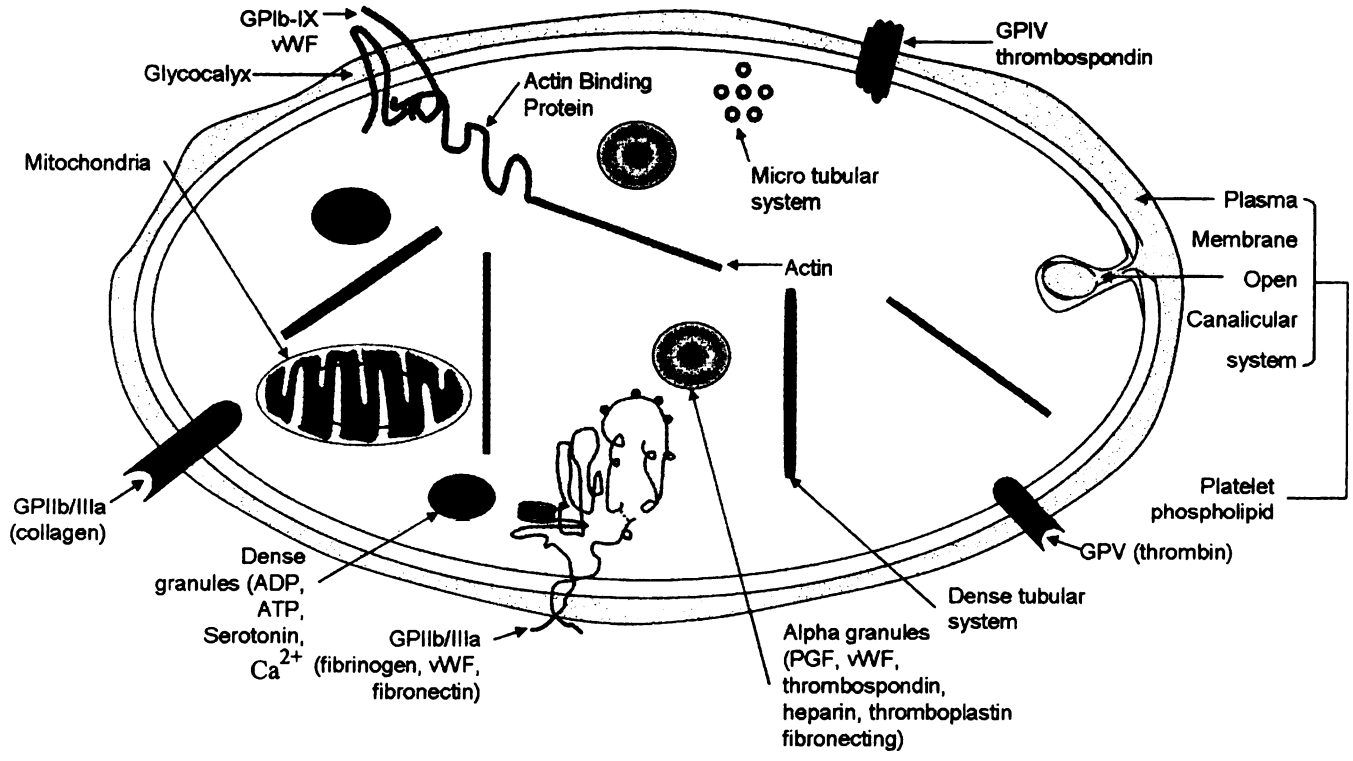

Figure 1.1. Detailed schematic of a platelet cross section, note the basic components- membrane structures, microtubules, and granules. Platelet membrane, overlying glycocalyx, and submembrane structures mediate platelet stimulation and express specific antigenic characteristics. The platelet canalicular system provides a window for extracellular/ intracellular exchanges. The dense tubular system stores Ca^{2+}, ATP, ADP, serotonin and the location of cyclooxygenase. Platelet α granules store factors required for further platelet activation.

1.2.2 Hemostasis: platelet in bleeding prevention

Hemostasis is the mechanism that prevents bleeding during vessel wall injury. Upon vessel wall injury, an immediate reflex of vessel constriction and platelet adhesion to the exposed collagen occurs to reduce blood loss. Subsequent activation and platelet granular release of ADP, TXA_2 and serotonin further activate platelets and recruit more platelets to the site of injury. Serotonin constricts the vessel, thereby facilitating the formation of platelet plug.

Coagulation is the final step of the hemostasis and has two mechanisms, extrinsic and intrinsic. In brief, the extrinsic mechanism starts with the release of factor III[18] from damaged tissues that activate factor VII by converting it to factor VIIa with the help of tissue factor, a protein present in sub endothelial matrix.[19] The intrinsic mechanism forms the active factor XI with a cascade of reactions.[20] Eventually, both intrinsic and extrinsic mechanisms work together to activate factor X.[21,22] Both active factor VII and active factor XI will promote cascade reactions, finally activating factor X. Active factor X, along with factor III, factor V, Ca^{2+}, and platelet thermoplastic factor (PF_3), will activate the prothrombin activator that converts prothrombin to thrombin.[23,24] Finally, thrombin converts fibrinogen to fibrin and forms a loose mesh.[25,26] This loose mesh of fibrin network is covalently modified by factor XIII and converts to a dense mesh of fibers, trapping activated platelets and red cells.[27,28]

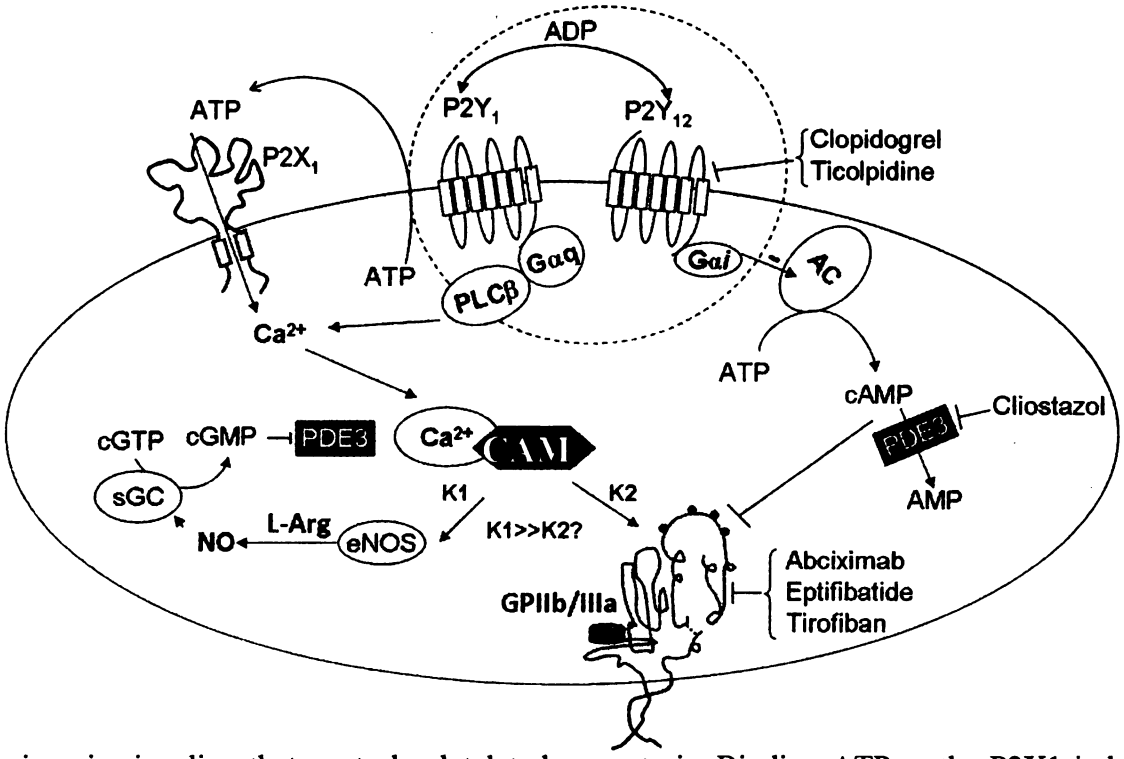

Figure 1.2. Platelet purinergic signaling that controls platelets homeostasis. Binding ATP to the P2X1 induced Ca^{2+} flux in to the cytosol. ADP activation of P2Y1 promotes Phospholipase C (PLCβ) and increase $Ca^{2+}i$. Therefore both P2X1 and P2Y1 increase $Ca^{2+}i$. Increased $Ca^{2+}i$ stimulates NO production as well as GPIIb/IIIa activation. Also, ADP-P2Y12 interactions retards the adenylyl cyclase activity, decreases the formation of cAMP. As low cAMP favors high $Ca^{2+}i$, ADP is a dual promoter of platelet activation. However, NO stimulates cGMP and thereby inhibits phosphodiesterase 3 (PDE3), preventing cAMP dissociation. Therefore, NO favors low $Ca^{2+}i$ and inhibits platelet activation. Depending on the extent of the $Ca^{2+}i$ increase, platelet inhibition or activation dominates.

1.2.3 Mechanisms of platelet function regulation

Platelet activation, aggregation and inhibition are highly regulated mechanisms.[29-32] Extensive activation or delayed response to activation stimuli would be fatal. Platelet activation is a multi step process involved in multiple signaling cascades triggered by binding soluble factors (mobilized by the vascular injury) or adhesive molecules to the platelet surface. Most platelet activators (agonists) bind to the platelet membrane receptors coupled to G proteins, which initiate the mobilizations of secondary messengers leading to platelet activation. Purine nucleotides such as ADP and adenosine have been recognized as critical platelet agonists. ADP released from initial collagen-induced platelet activation propagates further platelet activation and aggregation, until platelet inhibitors such as NO dominate and inhibit the process.

In brief, platelet dense granular ADP release dramatically intensifies platelet activation and aggregation inducing the activation of P2Y1 and P2Y12, two G protein coupled receptors (GPCR). Activation of Gq, a smaller G protein, coupled to P2Y1 promotes the hydrolysis of Phospholipase C (PLCβ) and generates inositol (1,4,5)-triphosphate (IP3) and diacylglycerol (DAG).[9] IP3 mobilizes the intercellular calcium stores to initiate activation of the Ca^{2+}-dependent integrin; GPIIb/IIIa. DAG activates protein kinase C (PKC), phosphorylates myosin light chain kinase (MLCK) and eventually activates GPIIb/IIIa.[33] Activation of P2Y12 has an inhibitory action on adenylyl cyclase and hence reduces the formation of cAMP (Figure 1.2).[17] Binding ATP to its receptor, P2X, can also increase the cytosolic calcium, $Ca^{2+}i$, by allowing

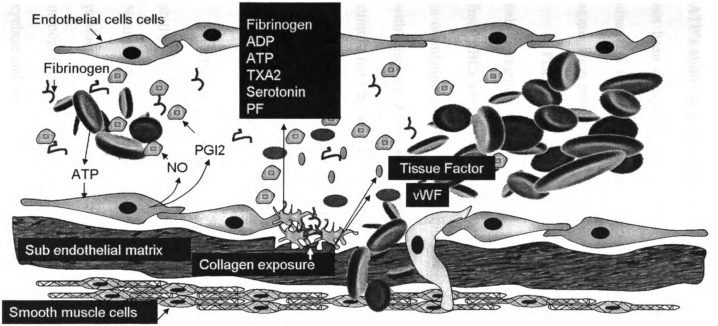

Figure 1.3. Left side of the figures shows the platelet function regulation under normal conditions. In brief, upon deformation, RBC releases ATP which stimulates endothelial and platelet NO production. NO reaches smooth muscle cells relax them and luminal NO prevent platelet activation. Right side of the figure shows the hemostasis under vascular insult. Exposed subendothelial collagen activates platelets and activated platelets cover the site of the injury. They also release numerous factors required for further platelet recruitment, activation and the initiation of the coagulation cascade. Activated platelets release considerable amounts of NO which regulates hemostasis. This NO further prevents vascular thrombosis.[1-3]

extracellular Ca^{2+} to flow in to the cytosol (Ca^{2+} influx) through the opened receptor channel. Due to the issues related to the fast desensitizing nature of this receptor and ATP's ability to easily break down to ADP, the role of ATP in platelet aggregation has not been completely explained. However, hyperactive platelets in conditions where abnormal levels of ATP exist suggest a possible involvement of ATP in the platelet aggregation process.

Platelets maintain continuous interaction with the endothelium and RBCs that help to regulate the vascular properties. We have previously shown that ATP released from RBCs while traversing through a resistance vessel mimic can elicit NO production in circulating platelets and endothelial cells.[1,34] It has also been reported that people with diseases where RBCs release less ATP upon deformation or pharmacological stimuli, have hyperactive platlets.[2,4,35-37] A possible implication of this decreased RBC-derived ATP release arises when considering that ATP is a recognized stimulus of NO production in platelets and endothelium. Importantly, NO is a well-established inhibitor of platelet aggregation.[38-40]

Therefore the interesting features of the purinergic platelet activators are their ability to stimulate NO production and induce platelet activation at the same time. Natural platelet antagonists such as NO and prostacyclin (PGI2) up regulate the production of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). PGI2 activates G_s coupled IP receptor, stimulates adenylyl cyclase and increases the concentration of cAMP.[41] NO increases cGMP and thereby increases the cAMP concentration as illustrated in Figure 1.2.[42] NO in the circulation is mainly generated through endothelial nitric oxide synthase (eNOS) (NOS III, a

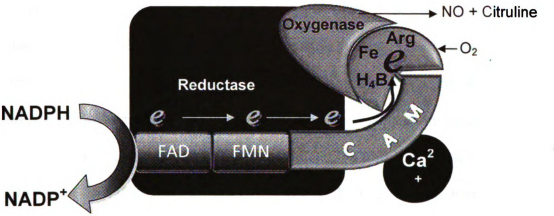

Figure 1.4 Electron transfer pathway and NO production in eNOS. Electrons (e-) are donated by NADPH to the reductase domain of the enzyme (eNOS) and proceed via FAD and FMN redox carriers to the oxygenase domain. There they interact with the heme iron and BH4 at the active site to catalyze the reaction of oxygen with L-arginine, generating citrulline and NO as products. Electron flow through the reductase domain requires the presence of bound calcium-calmodulin (Ca^{2+}/CaM).[43]

constitutively expressed NOS) and the main source is the endothelium. Other cell types with eNOS in the cardio-vascular system are red blood cells (RBCs), platelets, cardiac myocytes and megakaryocytes.[42,44] Endothelium derived NO prevents platelet adhesion to the endothelium and helps in vascular tone regulation. However there is also a considerable amount of evidence suggesting that platelet derived NO plays numerous roles in platelet function and vascular tone regulation.[1,45-49] NO life span in the circulation is limited to 3-10 seconds.[50] NO bio-reactivity should rapidly dissipate and only therefore affects cells within close diffusible range of the site of production. Therefore the diffusion-mediated delivery of NO from the endothelium to circulating platelets may be limited implying that platelet derived NO should be significant in platelet behavior. Therefore, purine nucleotides like ATP and ADP, which are stimuli for NO production and platelet activation, may be able to inhibit platelet activation or activate platelets depending on the local concentration of each.

eNOS regulation and NO production in platelets: Platelet eNOS is thought to be regulated in a manner similar to the eNOS in endothelial cells, which is primarily activated an increase in $Ca^{2+}i$ and the formation of the calcium calmodulin complex (Ca-CAM). Ca-CAM binding facilitates electron transfer from the reductase domain to the oxygenase domain of eNOS.[51] The reductase domain consists of FAD and FMN cofactors and transfers electrons from NADPH to the oxygenase domain. The oxygenase domain has binding sites for heme and arginine and facilitates the conversion of L-arginine to L-citrulline producing NO as a byproduct (Figure 1.4).[43,52,53]

It has been shown that increased NO production by the endothelium exposed to shear stress is Ca^{2+} independent.[54,55] Bradykinin induced endothelium NO production can be blocked by calmodulin inhibitors and seems to be calcium dependent.[56] Also ATP and ADP induced $Ca^{2+}i$ increase in platelets stimulate platelet NO production. Therefore, eNOS is possibly regulated by both the Ca-CAM dependent mediated electron flux as well as the shear stress induced activation of eNOS.[54,57] eNOS is a membrane associated protein and its localization depends on the co-translational modifications and post translational modifications.[55] This facilitates eNOS attachment to golgi and caveolae regions. Golgi and caveolae are frequently subjected to movements and therefore these localizations of eNOS provide more evidence for shear induced eNOS activation.[58]

1.2.4 Platelet associated disorders

Platelet associated disorders can be can be attributed to unusual platelet availability, unusual platelet function or both. Some of these conditions are acquired while others are congenital. Broadly, defined conditions where unusually low platelet counts prevail are called thrombocytopenia[59,60] while high count conditions are classified as thrombocytosis. [61,62] Platelet function disorders are very diverse and heterogeneous. The dysfunction can be a result of the receptors, signal transduction pathways, granule contents, cytoskeletal proteins or platelet procoagulant activity.[63-66] A detailed classification of platelet function disorders id shown in table 1.1.

Disorders with known or presumed defects intrinsic to platelets

Primary defects of adhesion	(a) Bernard–Soulier Syndrome (b) platelet-type von Willebrand disease
Primary defects of aggregation	Glanzmann thrombasthenia Defects of secretion and/or signal transduction (excluding granule deficiencies) affecting: (a) platelet-agonist receptors for thromboxane A2, ADP or collagen (b) thromboxane generation (impaired liberation of arachidonic acid, or deficiencies of cyclooxygenase-1 or thromboxane synthase) (c) G-protein activation (d) phosphatidyl inositol metabolism defects, such as phospholipase C deficiency (e) calcium mobilization (f) protein phosphorylation
Disorders affecting platelet granules or their contents	(a) d-granule deficiency, including forms with pigment abnormalities (e.g. Hermansky–Pudlak Syndrome) (b) gray platelet syndrome (a-granule deficiency) (c) combined ad-granule deficiency (ad-storage pool deficiency) (d) Quebec platelet disorder (increased platelet u-PA and a-granule protein degradation)
Defects of platelet procoagulant function	(a) Scott Syndrome (b) factor V New York
Defects in structure or in cytoskeletal proteins	(a) MYH9-related disorders (formerly known as May Hegglin anomaly, Sebastian syndrome, Alport's or Fechner syndrome) (b) Wiskott–Aldrich Syndrome protein defects (includes some forms of X-linked thrombocytopenia) (c) platelet spherocytosis (d) macrothrombocytopenia with cytoskeletal abnormalities and absent shape change (e) microvesicle generation defects
Miscellaneous disorders	(a) Montreal Platelet Syndrome (b) X-linked macrothrombocytopenia (not linked to the WASP gene) (c) Paris-Trousseau Syndrome (d) thrombocytopenia with absent radius syndrome (e) familial thrombocytopenia with predisposition to leukemia (f) congenital amegakaryocytic thrombocytopenia

Disorders with defects extrinsic to platelets

	von Willebrand factor deficiency afibrinogenemia

Table 1.1 Classification of platelet disorders[63]

1.3 PLATELETS IN CARDIOVASCULAR DISEASES AND ANTIPLATELET THERAPY

Platelets play a critical role in the development and pathology of cardiovascular disease including atherosclerosis, coronary artery disease (CAD), hypertension, heart attack and stroke. Conditions such as diabetes mellitus, of which a symptom is hyperactive platelets,[67] has been shown to have a strong link with cardiovascular disease.[68]

Hyperactive platelets in arterial thrombus formation are one of the major concerns in cardiovascular therapy.[69-71] Antiplatelet drugs are frequently used to reduce the platelet hyperactivity and the cost of antiplatelet therapeutics in the year 2005 was US$ 6.2 billion.[72] Existing pre and post thrombosis treatments commonly, known as antithrombotic drugs, can be classified in to three basic classes: anticoagulants, thrombolytics and antiplatelet.[73] Anticoagulants are used to prevent blood coagulation mechanisms often used in blood transfusion and surgical procedures. Thrombolytic drugs are used to dissolve thrombi after a thrombotic condition in the circulation such as myocardial infarction or ischemic strokes. Out of the three, antiplatelet therapy is of greater concern as it is used not only to prevent the initiation of thrombus formation but also regulate platelet mediated inflammatory conditions.

1.3.1 Antiplatelet therapy associated with purinergic signaling

Despite the fact that current antiplatelet therapeutics serve remarkably in vascular disease management, there are limitations due to drug efficacy in different clinical circumstances. Lack of direct evidence for cardiovascular event prevention and insufficient signal transduction information of currently marketed antiplatelet drugs are the major concerns in novel antiplatelet target identification and drug development. Current antiplatelet drugs can be broadly categorized to four different groups based on their targeted signal transduction pathway: cyclooxygenase1 (COX1) inhibitors, P2Y12 inhibitors, αIIβIII inhibitors and PDE inhibitors.[71] Except COX1 inhibitors, the others directly regulate purinergic signal transduction pathways.

P2Y12 inhibitors: P2Y12 is GPCR associated with Gαi which inhibits adenylyl cyclase upon ADP binding, regulating cAMP generation. Inhibition of this receptor results in increased cytosolic cAMP, which eventually inhibits $Ca^{2+}i$ mediated platelet activation and aggregation.[33,74] Even under P2Y12 inhibition, P2Y1 still mediates platelet dense granular Ca^{2+} mobilization leading to $Ca^{2+}i$ increase. Increased adenylyl cyclase activity and subsequent cAMP increase results in $Ca^{2+}i$ granulation preventing αIIβIII activation. An example of a P2Y12 inhibitor is ticlopidin, an older drug belonging to the thienopyridine family, it was later replaced by Plavix (clopidogrel).[75,76]

PDE inhibitors (dipyridamoles): Clinical usage of dipyridamole started in the early 1960s as a coronary vasodilator. Dipyridamole's ability to inhibit platelet adhesiveness

to glass (ex-vivo) in patients with coronary artery disease led Boehringer Ingelheim to introduce it as an antithrombotic agent.[77] By inhibiting the enzyme cyclic guanine monophosphate (cGMP) phosphodiesterase (PDE), bipyridamole inhibits the breakdown of cGMP and cAMP, resulting in increased levels of cAMP and cGMP.[71,78-80] Also combinatorial therapy of bipyridamole in combination with acetyl salicylic acid has been found much more effective as an antiplatelet agent than individual therapies.[81]

αIIβIII inhibitors: Glycoprotein IIb/IIIa inhibitors prevent platelet aggregation and adhesion by preventing fibrinogen, vWF and fibronectin binding to αIIβIII. GP IIb/IIIa heterodimeric protein belongs to the family of integrins consist of a α2- and β3-subunit.[82] Binding three or four cations (Ca^{2+}) to the α2 sub unit initiates conformational changes required to stabilize the GPIIb/IIIa heterodimer. Ability of GPIIb/IIIa antagonists to bind to both resting and activated platelets is a very important property to retard ongoing platelet activation and prevent activation initiation.[83] GPIIb/IIIa inhibitors can broadly be divided two categories, inhibitors with affinity to the receptors and competitive inhibitors that compete with natural ligands such as fibrinogen for binding.[84]

1.3.2 Failures/insufficiency in current antiplatelet therapy- necessity of new drug targets

Existence of hyperactive platelets in entirely different clinical situations such as type 1 and type 2 diabetes, cystic fibrosis, and primary pulmonary hypertension[85-88],

may explain the lack of universal antiplatelet therapeutics and failures of current therapeutics in some clinical situations. For an example, aspirin and clopidogrel resistance in diabetes is a growing concern in the clinical community.[89-92] Although there are different explanations for this phenomena, it has never been viewed as preventing ADP-induced essential NO production. In diabetes, RBCs are less deformable and release less ATP in to the circulation.[35] Lack of bioavailable ATP leads decreased NO production in endothelial cells and in platelets, making platelets more susceptible to activation.

When clopidogrel binds to the P2Y12 ADP receptor, $Ca^{2+}i$ concentrations may be decreased even below the basal level. Although clopidogrel would work as an effective antiplatelet agent in a clinical situation where bioavailable ATP is abnormally high, in a diabetes-like situation, it would make the situation worse. Therefore, antiplatelet therapy should be developed as customized therapy after evaluating certain parameters like RBC deformability and ATP bioavailability. However, this only can be achieved by identifying new targets of signal transduction pathways that are involved in platelet function. ATP activated P2X signal transduction pathway has never been investigated as a potential drug target in antiplatelet therapy.

1.4 A BRIEF INTRODUCTION TO THE EFFECT OF DEFECTIVE CFTR ON MACROPHAGE FUNCTION IN CYSTIC FIBROSIS

Cystic fibrosis (CF) is a disease where bacterial infections in the lungs and associated inflammations are the main causes of death (CF lung disease).[93] Lung macrophages (alveolar macrophages) are mainly responsible in protecting lungs from pathogen invasion and harmful substances.[94] CF is a result of a genetic mutation in the fibrosis transmembrane conductor regulator (CFTR) gene and its product, CFTR protein.[95] To date, CFTR mutations in CF macrophages have not been correlated to alveolar macrophage function linked to CF lung disease.

A portion of this dissertation describes the correlation between cellular glucose uptake and subsequent ATP release that influences the phagocytosis process of alveolar macrophages, providing the significance of defective macrophage-CFTR in this process. CFTR is a protein directly facilitating or assisting cytosolic ATP release to the extracellular matrix.

1.4.1 CF pathogenesis and CFTR

Cystic fibrosis (CF) is one of the most common lethal autosomal recessive disorders especially, in the Caucasian population[96]. Statistical data shows that nearly 1 in every 2500 Caucasian children is diagnosed with CF in their early childhood. People

of European descent carry one gene for CF, making it the most common genetic disease among this group.

CF is a genetic disorder caused by a mutation in a gene, Cystic Fibrosis Transmembrane Conductance Regulator or commonly known as CFTR gene[96]. CFTR protein is identified as a cAMP regulated chloride channel present in the cell membrane[97,98]. Certain mutations of the CFTR protein make it inactive, which affects its functions, including chloride and adenosine triphosphate (ATP) conductance. ENaC, an epithelial sodium ion channel, regulated by CFTR is essential for the re-absorption of electrolytes in the sweat duct and the respiratory epithelia[99]. Disturbances to this re-absorption process in CF result in complications in the epithelial lining of organs such as the lungs, pancreas, intestine, liver, male reproductive tract, and sweat glands. As water follows the same direction as salts, CF patients are found to have airways with unusually dehydrated mucous secretions that obstruct the luminal space.

Lungs are the largest exposed organs in the body to the outside environment. Innate immunity is the mechanism that keeps lungs free from infections, which is primarily governed by macrophages. Defective CFTR in pulmonary epithelia disturbs the salt balance, reduces airway surface liquid (ASL) hydration.[100] Reduction of ASL volume leads to accumulation of thick mucus layer on the top of the epithelia and prevents airways surface clearance making it more vulnerable to infections. As a result of decreased mucus clearance, CF airways become fertile ground for bacteria and viruses. Bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* have a fertile environment in these mucus layers and grow into infections and cause inflammations in CF lungs.[101,102] These infections are the prime cause of death in people

with CF, although advancements in CF therapy has significantly increased the life span of CF patients.[103]

1.4.2 Defective CFTR and alveolar macrophages

Macrophages are mononuclear phagocytes derived from blood monocytes.[104-106] Alveolar macrophages are the front line defense system in the lungs against pathogen and particulate matter invasion. Other than its main function, phagocytosis, alveolar macrophages are involved in numerous functions, such as bactericidal activity, antigen presentation, tumor cytotoxicity, removal of aged or damaged cells, repair of injured tissue, bone resorption, and special lipid metabolism.[105] A large number of macrophage destruction due its defense activities is predicted by considering 1.5 X 10^6 monocyte production in mouse bone marrow a day.[107]

Ingestion of pathogens or phagocytosis by alveolar macrophages is the one of the foundation mechanisms of pulmonary innate immunity against pathogen invasion. There are conflicting evidences in the literature about the effect of CFTR on macrophage bacterial killing ability. One such study demonstrated the requirement of CFTR for phagosomal acidification and subsequent bacterial killing in 1996.[108] Similar kind of studies performed later denied the involvement of CFTR in macrophage phagosomal associated bacterial killing.[93,109] In brief, the role of defective CFTR in alveolar macrophage on its function as well as if such dysfunction exists, the effect of that particular macrophage dysfunction on CF pathogenesis has not been explained.

It has been shown that the macrophage glucose transport mainly mediated through facilitative glucose transporter GLUT1 and *P.aeruginosa* ingestion is glucose dependent.[110] Reduced glucose uptake in any cell should results in decreased reducing power which may results in reduced bactericidal ability in macrophages. Failure to release ATP into the extracellular matrix due to the CFTR dysfunction can create a negative feed back on glucose uptake and also it has been shown that the accumulation of ATP in the cytosol leads to GLIT1 inhibition.[111] Theoretically it is clear that dysfunction if CFTR in CF may result in reduced ATP release which eventually inhibit macrophage glucose uptake leading to the decreased phagocytic activity. In an attempt to reconcile these diverse findings and to mimic the alveolar macrophage CFTR dysfunction in CF lung disease, investigations in chapter 4 were performed. Here we examined the effect of CFTR inhibition on rabbit alveolar macrophage (RAM) phagocytosis and glucose transport. Previously developed glucose sensitizer for RBCs, Zn activated C-peptide,[112] has been used to investigate whether it can enhance the glucose uptake and improve the opsonized bacteria particle ingestion by alveolar macrophages.

1.5 ANALYTICAL TOOLS TO EVALUATE PLATELET AND MACROPHAGE FUNCTION

Although the problems that are investigated in this dissertation are biological, highly selective analytical tools described here enabled us to take measurements earlier described as difficult measurements in the literature. The reason behind our success

may be due to the lack of customizing ability in most of the automated instruments available in conventional biomedical laboratories. Here, we briefly describe the basic principles of analytical techniques used to evaluate key components of the signal transduction pathways in platelets and macrophages such as ATP, intercellular NO, $Ca^{2+}i$, platelet aggregation and cellular glucose uptake.

1.5.1 Chemiluminescence assays for cellular ATP release measurements.

Evaluation of standard ATP assay and the ATP released from platelets or RBCs were conducted with the luciferin/luciferase chemiluminescence reaction related to the reaction shown in Figure 1.5.[113] ATP concentration is proportional to the chemiluminescence intensity. The sensitivity of the assay is enhanced by addition of 2 mg of D-luciferin (Sigma, St. Louis, MO) to the crude firefly extract (Sigma, St. Louis, MO). The luciferin/luciferase solutions were prepared by diluting the luciferin in 5 ml of distilled; deionized 18.2 MΩ water (DDW) and adding it to a vial containing 100 mg luciferase. The luciferin/luciferase mixture was prepared on the day of use.

1.5.2 Fluorescence determination of intracellular NO and $Ca^{2+}i$

Fluorescence imaging and spectroscopy is being used widely to study living cells due to its sensitivity, ease of use, rapidity, reproducibility and adaptability. Detection of picomolar concentrations of analytes in a single cell is currently achievable

with the appropriate fluorescence probe and the instrumentation.[114] A cell permeable florescence probe, DAF-FM diacetate (4-amino-5-methylamino- 2′, 7′-difluorofluorescein diacetate) has been used to detect intracellular NO production in platelets. Once in the cell, cellular esterases cleave the ester moiety and forms DAF-FM, which is the active probe. Although the quantum yield of DAF-FM is ~0.005, the fluorescence intensity increases about 160-fold, to ~0.81, after reacting with nitric oxide.[115] The ability of use in several instruments such as flow cytometers, microscopes, fluorescent plate readers and fluorometers with excitation/emission maxima of 495/515 nm, make DAF-FM DA a versatile fluorescence probe.

An acetomethyl ester of fluo 4, fluo 4 AM, (4-(6-acetoxymethoxy-2,7-difluoro-3-oxo-9-xanthenyl)-4'-methyl-2,2'-(ethylenedioxy)dianiline-n,n,n',n'-tetraacetic acid tetrakis (acetoxymethyl)ester) has been used to determine $Ca^{2+}i$ in platelets. The quantum yield of DAF-FM is ~0.14, K_d for Ca^{2+} of 390 nM, the fluorescence intensity increases about 100-fold after reacting with Ca^{2+} and the Ca^{2+} free indicator in non fluorescent (Figure 1.6).[116,117]

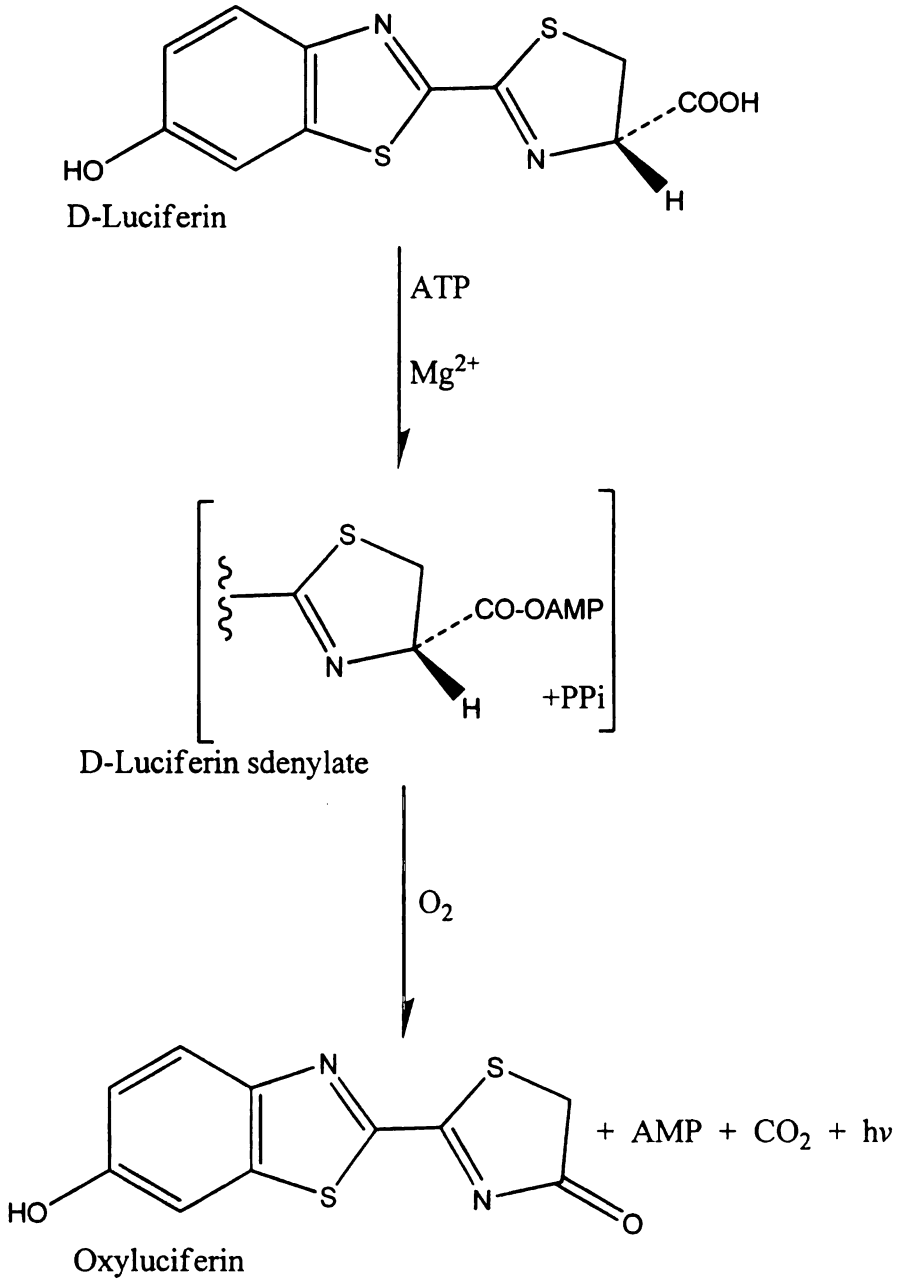

Figure 1.5 Schematic representation of luciferin luciferase bioluminescence reaction[113]

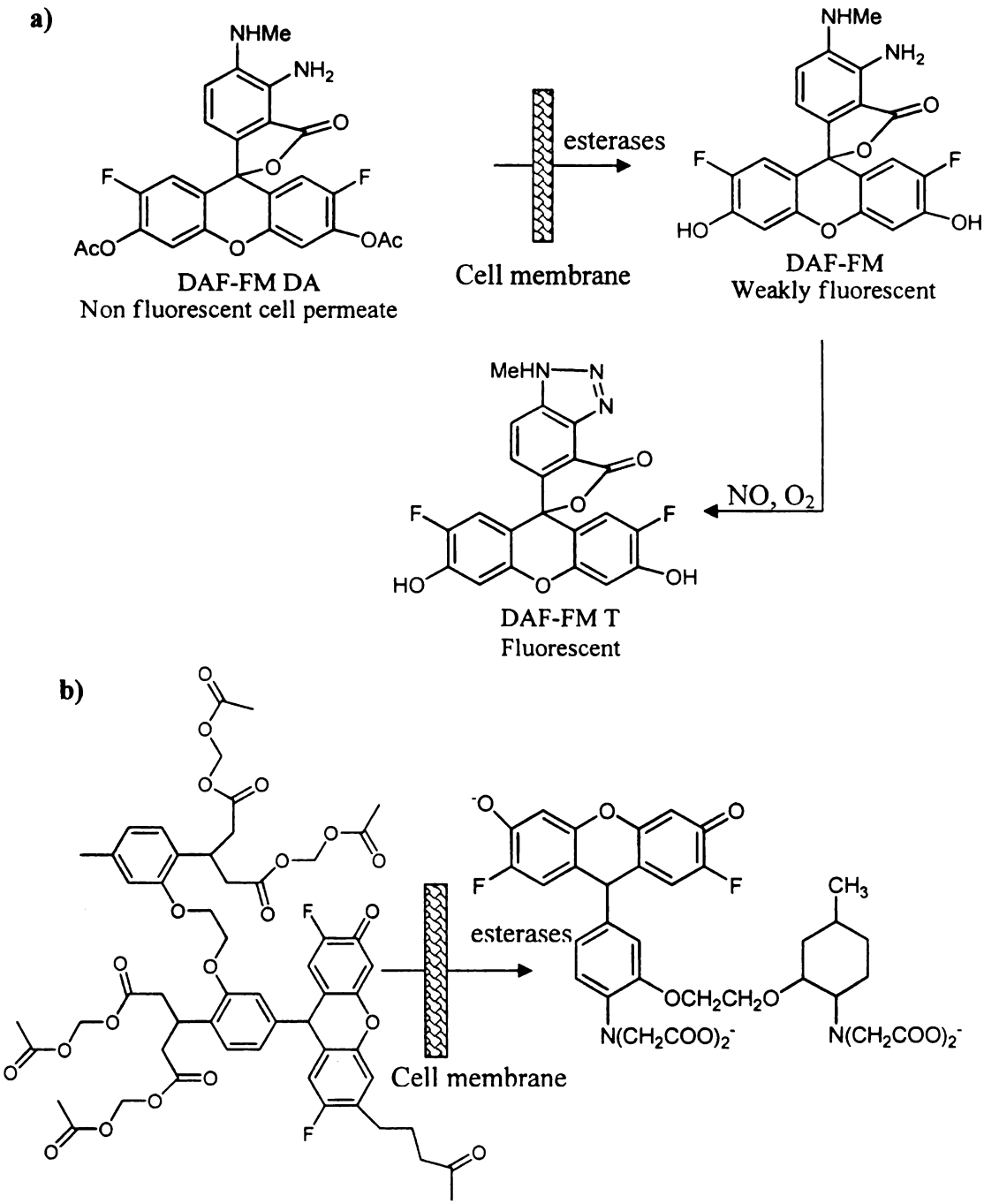

Figure 1.6 Fluorescence probes used in the determination of a) NO, and b) $Ca^{2+}i$, their cell permeation mechanism and active probe formed after cellular esterase mediated cleavage of diacetate moiety in DAF FM-DA and acetomethylester moiety in fluo 4 AM[115,116,117]

1.5.3 Light transmittance aggregometry for platelet aggregation

Platelet aggregometry is the most frequently used technique to screen patients for inherited or acquired defects of platelet function ever since its introduction in 1962 by Born [118,119].Optical aggregometry measures the increase in light transmission through platelet-rich plasma or washed platelet suspended in an appropriate buffer, that occurs when platelets are aggregated upon agonist addition (Figure 1.7).[120]

$$\text{Maximum aggregation [\%]} = 100\,\% \times \frac{\text{(aggregation at start-aggregation maximum)}}{\text{(aggregation at start-platelet poor plasma)}}$$

1.5.4 Liquid scintillation counting for cellular glucose uptake measurements

Liquid scintillation counting (LSC) was introduced in 1950 with the idea of improving counting efficiency of low energy β emitters. Currently, LSC offers the counting efficiencies of the order of *55%* for ^{3}H and 95% for ^{14}C.[121] In LSC, fraction of the energy of ionizing radiation emitted by the isotope is converted in to light by the scintillation cocktail (Figure 1.8). Scintillation cocktail basically consists of scintillator molecules dissolved in an organic solvent. Liquid scintillation cocktail composition should enable efficient transfer of energy between the solvent and scintillation molecules as well as coexistence of the aqueous radioactive solution with the organic solvent. Earlier benzene and toluene were used as the solvent but less toxic solvents like xylene or pseudocumene are being used. In the last twenty years, a new

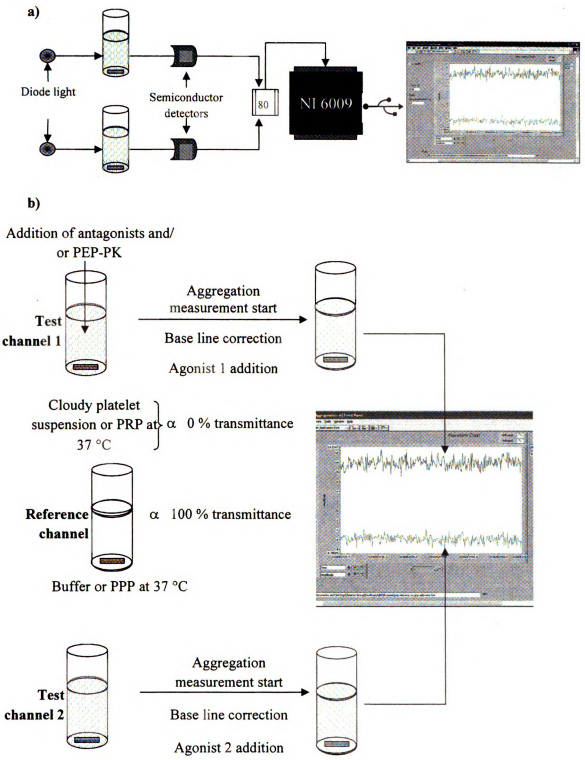

Figure 1.7 a) Schematic diagram of a Chronolog 490-2D aggregometer **b)** Working principle of an aggregometer, in brief, tests in each channel are considered as 100 % translucent suspensions compared to the reference sample which is considered as 100 % transparent.

-generation of aromatic solvents like di-isopropylnaphthalene (DIN), phenylxylylethane (PXE) or dodecylbenzene (LAB) has been developed. When radionuclide decay radiation passes through the solvent, incident electrons, or secondary electrons created by the interaction of radiation with the solvent. Approximately 10% of the energy is transferred to excited singlet and triplet states. Relaxation of excited singlet state to very quickly to their ground state S1. Triplet states lose their energy by internal conversion and cannot directly emit light. The energy migrates from one solvent molecule to another on a sub-nanosecond time scale until the energy is trapped by a solute molecule or dissipated as heat. Energy trapped in primary scintillation molecules than transfer to the secondary scintillation molecules which shift the wavelength and emit the light in the detection range of the PMT.(adapted from reference 122)[122]

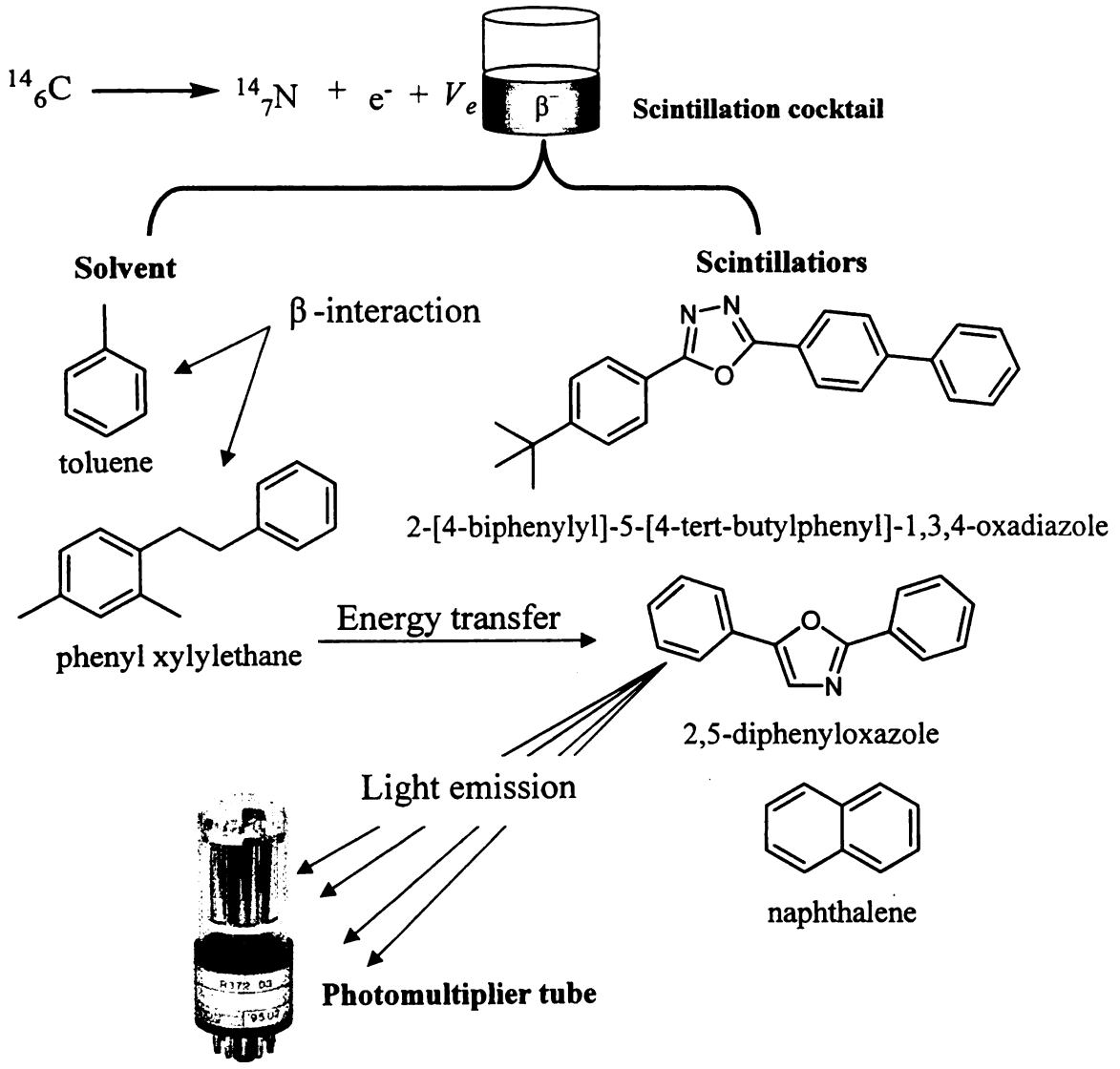

Figure 1.8 Basics of liquid scintillation counting (LSC), the solvent portion of an LSC cocktail comprises from 60 - 99% of the total solution. When a radioisotope dissolved in the cocktail undergoes an emission event, solvent molecules act as efficient collectors of energy. The energy from these molecules passes back and forth among the solvent ring systems, allowing efficient capture by dissolved phosphors (primary and secondary scintillation molecules). Primary Scintillatiors provide the conversion of captured energy to the emission of light which is detected at the photomultiplier tube.

1.6 THESIS OBJECTIVE

1.6.1 Role of ATP and its receptor, P2X1, in platelet function

Purinergic signaling plays a very important role in platelet homeostasis. Particularly the complex multiple signal transduction pathways associated with ATP gated P2X1 ionotropic (conduct ions) channel family receptors, and ADP gated heptahellical metabotropic(do not conduct ions) receptors are key regulators of platelet function.[123] However, the involvement of the ATP-gated P2X1 receptor in platelet homeostasis is controversial. ATP induced platelet shape change and ATP upregulated-collagen induced platelet activation reported in the literature are critical evidence for ATP-P2X1 aggregatory effect on platelets.[3,124-126] In vivo studies conducted on the P2X1 knockout mice ($P2X1^{-/-}$) has shown reduced mortality due to systemic thromboembolism (a Lodgement of a blood clot causing blockage of a blood vessel), decreased size of mural thrombi (a blood clot in a large blood vessel) upon laser-induced vessel wall injury and the rapid thrombus clearance indicating the importance of P2X1 in cardiovascular diseases.[127]

There are specific difficulties associated with P2X1 receptor function assessment that prevents a complete understanding of its behavior. The major constraint is the ligand occupied rapid desensitization of the receptor. In the presence of ATP, the reported desensitization time of the P2X1 receptor is 47-107 ms.[128,129] It has been shown that stirring washed human platelets at 37 °C for 10 minutes causes a release of 10 attomoles of ATP per platelet without adding any agonist.[130] Therefore it is fair to estimate that

the ATP release from resting washed platelet over a course of time can easily desensitize its own P2X1 receptors. One of our major concerns on previous P2X1-ATP functionality analysis was the utilization of α,β–methylene ATP, a stable analogue of ATP. Although the characterization data for α,β–methylene ATP on human urinary bladder cell showed high specific binding to P2X1 compared to ATP(binding affinity order, α,β-methylene ATP > β,γ-methylene ATP > suramin > 2-methylthio ATP > ATP > ADP >> adenosine),[131] the subsequent receptor recycling and the overall receptor kinetics have not been explored well. Furthermore, the inability of suramin, a potent P2X antagonist to overcome the receptor mediated α,β–methylene ATP induced depolarization of P2X1 in rat isolated vagus nurves explains that, this unusual binding of α,β–Methylene ATP which may retard the receptor function and the recycling.[132] The dilemma of the use of apyrase to keep the P2X1 receptor in the functional state and the use of apyrase-sensitive ATP to stimulate P2X1 encouraged scientists to use α,β–methylene ATP, instead of a form of ATP quite similar to the bioavailable ATP. It has been shown that α,β–methylene ATP ATP is able to induce a small calcium influx just enough to elicit a platelet shape change but not the aggregation.[83,133,134]

Based on these findings, and others reporting a dual nature of ATP and its effects on platelet function, we anticipated that ATP has an anti-platelet effect at low levels of ATP due to its ability to increase platelet NO production and an aggregatory effect at higher concentrations of ATP due to increased $Ca^{2+}i$.[135,136] In addition we also provide data suggesting that the ATP gated P2X1 receptor activity is a major contributor to platelet aggregation.

In the present study also we investigated the behavior or P2X1 in the presence of exogenous ATP very closely. We have optimized conditions to complete platelet P2Y1 receptor inhibition, employed an enzymatic system to eliminate the accumulation of ADP due to ATP break down, and sensitize the platelets P2X1 receptor to evaluate its natural performance in the circulation. We also extended our study to evaluate the clinical significance of bio available ATP on platelet function and as well as the cross talk between P2X1 and P2Y types receptors.

Findings of the investigations described in this chapter 2 and 3 of thesis would bring more attention to the underestimated clinical significance of bioavailable ATP and the P2X1 receptor. We also believe that our finding will be very useful in future patient specific and customized antiplatelet therapy development. Due to overwhelming dependency of platelet function with RBCs and endothelial cells described here may be useful for platelet function regulation by RBC targeted therapeutics.

1.6.2 Inhibition of CFTR on glucose uptake and phagocytosis by alveolar macrophages

In recent years, CFTR has been widely investigated as a dynamic regulator in the cardiovascular system due to its involvement in RBCs derived ATP release.[2,137] However, in most of the CF investigations, CF pathology has been linked to the defective salt balance due to CFTR dysfunction in the airway epithelia.[102] But, it is impossible to explain the pathophysiology of CF lung disease associated with extensive bacterial infections and inflammations only by considering defective CFTR in lung

epithelial cells. It is dubious that CF lung disease is almost independent of the alveolar macrophage function, which is generally considered as the front line defense in the lungs against pathogen and particulate material intake. Therefore the investigations described in the chapter 4 of this dissertation were primarily designed to examine the effect of the CFTR dysfunction in alveolar macrophage function.

A substantial decreased phagocytic activity has been observed when RAM CFTR is inhibited. This was just a result of the initial effort of correlate CF pathogenesis with alveolar macrophage activity. It was speculated that the lack of CFTR activity retards the cytosolic ATP clearance and led to ATP accumulation in the macrophage cytosol. Accumulated ATP may result in GLUT1 inhibition and lead to decreased macrophage glucose uptake. Therefore, CFTR inhibition on macrophage glucose uptake was investigated. Here, CFTR inhibition has retarded the macrophage glucose uptake to a large extent which is similar to the reduction observed in macrophage bacteria particle ingestion after CFTR inhibition. At this point, it is clear that CFTR function is essential for cellular energy dynamics and CFTR failure leads to reduced glucose uptake eventually retarding CFTR phagocytosis against pathogen invasion.

Application of previously reported glucose sensitizer RBCs, Zn activated C-peptide[112] to the CFTR inhibited macrophages revealed the ability of Zn activated C-peptide to completely reverse the CFTR blockage elicited glucose uptake inhibition as well as opsonized bacteria particle ingestion. Therefore, investigations described in the chapter 4 of this dissertation have been able to correlate the effect of the mutated CFTR in CF macrophages with their dysfunction and with the extensive bacterial infections

and colonization in CF lungs. Although the mechanism is so far unclear, the ability of Zn activated C-peptide to enhance the macrophage glucose uptake and phagocytosis may lead to a novel aerosol type therapeutic agent that can be used as a macrophages sensitizer in CF lung disease.

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CHAPTER 2

2.1 EXTRACELLULAR BIOAVAILABLE ATP AND PLATELET FUNCTION

Hyperactive platelets are associated with many different disease states. For example, people with type 1 and type 2 diabetes, cystic fibrosis, and primary pulmonary hypertension are reported to have hyperactive platelets.[1-5] It has also been reported that people with these diseases have red blood cells (RBCs) that release less adenosine triphosphate (ATP) upon being subjected to deformation or pharmacological stimuli.[6-9] A possible implication of this decreased RBC-derived ATP release arises when considering that ATP is a recognized stimulus of nitric oxide (NO) production in the platelet. Importantly, NO is a well-established inhibitor of platelet aggregation.[10-12]

Adenine receptors found on platelets are major determinants in platelet function.[13,14] P2Y receptor family is for adenosine diphosphate (ADP), while the P2X receptor family is for ATP.[15-17] These receptors can be subdivided into 7 distinct P2X receptors (P2X1–7) and eight different P2Y (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 and P2Y14) receptors.[18] Two ADP receptors, the Gq-protein-coupled P2Y1 and Gi-protein-coupled P2Y12, and one ATP receptor, the P2X1 ion channel, have been identified on platelets.[19] Both the P2X1 and P2Y-type receptors are thought to contribute to platelet aggregation, although the exact role for the P2X1 receptor is not as well-defined as those for the P2Y –type receptors. In fact, some reports suggest that the P2X1 receptor is not required for platelet aggregation.[20]

2.2 A RELATIONSHIP BETWEEN P2X1 P2Y1 PLATELET RECEPTORS

2.2.1 P2X, P2Y receptor synergy

Although cross-talk between P2X ionotropic receptors and P2Y type metabotropic receptors has been shown for other cell types such as cells in the central nervous system (CNS),[18,21] such a relationship has never been established for platelets. When P2X1 is co-expressed with P2Y1 in Xenopus oocytes, it elicited more pronounced P2X1-mediated currents, suggesting that P2Y may be assisting optimum performance of P2X1. This work further suggested that P2X1 activation may not result from direct phosphorylation, but more likely a staurosporine-sensitive (staurosporine is a natural alkaloid which inhibits protein kinases through the prevention of ATP binding to the kinase) phosphorylation of an accessory protein in the P2X1 receptor complex.[22,23]

Other than the cross-talk with P2Y, there is emerging evidence for P2X receptor cross-talk with other types of receptors. Reports suggesting the possibility of ATP or ATP derivatives as antagonists for P2Y type receptors appeared a decade ago at a time when understanding of these receptors was limited.[24-26] Application of these non-hydrolysable ATP analogues as P2Y antagonists suggest two basic possibilities. The first one would be P2X1 may act as an ATPase, which would generate ADP in close proximity to the P2Y receptors, which induce P2Y activation. The other possibility is that there may be not direct P2Y1 inhibition; instead the lack of ADP generation with these ATP analogues leads to non-responsive P2Y receptors. However there is no direct

evidence to deny the possibility of the blockage of both P2X and P2Y type receptors by these ATP analogues. Therefore it is possible that both P2X and P2Y receptors work together to increase the $Ca^{2+}i$, and also to regulate the $Ca^{2+}i$ by cAMP-mediated Ca^{2+} down regulation and PKC mediated PLCβ inhibition.[27,28]

2.2.2 Common components in platelet purinergic signaling

The first common component in P2Y1 and P2X1 signaling is $Ca^{2+}i$. ATP ligand binding associated conformational changes lead to P2X1 cation channel opening resulting in Ca^{2+} influx. ADP binding to the P2Y1 receptor activates a small G-protein, Gq. Activation of Gq leads to the activation of phospholipase-C-β2 (PLCβ2), which eventually results in inositol triphosphate (IP3) and diacyl glycerol (DAG) production. IP3 and DAG catalyze the mobilization of the stored Ca^{2+} in the dense tubular system to the cytosol.[29,30] Although DAG activates PKC mean while inducing Ca^{2+} mobilization, PKC-associated direct inactivation of PLCβ through a phosphorylation reaction has been observed in several cell types, which helps to regulate the entire mechanism.[31,32] Therefore, elevated concentrations of $Ca^{2+}i$ can inhibit further ADP-mediated $Ca^{2+}i$ increase, but there is no evidence for increase in $Ca^{2+}i$ or on associated signaling pathway that can inhibit ATP mediated Ca^{2+} influx. It seems the down regulation of $Ca^{2+}i$ is mainly controlled by cAMP-associated $Ca^{2+}i$ granulation or PKC-associated direct PLCβ inhibition.

The second common component for P2X1 and P2Y1 associated signal transduction is NO. Ca^{2+}-calmodulin (Ca-CaM) activates eNOS in platelets to produce

NO, which prevents platelet activation. Under normal conditions with basal $Ca^{2+}i$, all the $Ca^{2+}i$ available for CAM should be consumed by eNOS[33]. The resultant NO facilitates high cAMP concentrations in the cytosol that is associated with $Ca^{2+}i$ re-granulation. Therefore, the basal levels of $Ca^{2+}i$ seem capable of mediating enough NO production to prevent any GPIIb/IIIa activation by the CAM.[34-36] In the case of substantial $Ca^{2+}i$ build-up, even in the presence of maximum inhibitory conditions elicited by NO, the $Ca^{2+}i$ surplus can induce platelet shape change through a myosin light chain kinase (MLCK) mediated mechanism and binds to the GPIIb/IIIa, both through a CAM mediated mechanism(Figure 2.2).[37,38]

2.3 INTERCELLULAR COMMUNICATIONS OF PLATELETS IN THE CIRCULATION

When evaluating platelet function it is very important to consider its surrounding environment in the circulation and its communication with other cell types. RBCs and endothelial cells are the two most abundant, and interacting cell types with platelets. Therefore, platelet function may highly depend on the metabolic and physical characteristics of these two cell types. Under normal circulatory conditions, vascular endothelium is antithrombotic (favors prevention of thrombus formation) but under certain conditions, it is prothrombotic (favors thrombus formation). Coagulation factor XIII, a transglutaminase has been shown to modulate platelet GPIIb/IIIa and endothelial integrin, αVβIII, leading to increased platelet adhesion to the endothelium.[39,40] Both plasma and platelet XIIIs are activated by thrombin.[41] NO plays major role in

cardiovascular tone regulation by inducing vasorelaxation, inhibiting platelet activation and subsequent aggregation. Although NO synthesis occurs in a range of cell types and tissues, including the vascular endothelium, macrophages, and platelets, endothelium derived NO is the main platelet inhibitor.[42,43] Binding of ATP to P2Y type receptors in the endothelium increases $Ca^{2+}i$ and activates eNOS, which produces NO.[44] Endothelium derived NO may pay an important role in preventing the adhesion of platelets traversing much closer to the endothelium. However, there should be another source of NO for platelets traversing away from endothelium to prevent their activation and aggregation. It has been shown that platelets produce their own NO and even increase the NO production upon activation.[44] The ability of NO released from platelets to inhibit platelet activation and further platelet recruitment for the aggregation process has been shown.[45]

One source for the endothelium to increase $Ca^{2+}i$ and eventually produce NO is ATP. The main extracellular ATP source in the circulation is RBCs. RBCs contain membrane-bound glycolytic enzymes and hold millimolar amounts of ATP, and release ATP upon hypoxia and deformation.[46,47] When traversing through resistance vessels, RBCs are subjected to mechanical deformation, which results in ATP release.[48,49] ATP released from RBCs stimulates endothelial NO production, which eventually leads to smooth muscle relaxation essential to regulate blood pressure. This ATP also interacts with platelets, stimulate their NO production and prevent platelet aggregation. Therefore, it is very important to have proper chemical communication between RBCs, endothelium, and platelets, as RBCs possess a remarkable physiological sensitivity to the changes in the cardiovascular system.[50]

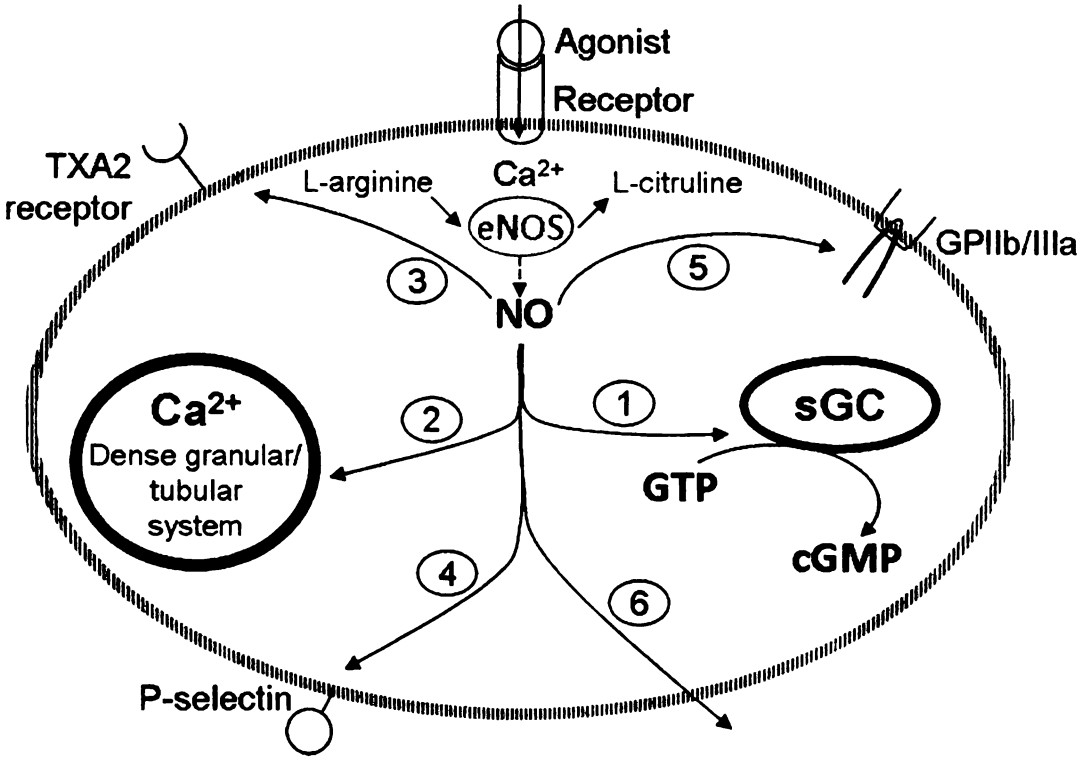

Figure 2.1 Effects of nitric oxide on platelet inhibition. Nitric oxide (NO) plays a central role in the inhibition of platelet function. NO, formed as a by-product of the conversion of L-arginine to L-citrulline, is involved in many mechanistic pathways contributing to platelet inhibition. In reaction (1), NO binds the heme moiety of soluble guanylyl cyclase, leading to the catalysis of guanosine triphosphate (GTP) to 3,5-cyclic guanosine monophosphate (cGMP): cGMP acts as NO's principal second messenger, leading to platelet inhibition. Nitric oxide has been associated with decreased intracellular Ca^{2+} levels through the inhibition of Ca^{2+} release from the dense tubular system (2). Decreased Ca^{2+} levels inhibit cytoskeletal rearrangement, α- and dense-granule release, and overall platelet activation. The thromboxane A2 (TXA2) receptor represents a complementary pathway involved in platelet activation. NO acts to catalyze the phosphorylation of the TXA2 receptor, preventing TXA2-mediated activation (3). The expression of P-selectin is required to facilitate platelet adhesion. NO leads to the down regulation of this protein, leading to inhibition of platelet adhesion (4). Fibrinogen, a bivalent molecule, promotes cross-linking between platelets via the GPIIb/IIIa receptors present on the platelet surface. NO attenuates platelet aggregation by decreasing the total number of GPIIb/IIIa receptors available to bind to fibrinogen (5), in addition to increasing the dissociation constant of these receptors for fibrinogen. Along with these various mechanisms through which NO exerts its inhibitory effects on platelets, it also diffuses through the platelet membrane (6) and inhibits neighboring platelets.[51]

Investigating one particular cell type would help to understand only a partial behavior of that particular cell type. It is very important to consider all possible combinations of effects that can be elicited by other cell types in the circulation simultaneously for a better understanding the behavior or one particular cell type. In order to predict the behavior of a platelet in traversing through an arteriole, it is necessary to consider the pressure exerted on the RBCs, RBC deformability, the extent of ATP release, and endothelial and platelet NO production, which eventually leads to vasodilation and platelet inhibition.

2.4 FATES OF $Ca^{2+}i$ MEDIATED BY ATP

Although its ATP-binding abilities may result in the subsequent production of NO, the P2X1 receptor has also been shown to participate in platelet activation and subsequent aggregation; through a mechanism which is not completely understood.[52] It has been reported that "priming" of the P2X1 receptor with a P2X1 agonist often sensitizes the platelet to expedited activation and aggregation through the P2Y–type receptors.[53] For example, it has been reported that addition of α-β Me-ATP, a stable ATP analogue, to the platelets will lead to an increased level of aggregation upon stimulation of the P2Y–type receptors.[54] While many theories exist for this phenomenon, definitive proof is elusive due to the difficulty in studying the P2X1 receptor and the numerous sub types from P2X1 to P2X7 in addition to the variety of methods by which platelets are prepared from whole blood.[55-57] Therefore, identifying all of the connections between the P2X1 and P2Y–type receptors has been difficult.

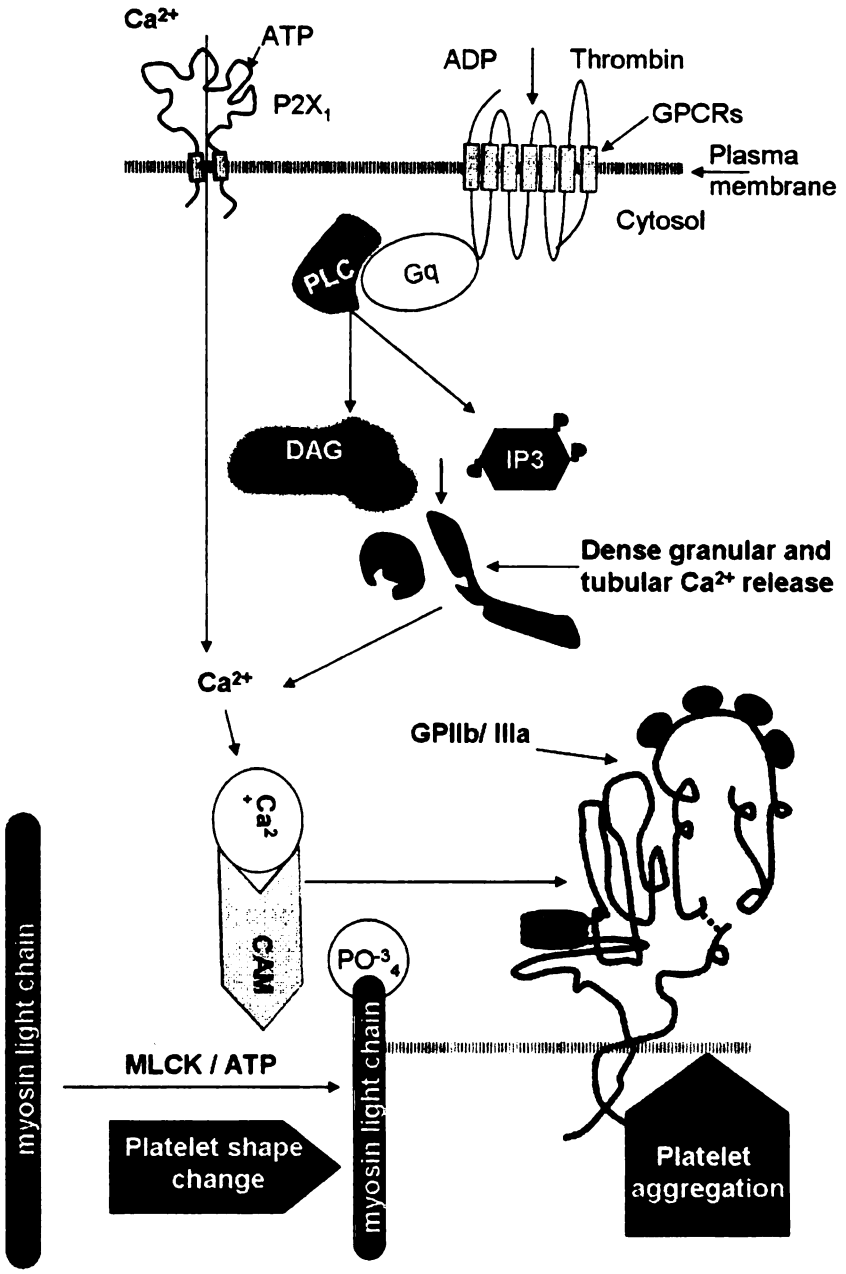

Figure 2.2 Both Ca^{2+} influx and stored Ca^{2+} mobilization increase $Ca^{2+}i$. Ca^{2+} carrier protein, calmodulin (CAM) then binds to the $Ca^{2+}i$ and transport and facilitate calcium binding to the myosin light chain kinase (MLCK) and glycoprotein IIb/IIIa (GPIIb/IIIa). Activated MLCK phosphorylates myosin light chains (MLC) eventually contacting them. MLC contraction results platelet shape change. Binding calcium activates GPIIb/IIIa. Activated GPIIb/IIIa provides binding sites for fibrinogen and vWF and initiates platelet aggregation.[37,38]

2.4.1 ATP mediated $Ca^{2+}i$ as a platelet antagonist

Recently, in a study designed to quantitatively determine platelet nitric oxide (NO) production using fluorescence spectrophotometry, an interesting feature of the NO production by platelets was observed.[58] Specifically, when the platelet NO production was stimulated using ATP, a steady increase in the fluorescence emission intensity (indicative of the NO production) was measured as a function of the concentration of ATP added to the platelets.

However, beyond a certain level of added ATP, the platelet NO production did not increase, rather, it reached a steady value, even in the presence of additional ATP. It has been shown previously that activated platelet derived NO can prevent neighboring platelet activation.[45] Substantial and sudden increases in $Ca^{2+}i$ is one of the features of platelet activation and aggregation. Considering the fact that platelets produce NO through Ca^{2+} dependent eNOS activation[59], it is easy to correlate the activation induced by $Ca^{2+}i$ increase and the subsequent NO production.[60] Therefore, under normal circulatory conditions, at a certain level of basal NO production, platelets may be able to inhibit their own aggregation.

2.4.2 ATP mediated $Ca^{2+}i$ as a platelet agonist

ATP induced and P2X1 mediated transient $Ca^{2+}i$ increase and the reversible decrease in light transmission corresponding to platelet aggregation[61] suggested the possibility of P2X1 mediated platelet aggregation. However, the extent of the $Ca^{2+}i$

increase and the reversible nature of the aggregation excludes the possibility of P2X1 to be a receptor responsible for platelet aggregation. Furthermore ATP induced P2X1 mediated $Ca^{2+}i$ increase and platelet shape change, but not the aggregation, have also been observed.[61] Interestingly in all these investigations, instead of ATP, a stable analogue of ATP, α,β-methylene ATP has been employed. Reasons to avoid ATP have been described in chapter 1. Here it is suspected that although α,β-methylene ATP has a higher binding affinity towards P2X1 than ATP, it may not serve as an effective P2X1 agonist. Therefore, if ATP is a more effective agonist than α,β-methylene ATP and capable of inducing higher $Ca^{2+}i$ increase, it is quite possible for ATP to be a more potent platelet agonist, rendering P2X1 to be an important target in antiplatelet therapy.

Based on these facts and other reports that suggests a dual nature of ATP toward platelet function,[62] we suspected that ATP has an anti-platelet effect at low levels of ATP (due to its ability to increase platelet NO production) and an aggregatory effect at higher concentrations of ATP.[63] Here, we provide evidence suggesting that the ATP-gated P2X1 receptor activity contributes to platelet inhibition and aggregation.

2.5 EXPERIMENTAL

Platelet purinergic signal transduction pathways seem to be interconnected with numerous secondary messengers like Ca^{2+}, Ca-CaM and NO. Evaluation of changes in these secondary messengers upon binding agonists such as ATP and ADP to their corresponding receptors on platelets would be important in understanding platelet homeostasis properly. Furthermore, the quantification of agonist induced platelet

granular secretions and the effect of platelets inhibitors like NO on platelet activation in terms of granular secretion would also assist the above purpose. Application of P2X1 specific inhibitors and evaluation of the concentration dependent effects of ATP on platelet $Ca^{2+}i$, NO and aggregation would provide more complete picture of the roles ATP and P2X1 in platelet function.

2.5.1 Platelet and RBC preparation

Rabbits were anesthetized with ketamine (8 ml/kg, i.m.) and xylazine (1 mg/kg, i.m.) followed by pentobarbital sodium (15 mg/kg i.v.). A cannula was placed in the trachea and the animals were ventilated with room air. A catheter was then placed into a carotid artery for administration of heparin and for phlebotomy. After heparin (500 units, i.v.), animals were exsanguinated and the whole blood collected in 50 ml tubes. Generally, 70 ml of blood was collected from the animal. Blood was centrifuged at 500 x g at 37 °C for 10 min. Packed RBCs were resuspended and washed three times in the physiological salt solution (PSS; in mM; 4.7 KCl, 2.0 $CaCl_2$, 1.2 $MgSO_4$, 140.5 NaCl, 21.0 tris(hydroxymethyl)aminomethane, and 11.1 dextrose with 5% bovine serum albumin, pH adjusted to 7.4). The platelet rich plasma (PRP) was decanted for the subsequent isolation of platelets. Platelets were isolated from the PRP by adding 1 ml of acid citrate dextrose (ACD) to 9 ml of the PRP and centrifuging at 37 °C for 10 min at 1500 x g.

2.5.2 ADP induced platelets activation in terms of platelet granular ATP release

Binding ADP to receptors P2Y1 and P2Y12 results in an increase in platelet $Ca^{2+}i$ that subsequently initiates platelet shape change and aggregation through the mechanisms shown in Figure 2.2. As a result of ADP induced platelet activation, the contents of platelet storage granules such as ATP stored in platelet dense granules are released in to the extracellular matrix. Therefore, the concentration of ATP released from platelets is a measure of platelet activation.

The amount of ATP released from platelets activated with ADP was determined by using fused-silica tubing that was intended to resemble vessels *in vivo*. The instrumentation employed consisted of a syringe pump (Harvard Apparatus, Boston MA) equipped with two 500 all syringes (Hamilton, Fisher Scientific), two pieces of ~40 cm long microbore tubing with an internal diameter of 75 μm and an outer diameter of 362 μm (Polymicro technologies, Phoenix, AZ). The platelet suspensions or ATP standards loaded in one of the syringes was pumped through the tubing at a rate of 3.35 μl min^{-1}. At the first tee, a stream of HBSS (for ATP standards) or ADP standard solutions (10-40μM) in HBSS (for platelet suspensions) was combined. This stream was then mixed at the second mixing tee with luciferin/luciferase (6.7 μL/min), which resulted in a chemiluminescence reaction that was detected with a photomultiplier tube (PMT) housed in a light excluding box. Two sets of experiments were conducted on each day using two different lengths of mixing tubing (100 μm ID), 12.5 and 18.5 cm, from first tee to second tee. The length of the tubing (100 μm ID) from second tee to the PMT window was kept at 5 cm. All other parameters in Figure 2.3 were kept constant. A working curve was constructed using five ATP standards between 0 and 1.5 μM. The

ATP standards were prepared from a stock 100 μM ATP in HBSS. All solutions were prepared on the day of the experiment.

2.5.3 Extracellular NO mediated platelet inhibition-as a measure of ADP induced ATP release

Although the NO mediated inhibition of platelet activation is well-known, quantitative estimation of such inhibition in terms of the reduction of ATP release from ADP activated platelets in a microflow system has never been reported. Nitric oxide was prepared as a 38 mM stock solution from spermine NONOate (Cayman Chemical, Ann Arbor, MI) by dissolving 10 mg of solid spermine NONOate in 1 ml of 0.01 M sodium hydroxide (NaOH) solution. Working solutions were prepared by dilution of the alkaline NONOate solution in 0.1 M phosphate buffer saline (PBS, pH 7.4). Spermine NONOate is reported to have a half-life of 39 min at 37°C in 0.1 M phosphate buffer and follows first order kinetics for its rate of decay.[36] The 0.38 μM working NONOate solutions were incubated in a water bath at 37°C for 15 min to ensure NO release from the donor

The experimental setup was designed to let the platelets to incubate in a solution that contains NO and then activate them with ADP while traversing through the micro bore tubing. A platelet suspension was mixed with an NO donor (spermine NONOate) at the first tee and then pumped through the 75μm diameter, 0.5 m long fused silica coil (Figure 2.4). The tubing segment connecting second and the third tee was to activate the

platelets with ADP. This activated platelet suspension was then combined with luciferin/luciferase at the third tee prior to moving through the detector assembly.

2.5.4 Effect of RBC derived ATP on platelet NO production in a micro flow system

Although evaluation of individual cellular responses upon the addition of an agonist is useful, multi cellular experimental platforms are more dynamic and provide real time information about cell behavior. As discussed in section 2.3, RBCs interact with the platelet in the circulation during normal hemostasis. In this experiment, the objective was to investigate the effect of deformation-induced ATP release from RBCs on platelet NO production. Demonstration of ATP mediated intracellular chemical communication between RBC derived ATP and platelet, is a critical part of mimicking the blood circulation in vitro. This experiment not only evaluates the effect of ATP on platelet-derived NO, which can easily be performed with ATP standards, but also many other known or unknown interactions between RBCs and platelets that might have an influence on platelet NO production. Therefore, we anticipated the changes of NO production in platelets interacting with RBCs to be proportional to the extent of the ATP release from interacting RBCs.

Measurements were performed using the diaminofluorofluorescein diacetate (DAF-FM DA) fluorescent probe for NO. A stock solution of DAF-FM DA (Molecular Probes/Invitrogen, Eugene, OR) was prepared by dissolving the DAF-FM DA in 20 μL of DMSO. Next, a working solution of DAF-FM DA was prepared by diluting 2 μL of

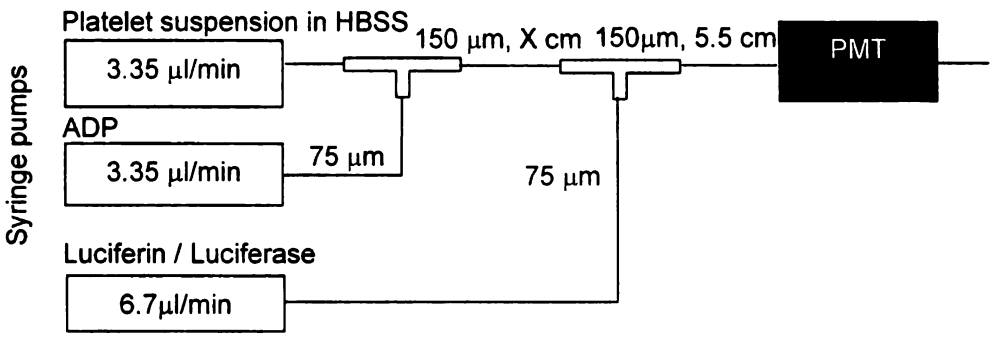

Figure 2.3 Two tee setup for investigation of platelet ATP release upon ADP addition. Platelets were allowed to mix with the agonist ADP at the first tee. Depending on the experiment, the length of the output tubing of the first tee was changed and platelets were expected to release ATP depending on the mixing time/the length of the mixing tubing. The released ATP was then reacted with luciferin/luciferase to produce chemiluminescence and the length of this tubing was kept 5.5cm, the least possible length which could go through the PMT window. All solutions were prepared on the day of the experiment.

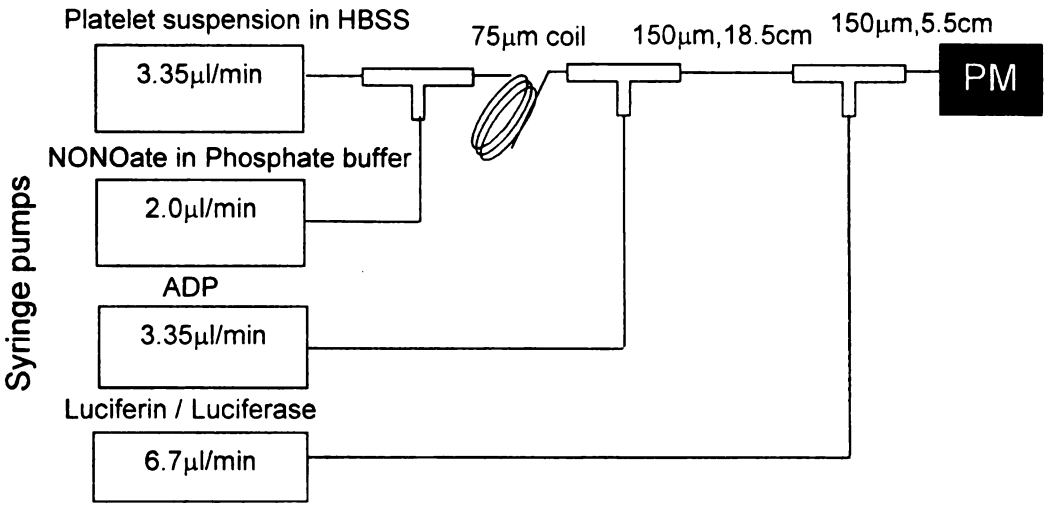

Figure 2.4 Three tee setup for the investigation of the nitric oxide inhibition of ADP-induced platelet activation. This setup facilitates platelets to initially interact with nitric oxide and then react with the ADP. ADP concentrations are 5-40 µM. In control experiment 0.1M phosphate buffer is pumped in place of NONOate.

the stock DAF-FM DA solution to 1 ml in HBSS. Next 100 μL of $\approx 10^8$ platelet suspensions were individually mixed with 50 μL of 10 μM DAF-FM DA in 2ml vials, diluted up to 500 μL and incubated for 10mins. Platelet mixtures without DAF-FM DA were prepared by mixing 100 μL of $\approx 10^8$ platelets in suspension with 400 μL of buffer. RBCs were separately incubated in the absence or presence of 80 nM iloprost.

Platelets were passed through a microbore tubing system consisting of three major components, namely, a dual syringe pump, the sections of microbore tubing, and a flow-through fluorescence detector (Jasco, Easton, MD) housed with a capillary flow cell. DAF-FM DA-loaded platelets and RBCs were pumped through a section of microbore tubing using a syringe pump to propel the solution at a flow rate of 6.7 μl min^{-1}. The pump is a conventional syringe pump where the syringe is easily accessible. Previous experience with this type of syringe has shown that platelet sedimentation during the measurement portion of the analysis is not problematic because the platelets are usually in solution as a suspension. The fluorescent signal that is produced in the platelets due to NO production was determined using the aforementioned flow-through fluorescence detector and monitored with a program written in-house with Lab View (National Instruments).

2.5.5 Fluorescence determination of platelet NO

As experiments in section 2.5.4 aimed to demonstrate the effect of RBC derived ATP on platelet NO production and the platelet sensitivity to the changes in

extracellular ATP, investigations described in this designed to correlate the interaction of ATP with its receptor, P2X1, and stimulation of platelet NO production.

Measurements were performed using the diaminofluorofluorescein diacetate (DAF-FM DA) fluorescent probe for NO. A stock solution of DAF-FM DA (Molecular Probes/Invitrogen, Eugene, OR) was prepared by dissolving the DAF-FM DA in 20 μL of DMSO. A working solution of DAF-FM DA was prepared by diluting 2 μL of the stock DAF-FM DA solution to 1 ml in HBSS. Aliquots of 100 μL of this working solution of DAF-FM DA were added to 400 μL of the platelet solution (3×10^8 platelets mL^{-1}) and allowed to incubate for 15 min. These platelets were washed twice with HBSS by centrifuging at 1500 g for 10 min. Finally, 100 μL of the washed, DAF-FM DA-loaded platelets were then resuspended in 400 μL of HBSS that contained 10 μM ATP. After 20 min, the fluorescence emission, resulting from NO production and the subsequent reaction with the fluorescence probe in the platelet, was determined. Fluorescence measurements were performed on a Fluoromax-4 spectrofluorometer (HORIBA Jobin Yvon, Edison, NJ) at room temperature. Emission spectra were obtained in a quartz cuvette with excitation at 495 nm and emission at 515 nm. Both excitation and emission slit widths of 2 nm were used in all experiments with sampling interval of 0.2 nm.

2.5.6 Fluorescence determination of platelet intracellular Ca^{2+}

Although sections 2.5.4 and 2.5.5 correlate the ATP binding to P2X1 and the subsequent platelet NO production, it does not provide the information about ATP

mediated P2X1 activation and related extracellular Ca^{2+} flux in to the cytosol. In this experiment, intracellular Ca^{2+} concentrations were determined using fluorescence spectroscopy. The expected result of this experiment was an increase in $Ca^{2+}i$ upon ATP binding and decrease in $Ca^{2+}i$ when platelet P2X1 is inhibited.

The fluorescence determinations were performed using Fluo 4 AM, an intracellular fluorescence probe for Ca^{2+}. The Fluo 4 AM stock solution was prepared by dissolving in 20 μL of DMSO. The working solution was prepared by diluting 5 μL of the stock solution in 1 ml of Ca^{2+}- free HBSS. To prepare the platelets for the determination of Ca^{2+}, the harvested platelets were sensitized with 0.28 U/ml apyrase in Ca^{2+} free HBSS. The sensitized platelets were then washed 2 times in a Ca^{2+}- free HBSS solution that did not contain apyrase in order to remove any residual apyrase from the initial wash. A 1 ml portion of these platelets (in the Ca^{2+}- free HBSS) was incubated with 500 μL of the working Fluo4-AM solution for 2 hours, centrifuged and again washed twice with Ca^{2+}- free HBSS. Next, 750 μL of this platelet sample were combined with 300 μL of Ca^{2+}- free HBSS that contained 0.67 mM NF 449. The remaining 750 μL of the platelet sample were treated with 300 μL of Ca^{2+}- free HBSS alone. Finally, 100 μL of NF 449-treated or non-treated platelets were added to 250 μL of HBSS that contained 40 mM Ca^{2+} and 10 μM ATP and allowed to incubate for 30 min before measuring the fluorescence emission from the sample at 516 nm (with 496 excitation) using the fluorescence parameters described in 2.5.6

2.5.7 Atomic absorption spectroscopy for platelet total Ca^{2+}

Although with fluorescence spectroscopy, we were able to see the reduction in $Ca^{2+}i$ upon P2X1 inhibition, we were not able to observe a significant increase in $Ca^{2+}i$ upon ATP binding. It was speculative that this may be a result of the fast kinetics of $Ca^{2+}i$ regulation in the platelet cytosol. Therefore, to further verify the effect of ATP on platelet $Ca^{2+}i$, atomic absorption spectroscopy has been used to detect changes in total platelet Ca^{2+} concentration.

For Ca^{2+} influx measurements using atomic absorption, platelet suspensions were prepared in Ca^{2+}- free HBSS as described above for those measurements involving fluorescence determination of Ca^{2+} influx. The platelet samples were fixed with buffered Para formaldehyde (1.8%) and washed three times with Ca^{2+}- free HBSS by centrifugation at 2200 *g*. Before the final spin, the platelets were counted to ensure the platelet number for subsequent data analysis. After the final spin, platelet pellets were dissolved in 2.5 ml of distilled de-ionized water (DDW) and subjected to freezing with liquid nitrogen and thawing at 37°C three times to ensure complete lysis of the platelets. The lysed platelet samples were centrifuged at 3000 *g* for 15 min and the supernatant was analyzed for Ca^{2+} using a Hitachi Z-9000 atomic absorption spectrophotometer.

2.5.8 Platelet aggregation measurements

Experiments 2.5.4-2.5.7 were designed to asses the purinergic signal transduction pathway associated with ATP binding to P2X1. Although increases in

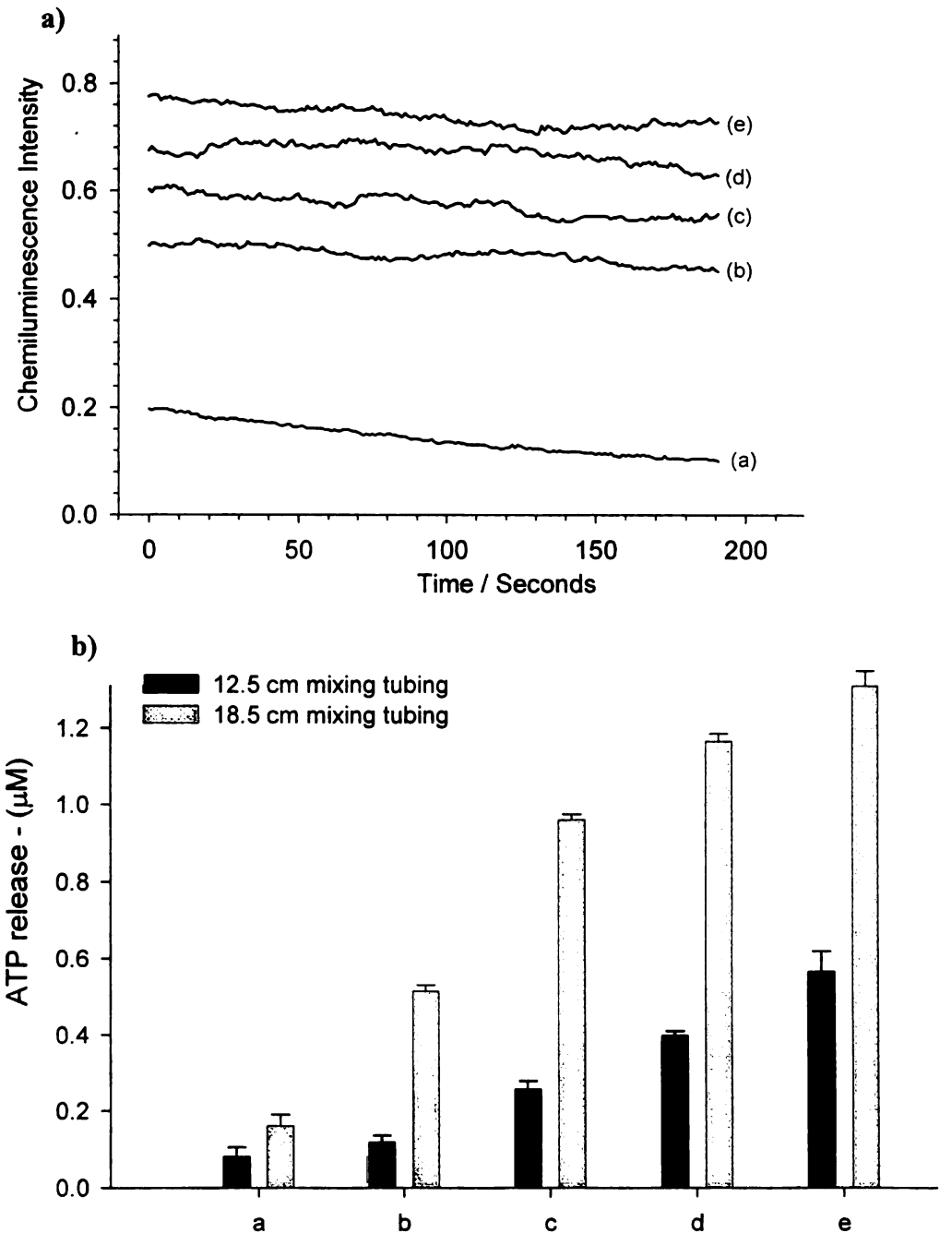

Figure 2.5 a) Real time curves corresponding to the platelet ATP release upon ADP addition. (a)-HBSS only (b) to (e) are platelets incubated with 10-40μM ADP respectively. **b)** Averaged standardized ATP release from platelet activated with ADP, a- + buffer, b- + 10μM, c: + 20 μM, d: + 30μM, e-+ 40 μM ADP. Here the results are presented, using ADP as an efficient agonist for platelet activation demonstrating the ability to use platelet derived ATP as a measure of platelet activation. *n=5*, error bars: standard error of the mean

platelet $Ca^{2+}i$ and subsequent NO production correlates the extracellular ATP concentration with platelet inhibition, the outcome of the same $Ca^{2+}i$ on MLCK induced platelet shape change and GPIIb/IIIa mediated aggregation were not conclusive. As platelet aggregometry is the most frequently used technique to screen patients for inherited or acquired defects of platelet function in terms of MLCK induced platelet shape change and GPIIb/IIIa mediated aggregation, platelet aggregation is used to interconnect extracellular ATP concentration with platelet activation. Due to the ability of both ATP and ADP to induce an increase in platelet $Ca^{2+}i$, increased aggregation was anticipated for both agonists. Inhibition of the P2X1 receptor was expected result in reduced ATP induced aggregation but not with ADP. The ability of increased $Ca^{2+}i$ to induce NO production, as well as integrin activation, made predictions very difficult for concentration dependency of ATP on platelet aggregation.

A Chronolog 490-2D aggregometer was employed for all platelet aggregation measurements. For each aggregation study, a 100 μL aliquot of platelets (3 x 10^8 platelets mL^{-1}) were added to 400 μL of buffered reagents (described for each study below). Reference and test cuvettes were analyzed during each aggregation trial; the contents were constantly stirred using P/N 311 stir bars and all studies were performed at 37 °C. Data was obtained at 10 data points per second for 30 min. For studies involving P2X1 inhibition, HBSS with 10 μM ATP was prepared. When investigating the effect of P2X1 inhibition, the HBSS also contained 20 μM NF 449, a competitive inhibitor of the P2X1 receptor. In separate studies involving the concentration dependency of ATP on platelet aggregation, the HBSS contained ATP concentrations ranging from 0 to 4 μM in ATP. For those studies involving the effect of ATP on

platelet aggregation in the absence of Ca^{2+}, Ca^{2+}- free HBSS was used to prepare the 10 μM ATP-containing solutions.

2.6 RESULTS AND DISCUSSION

2.6.1 ADP induced platelet activation and granular ATP release

ADP is one of the most potent agonists for platelet activation and aggregation.[64] Platelet activation has long been measured in terms of platelet ATP release.[65-68] Although there are different methods employed for platelet ATP detection,[65] the luciferin/luciferase ATP specific enzymatic assay is widely accepted as the most reliable method.[66-68] Figure 2.5 shows the platelet ATP release data obtained by using the two tee microbore tubing assembly described in Figure 2.3. When traversing through a 75 μM microbore fused silica tubing for 32.9 seconds, while reacting with 10 μM ADP, platelets release 42.7% higher ATP than platelets interacting with buffer alone. Increasing ADP exposure time from 32.9 to 42.4 seconds by lengthening the tubing resulted in a 2.4 fold increase in ATP release.

2.6.2 Inhibition of ADP induced platelet activation by extracellular NO

The effect of NO on platelet inhibition has been discussed under sections 2.3 and 2.4. Although the involvement of extracellular NO on platelet endothelial adhesion and changes in the components of signal transduction pathways have been reported

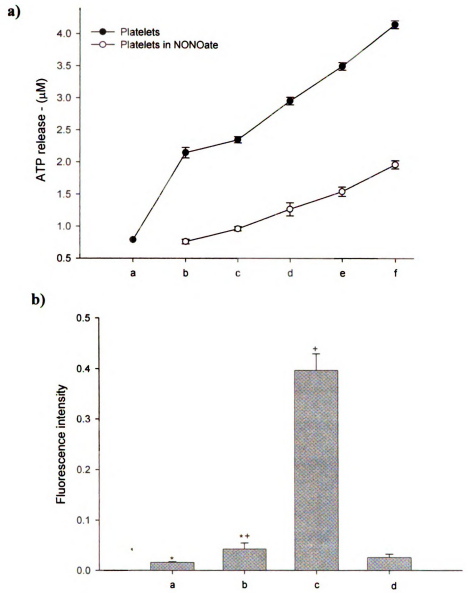

Figure 2.6 a). Inhibition of ATP release from ADP activated platelets in the presence of ~200 nM NO. Experiment was conducted with the use of the "3" microbore tubing assembly described in figure 2.4. a- platelets, b- platelets + 5 μM ADP, c- platelets + 10 μM ADP, d- platelets + 20 μM ADP, e- platelets + 30 μM ADP, f- platelets + 40, 200 nM NO was able to inhibit the ADP induced ATP release by 43%. Platelets concentration ~10^8 /ml **b).** RBC derived ATP on platelet NO production in a flow through system: a- Platelets, b-RBCs + Plt, c-RBCs (incubated with trental) + Platelets, d-RBCs (incubated with Diamide and then Trental) + Platelets, *n=3*, error bars: standard error of the mean

previously,[69,70] the effect of extracellular NO on ATP release from ADP activated platelets in a flow system has never been investigated. The three tee assembly in Figure 2.4 provided the essential elements to create a multi component flow base reactor.

2.6.3 ATP, a communicator between RBC and platelet

Previously, we described a fluorescence based method that enabled the quantitative determination of basal levels of NO in platelets and the amount of NO produced after stimulation with the platelet aggregation agonists ADP or ATP.[58] The collaborative study in this paper suggests a possible unique relationship between RBCs and platelets in the circulation. Specifically, RBCs may be able to communicate with the platelet through RBC-derived ATP, stimulating NO. In order to verify this relationship, a fluorescence based microflow experiment described in 2.2.4 was conducted.

Here, the ability to measure platelet-derived NO upon stimulation with ATP released from deformed RBCs in a microflow system is demonstrated. The percent increase or decrease in platelet-derived NO (measured spectrofluorometrically with the DAF-FM DA probe) is presented for RBCs in the presence or absence of iloprost. Iloprost is a prostacyclin analog that has been shown to increase the ATP release from RBCs and here iloprost resulted in a 9 fold increase in platelet NO production compared to the untreated RBCs. When RBCs were treated first with diamide, a known oxidant that reduces RBC membrane deformability, and then with iloprost, a 60 % reduction of platelet NO compared to the untreated RBCs, has been observed.

2.6.4 ATP and P2X1 in the regulation of platelet NO and cytosolic Ca^{2+}

The increase in platelet NO production upon addition of ATP was measured using DAF-FM DA, a fluorescent probe for NO (Figure 2.7). The percent increase, normalized to the fluorescence intensity for platelets with the probe, but no agonist, was 26.7 ± 7.7% for ATP, a value representing a significant increase in platelet NO production ($p < 0.001$). Though not quantitatively determined here, this 26.7 ± 7.7% increase corresponds to approximately 2.2×10^{-8} moles of NO per 3×10^{8} platelets based on a previous report.[58] A decrease in fluorescence intensity was measured when the platelets were incubated with L-NAME, a competitive inhibitor of nitric oxide synthase (NOS), to verify that the increase in fluorescence intensity was indeed due to the production of NO. The black bars in Figure 2.7 demonstrate the ability of the ATP to increase NO production in the platelets.

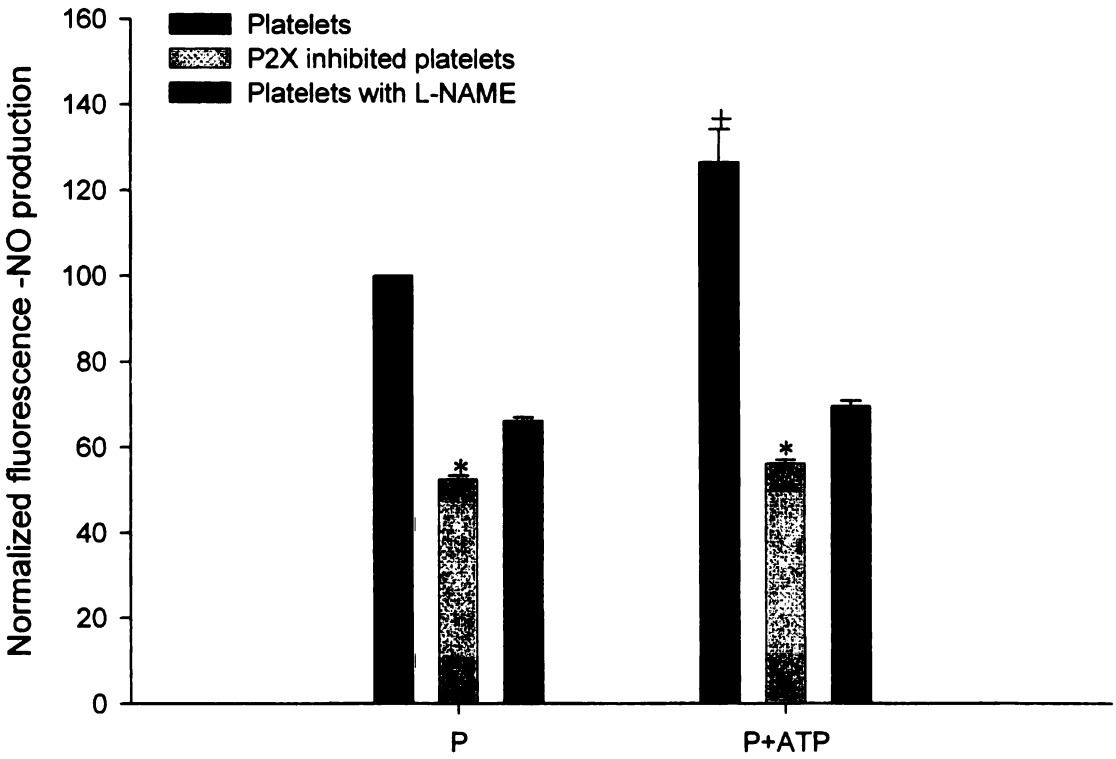

Figure 2.7 Effect of P2X1 inhibition on platelet derived NO. Platelets treated with DAF FM DA were investigated for intracellular NO with (gray bars) or without (black bars) P2X1 inhibition. As expected, the basal NO production significantly increased in the presence of ATP ($^{+}P < 0.001$). Inhibition of P2X1 significantly reduced NO production in the platelets, even in the presence of this agonist ($*P < 0.001$). L-NAME, a competitive inhibitor for NOS has been used to demonstrate that the measured fluorescence signal is due to NO. *n=3*, error bars: standard error of the mean

When the platelets were incubated with the NF 449, a selective P2X1 receptor inhibitor, the NO production was significantly decreased ($p < 0.001$) upon addition of ATP. Based on the results shown in Figure 2.7 suggesting a Ca^{2+} influx in the presence of ATP, experiments were performed to examine the Ca^{2+} influx into the platelets in the presence of various agonists and antagonists of the P2X1 receptor. It is known that stimulation of this receptor will result in Ca^{2+} influx into the platelets,[71-73] and the data in Figure 2.8 demonstrates that this increase in Ca^{2+} flux into the platelet only occurs when the P2X1 receptor is open. Specifically, the addition of Ca^{2+} to the platelet suspension when the P2X1 receptor is inhibited with NF 449 resulted in a decreased Ca^{2+} flux into the platelet (even in the presence of ATP).

While inhibition of P2X1 significantly decreased Ca^{2+} influx, the addition of ATP to the platelets in the absence of the NF 449 did not significantly enhance the Ca^{2+} influx into the platelet. It was anticipated that this may be due to Ca^{2+} binding to calmodulin (resulting in no increase in fluorescence intensity) as opposed to the fluorescent probe. Therefore, Ca^{2+} influx studies were performed with atomic absorption spectrophotometry in order to measure the total Ca^{2+} influx into the platelet. This technique could measure influx even if the Ca^{2+} were to bind to calmodulin or any other substance in the platelet. As shown in figure 2.9, measurement of Ca^{2+} by atomic absorption did reveal a significant increase in Ca^{2+} flux into the platelet. Furthermore, this Ca^{2+} influx measured by atomic absorption was inhibited in the presence of the NF 449 inhibition of P2X1.

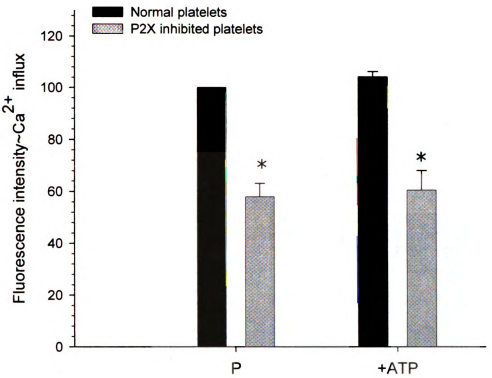

Figure 2.8 Influence of ATP on P2X1 mediated Ca^{2+} influx in platelets. Platelets treated with fluo-4 AM were investigated for Ca^{2+} influx into the platelets with (gray bars) or without (black bars) P2X1 inhibition.. In (A) the Ca^{2+} influx did not significantly increase in the presence of ATP. However, inhibition of P2X1 significantly reduced Ca^{2+} influx (*$P < 0.001$). *n=3*, error bars: standard error of the mean

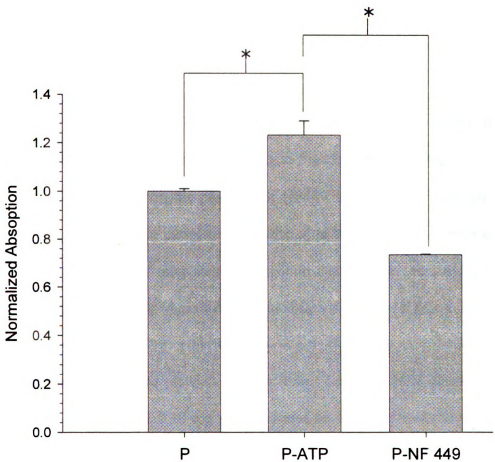

Figure 2.9 Influence of ATP on P2X1 mediated Ca^{2+} influx in platelets. The total intracellular concentrations of Ca^{2+} were determined with atomic absorption spectroscopy. Here, a significant increase in Ca^{2+} influx into the platelets was determined using either ATP as the agonist (*P = 0.004). As expected, measurement of P2X1 inhibition of Ca^{2+} influx into the platelets could also be determined with atomic absorption (last bar, P = 0.025). *n=3*, error bars: standard error of the mean

2.6.5 ATP and P2X1 in the regulation of platelet aggregation

The results in Figures 2.9 and 2.10 suggest that ATP activation of the P2X1 receptor is required for both Ca^{2+} influx into the platelet and NO production in the platelet, implying that significant platelet aggregation would only occur when the P2X1 channel is activated. Platelet aggregation was measured by adding ATP to the platelets in the presence and absence of the NF 449, the P2X1 receptor antagonist.

The presence of 10 μM ATP in the HBSS solution to which the platelets were added resulted in the expected increase in aggregation of the platelet solution, as shown in Figure 2.10. In accordance with the results in Figures 2.8-2.10, when 10 μM ATP was added to the platelets in the presence of NF449, the extent of aggregation was decreased to level similar to the platelets incubated in buffer alone. Further evidence for the importance of Ca^{2+} in platelet aggregation can also be seen in Figure 2.10. Platelets in the bottom three traces were prepared in a Ca^{2+}- free buffer and stimulated with a solution of ATP, ADP or buffer that was also Ca^{2+}- free. A comparison of the aggregation profiles of these platelets in a Ca^{2+}- free buffer to those platelets in Ca^{2+}- buffer (as shown in Figure 2.10 top 4 traces) reveals the importance of the Ca^{2+} for platelet aggregation. The aggregation increases when Ca^{2+} is present in the buffer, even in the absence of any type of P2X1 stimulus (ATP). The data in Figure 2.10 support the importance of Ca^{2+} in platelet aggregation and the role of the P2X1 receptor in this aggregation mechanism. In this experiment, ADP was used as a positive control for platelet aggregation. Application of 10 μM NF 449 resulted in the inhibition of this

aggregation to a greater extent. Although NF 449 is a selective P2X1 inhibitor, the concentration we used here is well above its IC50 value for P2Y1.

An appraisal of the results shown in Figures 2.8-2.11 does not explain the dual nature of ATP in platelet aggregation. For example, through its ability to stimulate Ca^{2+} influx through the P2X1 receptor, maintain increased Ca-CAM concentration in the cytosol and stimulation of eNOS activity, ATP can be regarded as a stimulus for NO production, an inhibitor of platelet aggregation.[74-76] However, ATP also has the ability to increase platelet aggregation through the same pathway, but with excessive Ca^{2+} influx.

Therefore, aggregation curves were measured in the presence of buffers containing ATP from 0 to 4 μM (Figure 2.12). The aggregation curve for platelets in buffer containing 0 nM ATP is labeled as (a) and was fairly constant for the entire 1500 seconds of measurement period. Interestingly, the addition of 10 and 20 nM ATP to the platelets resulted in an apparent decrease in the aggregation. The addition of 40 nM ATP increased aggregation in comparison to the 20 nM addition, although the value was still lower than that of 0 nM ATP. Subsequent additions of 0.10, 1.0 and 4.0 μM ATP resulted in significant aggregation of the platelets, which is summarized in Figure 2.13b as the final %T after 1200 seconds. The difference in aggregation profiles in Figures 2.11 and 2.13 are due to the increased concentration of ATP used in Figure 2.10 (10 μM) resulting in a more rapid progression of aggregation.

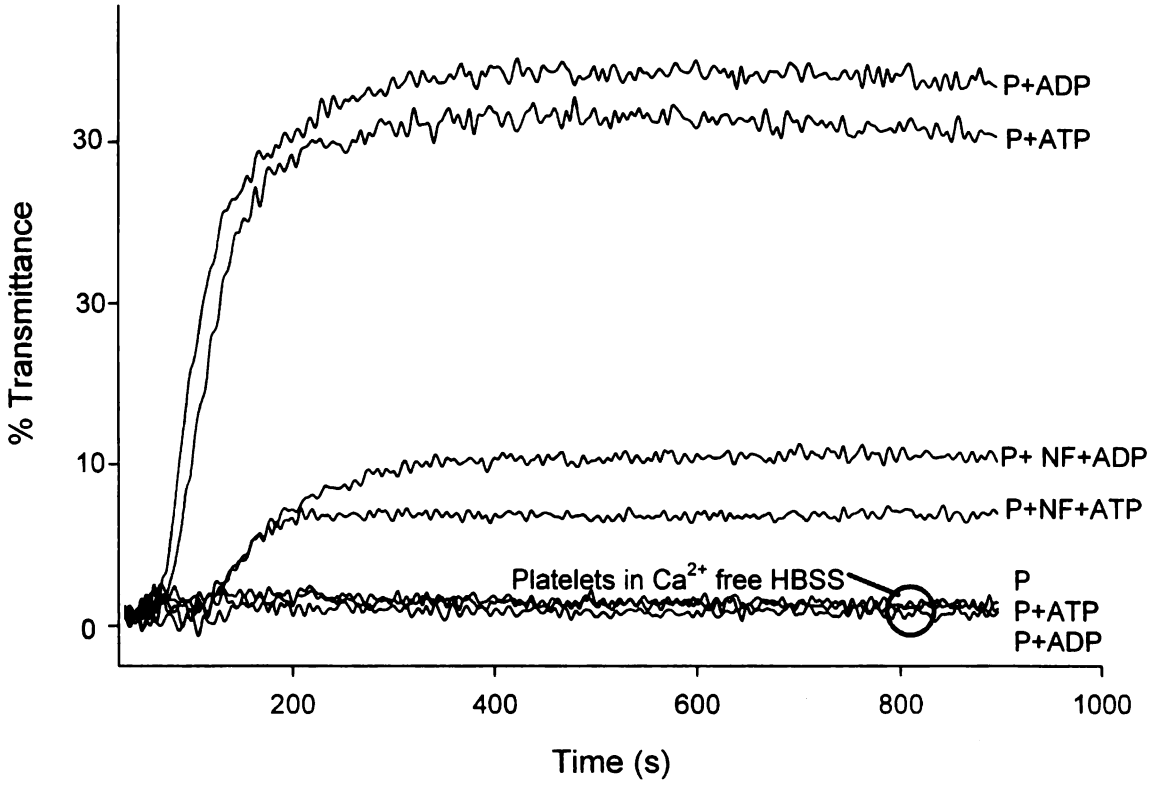

Figure 2.10 Representative curves for P2X1 inhibition on ATP and ADP induced platelet aggregation. Aggregation curves were measured for both normal and P2X1 inhibited platelets in the presence of ATP and ADP (10 μM).

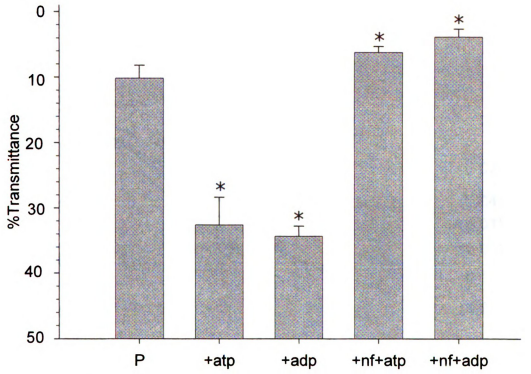

Figure 2.11 P2X1 inhibition on ATP and ADP induced platelet aggregation. Aggregation curves were measured for both normal and P2X1 inhibited platelets in the presence of ATP and ADP (10 μM). Here is the summary of the aggregation (reported as the % transmittance) from n = 3 rabbits. As expected, there was a significant difference in the % transmittance (aggregation) for platelets in the presence of ATP (*P < 0.001). P2X1 inhibition reversed the aggregation trend to values that were slightly lower than normal platelets without any agonist (*P < 0.0024). *n=3*, error bars: standard error of the mean

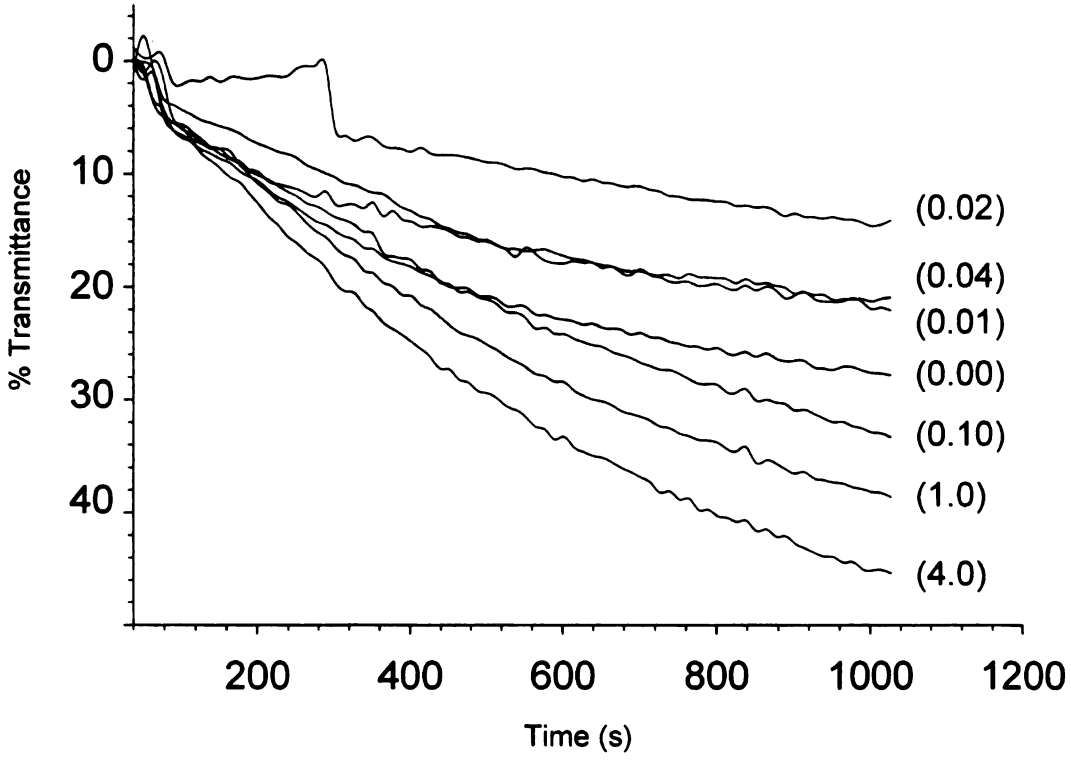

Figure 2.12 Concentration-dependent dual nature of ATP as a platelet antagonist and agonist. Representative aggregation curves for platelets in buffer containing varying concentrations of ATP

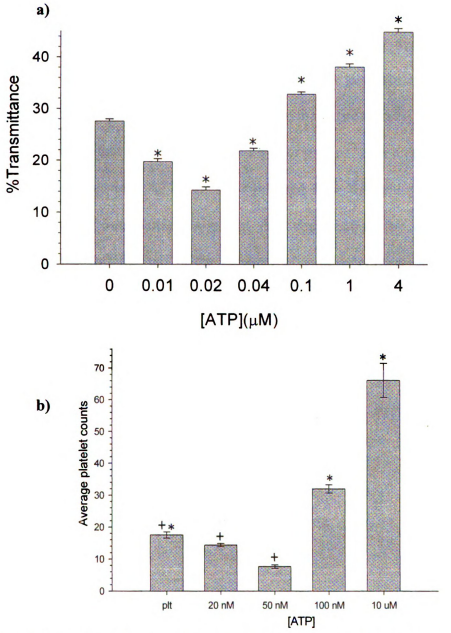

Figure 2.13 a) Concentration-dependent dual nature of ATP as a platelet antagonist and agonist. Averaged summary of these curves are shown in (B); note that the aggregation begins to significantly decrease with incremental ATP, prior to a significant rise as the ATP levels increase *$P < 0.00$. Error bars are standard error of the mean for n = 3 rabbits. **b)** The effect of exogenous ATP on platelet adhesion. Platelets were treated with various concentrations of ATP (and were centrifuged to remove excess ATP) prior to flow through a microfluidic channel coated with a confluent layer of bovine pulmonery artery endothelial cells-bPAECs (A). A stream of equilibrated medium was pumped over the endothelium to ensure any non-adherent platelets were removed before averaging the platelet count. $n = 5$ rabbits, error bars: standard error of the mean. ($^{+}p<0.05$; *$p<0.01$)

The data in Figure 2.13a demonstrate that platelet exposure to varying concentrations of ATP may play an important role in platelet aggregation. Therefore, subsequent studies were performed by in our laboratory to determine the role of ATP in platelet adhesion to an immobilized endothelium in the presence of flow. A microfluidic device, similar to that previously reported by our group,[77] was employed to investigate the effects of exogenous ATP, and ATP released from RBCs, on platelet adhesion to endothelial cells. Summarized averaged data representing the number of platelets adhering to the endothelium in the presence of various concentrations of exogenous ATP added to the platelets prior to pushing the platelets over the immobilized endothelium is shown in Figure 13b. It is clearly evident that the adhesion trend as a function of ATP concentrations is strikingly similar to the aggregation curve summary shown in Figure 2.13a. It is well-established that flowing RBCs subjected to shear-induced stress will release ATP.[78,79]

2.7 CONCLUSIONS AND OTHER CONSIDERATIONS

The main objective of the experiments performed here were to provide further evidence that the P2X1 receptor is a determinant in platelet aggregation and adhesion. The P2Y1 and P2Y12 receptors are generally thought to be important purinergic receptors for platelet aggregation. However, there have been recent reports that the P2X1 receptor may also be important. For example, Marhaut-Smith's group has reported that the P2X1 receptor does indeed participate in increase Ca^{2+} flux into the platelet independent of P2Y-type receptor activity.[80] Moreover, in vivo studies have

shown that P2X1 knockout mice have a decreased level of thrombus formation and increased bleeding times,[81] both indicators of lower platelet aggregation activity. Such studies with P2X1 deficient mice also suggest that the P2X1 receptor is an important component in platelet aggregation pathways.

However, there have been other reports that P2X1 activity is not required for platelet aggregation.[82] Further complicating the role of the P2X1 receptor in platelet function, there are reports suggesting ATP as a platelet inhibitor,[83,84] due to its ability to stimulate NO through eNOS while other suggested ATP as a platelet activator.

The work shown here demonstrates that both platelet NO production and aggregation are affected by P2X1 activation with ATP, but in a concentration-dependent manner (Figure 2.12 and 2.13). When ATP is added to the platelets, a significant increase in NO production is measured as shown in Figure 2.1 and elsewhere.[58] However, results here show that blocking the P2X1 receptor with NF 449, a potent P2X1 antagonist, results in NO production equivalent to that of platelets without any added activators.

Another interesting feature of the work presented here is the form and manner by which ATP was administered to the platelets. Most studies investigating the effect of ATP on platelet physiology employ α-β-methyleneATP, a stable analogue of ATP that does not break down even in the presence of enzymes known to break down ATP (e.g., apyrases). It is known that α-β-methylene ATP binds specifically to P2X1, although it does not result in platelet aggregation, and its effect on Ca^{2+} influx, while significant, is short-lived (5-10 s).[71,85] Moreover, α-β-methylene ATP is also known to "desensitize" the platelet P2X1 receptor, thereby decreasing the overall Ca^{2+} flux into the platelet.[86]

In addition to the form of ATP added (e.g., the α-β-methylene ATP), many researchers often use apyrase in their solutions when performing platelet studies. The apyrase is often employed to sensitize the platelets, making them responsive to purinergic agonists. In these studies, apyrase was also employed; however, after the addition of apyrase, the platelets were washed twice in apyrase-free buffer. This washing of the apyrase from the buffer was performed in order to guarantee that any authentic ATP added to the platelets would have an opportunity to bind to the P2X1 receptor before being converted to ADP or other nucleotides form by the apyrase. Such studies (in the absence of apyrase) need to be performed rather quickly (within ~ 2 hrs) or the platelets do become desensitized; however, if the studies are performed soon after washing from the plasma, the receptors respond quite strongly to purinergic agonists such as ATP even without washing in an apyrase-containing buffer.

The results presented here suggest that the effect of ATP on platelet behavior is concentration dependent, although the concentrations that have been reported are not consistent (Figure 2.13).[85] Here, our results suggest that zero to low levels of ATP result in aggregation that is more pronounced (Figure 2.12). It does appear that increments of ATP reduce platelet aggregation initially, only to begin increasing with continued increments in ATP. We believe this may be due to the production of Ca^{2+}-calmodulin and saturation of eNOS within the platelet. Specifically, previous investigation on a fluorescence method to quantitatively determine the amount of NO that was produced and released from platelets as a result of ADP or ATP added to the platelet solution.[58] The results from this particular study showed that NO production in the platelets increased upon stimulation with ATP up to a value corresponding to 1.6×10^{-19} moles

of ATP added per platelet; beyond this amount of added ATP, the NO production remained constant. Collectively, it may be possible that levels of RBC-derived ATP that are abnormally low (resulting in low Ca^{2+} influx into the platelet) may subsequently lead to insufficient eNOS activation and insufficient production of NO. Conversely, if the Ca^{2+} influx into the platelet is abnormally high (due to exposure of the platelets to large increments of ATP, such as hemolysis), our previous studies suggest that the eNOS becomes saturated. If this saturation does occur, formed Ca^{2+}-calmodulin may then have an opportunity to diffuse to the platelet membrane and activate certain integrins, resulting in an increase in aggregation. However, such a proposed mechanism has yet to be validated.

This dual effect of ATP on platelet aggregation was verified by platelet adhesion studies using microfluidic technologies. The data in Figure 2.13b clearly demonstrates that there is an effect from ATP on the platelet's ability to adhere to an immobilized endothelium. The ATP that was added to the platelets was washed prior to adding to the microfluidic device, thus ensuring that the effect seen is from the ATP acting on the platelet as opposed to the ATP stimulating endothelium-derived NO that could also inhibit the platelet production. Importantly, the effect of ATP on both the aggregation and adhesion are strikingly similar and both show a bi-phasic effect of ATP on platelet function.

Another possible fate of the RBC-derived ATP is that it may be broken down by ectopyrases located on the endothelium. A breakdown of ATP to other adenine nucleotides (such as ADP) may lead to participation in platelet activation. However, if this were occurring in the system described here, an increase in platelet adhesion would

be expected even at lower ATP concentrations, as opposed to the decreased levels of platelet aggregation and adhesion shown in Figures 2.13a and 2.13b. Therefore, even if some of the ATP released from the RBC is enzymatically degraded to ADP, it is not enough to overcome the platelet inhibiting action of the NO production.

The work presented here involving the dual role of ATP as a platelet inhibitor and platelet activator is in concert with established findings involving hyperactive platelets, clinical outcomes, and certain types of disease. For example, people with diabetes, cystic fibrosis, and primary pulmonary hypertension all have RBCs that release less ATP than healthy controls.[7,8] Furthermore, all of these patient groups have platelets that are more hyperactive than platelets obtained from controls. The data in Figure 2.13b, which employs three different cell types in a single microfluidic channel, suggests that a decrease in the RBC-derived ATP has a direct effect on platelet adhesion to an endothelium. However, the authors also believe that deformation-induced ATP release is not the sole source of ATP from the RBC. Other reagents (e.g., iloprost, C-peptide) have been shown to induce ATP release in the absence of deformation.

It should be noted that the cell sources used in these studies are mixed (rabbit RBCs and platelets with bovine endothelial cells); however, human RBCs have been shown to produce NO in rabbit pulmonary beds.[87] Therefore, while not the ideal scenario, the cells employed here are functional for the studies reported.

The data reported here suggests that there is indeed an "optimal" level of ATP for reducing platelet aggregation. It may be somewhat difficult to determine that exact level from the results presented in Figures 2.12 and 2.13a; specifically, in Figure 2.12, the ideal concentration seems to be around 20 μM for the number of platelets studied.

However, this value appears to be higher when in the presence of RBCs. A possible explanation for these results is that, in Figure 2.13a, the ATP was added to the platelets as authentic ATP standards prior to pumping across the endothelium. In this construct, there is really no "competition" for the ATP between the platelets and the endothelium. In figure 2.5, the ATP source is the RBC and this ATP is not available until the pumping mechanism is started. Therefore, in this scenario, ATP would be available for platelets *and* the endothelium. Therefore, a 20 nM addition of authentic ATP to platelets alone would quite possibly need to be increased in the presence of added cells. A second important piece of information to consider is that the authors used a 3.5% hematocrit, which is roughly a 50% decrease from studies to date employing RBCs through such channels. Typically, the ATP release levels are in the low hundreds of nanomolar range; here, that value may be less.

Importantly, the increase in platelet adhesion due to inhibition of RBC-derived ATP does provide a model of "low" ATP release as would occur with the RBCs from people with diabetes, cystic fibrosis, or primary pulmonary hypertension. Interestingly, there are other patient groups with hyperactive platelets who may have excessive extracellular ATP levels. For example, people with sickle cell disease whose cells are prone to hemolysis, are known to suffer from complications (e.g., stroke) associated with hyperactive platelets. Moreover, our group has found that people with multiple sclerosis release excessive amounts of ATP from their RBCs in comparison to controls (unpublished results) and also have been reported to be more susceptible to deep vein thrombosis. Therefore, the changes in platelet behavior as a function of ATP

concentrations monitored with microfluidic technologies, may serve as a starting point for explaining some of the clinical observations involving hyperactive platelets.

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CHAPTER 3

3.1 PLATELET PURINERGIC RECEPTORS

Purinergic signaling plays a very important role in platelet homeostasis by acting as a platelet agonist or antagonist depending on concentration of the binding molecule. Particularly, the complex multiple signal transduction pathways associated with ATP gated P2X ionotropic channel family receptors, and ADP gated heptahellical metabotropic (those that do not form an ion channel pore) receptors are the key regulators of platelet function.[1] However, the involvement of ATP in P2X activity in platelet homeostasis is controversial. There are number of published investigations suggesting the involvement of ATP and P2X1 receptor in platelet function.[2-4] Among these are in vitro studies on ATP depletion attenuating platelet aggregation, ATP / von Willebrand factor (vWF) mediated platelet aggregation due to shear forces, ATP mediated enhancement of collagen induced platelet activation and, more prominently, ATP induced platelet shape change.[5-8] All of these studies provide very strong evidence for the involvement of ATP and P2X1 in platelet homeostasis. Interestingly, in support of these in vitro studies, in vivo studies conducted on the P2X1 knockout mice ($P2X1^{-/-}$) have shown reduced mortality due to systemic thromboembolism, decreased size of mural thrombi upon laser induced vessel wall injury, and rapid thrombus clearance.[9]

Although the receptor structure and function are not well defined, there is emerging evidence suggesting a trimeric architecture for the heteromeric P2X1 receptor.[10-12] Trimerization of the P2X1 monomers occur in endoplasmic reticulum membranes and this polymerization seems to be occurring through disulfide bonding.

ATP binding induces conformational changes within and between subunits (Figure 3.1). These changes propagate to the ion channel by conserved residues located at the interface between the transmembrane domains leading to the opening of the P2X1 ion channel pore (Figure 3.2).[13]

There are specific difficulties associated with P2X1 receptor function assessment that prevent a complete understanding of its behavior. The major constraint is rapid ligand occupied desensitization. In the presence of ATP, the reported desensitization time is 47-107 ms.[14,15] Stirring at 37 °C for 10 minutes caused washed human platelets to release 10 attomoles of ATP per platelet without adding any agonist.[16] Therefore, prolonged storage of washed platelets in buffer results in platelet P2X1 receptor desensitization. A major concern with previous P2X1-ATP functionality analysis is the application of α,β–methylene ATP, a stable analogue of ATP, to evaluate the P2X1 activity. Although the characterization data for α,β–methylene ATP in human urinary bladder cells showed high specific binding compared to ATP,[17] the receptor recycling after ligand binding and overall receptor function has not been reported. Also the inability of suramin, a potent P2X competitive antagonist, to overcome the α,β–methylene ATP mediated depolarization of rat isolated vagus nerves suggests an unusually strong binding of α,β– methylene ATP to the P2X1 that may retard the receptor function and the recycling.[18] Difficulties in using ATP in the presence of apyrase, an ATPase used to keep the purinergic receptors in the sensitized state, is the reason behind α,β–methylene ATP application in platelet investigation. Studies using α,β–methelene ATP suggest that this particular ATP analogue can induce a small calcium influx to elicit a platelet shape change, but not full aggregation.[8,19,20]

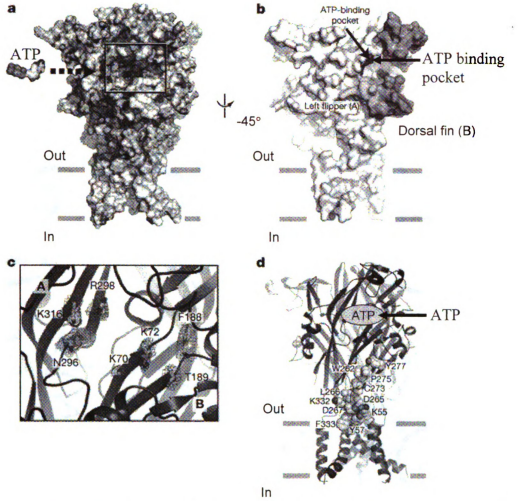

Figure 3.1 **a**,"A plausible ATP-binding pocket located between two neighboring subunits is highlighted in the black rectangle on an electrostatic potential surface representation of the trimeric $\Delta P2X_4$-B receptor. The surface is colored on the basis of the electrostatic potential contoured from -30 kT (red) to +30 kT (blue). White denotes 0 kT. An ATP molecule, scaled appropriately, is also shown. **b**, A surface representation viewed ~45° from panel **a**. The head, dorsal fin and left flipper domains forming the jaw-shaped ATP-binding pocket are colored. The putative ATP-binding residues are in blue for subunit A (Asn 296, Arg 298 and Lys 316) and in red for subunit B (Lys 70, Lys 72 and Thr 189). **c**, Close-up view of the highlighted region in **a** illustrating subunits A (blue) and B (red). Conserved residues implicated in ATP binding are labeled, and side chains are in stick representation. Contours from a $2F_o$ - F_c electron density map drawn around the side chains are in green. The electron density for the side chain of Lys 70 is weak and it has been built as an alanine. **d**, conserved residues, shown in space-filling representation, are located between the ATP-binding site and the transmembrane domain–extracellular domain interface. Only residues for a single subunit are shown" [21] T Kawate *et al. Nature* **460**, 592-598 (2009) doi:10.1038/nature08198

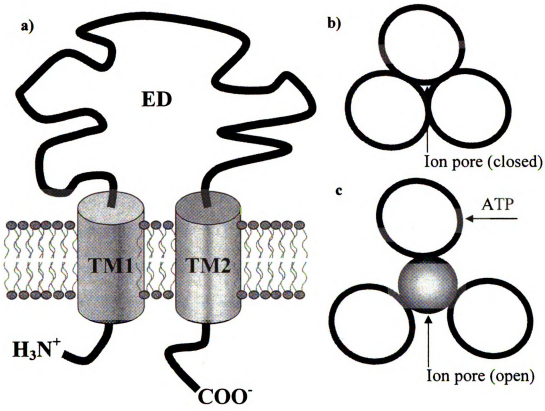

Figure 3.2 a) Heterotrimeric model of P2X1 receptor. **b)** and **c),** binding ATP to the extracellular domain (ED) leads to conformational changes in all three domains and opens up the channel.

3.2 POSSIBLE EXPLANATIONS FOR ATP-P2X1 FUNCTION UNDERESTIMATION

Current understanding of P2X structure and function is not sufficient to explain the behavior of the ATP binding cassette. It is not clear whether binding of ATP to P2X1 phosphorylates the receptor that induces the conformational changes for channel opening, or if ATP simply acts as a ligand for the P2X1 binding site that induces the channel opening. Speculation suggests that binding of ATP to the putative binding pocket, a jaw like conformation, closes the pocket inducing the conformation changes necessary for the channel opening.[21] It has not been explained how the channel is again closed. It is possible that the ATP binding and the subsequent conformational changes may lead to ATP hydrolysis, leaving ADP in the binding pocket. During this process the channel may be sensitized, opened and closed and desensitized. This may be the opportunity for apyrase to break down ADP and bring the receptor back to the sensitized state.

Note the –CH2 group between first and second phosphate groups in α,β–methylene ATP in Figure 3.3. Binding α,β–methylene ATP to the P2X receptor would open the channel and induce Ca^{2+} influx, but in order to be re-sensitized, apyrase may have to cleave it from the binding cassette. It is not clear how many phosphate groups need to be cleaved from the ribose in the nucleotide in order be detached from the P2X binding cassette. It is quite possible that once desensitized with α,β–methylene ATP, P2X would not be sensitized with apyrase.

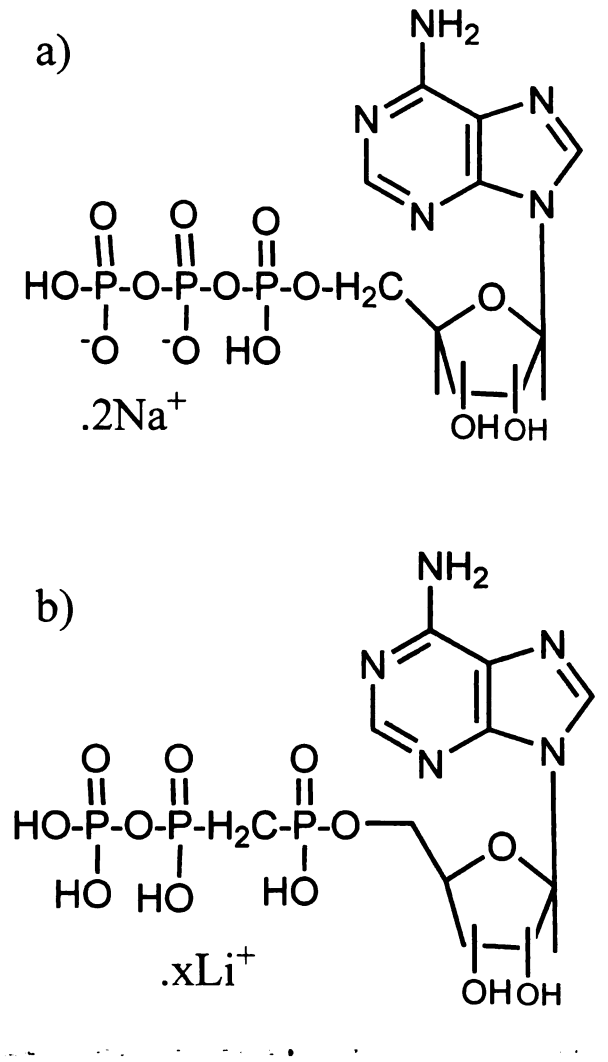

Figure 3.3 a) Adenosine 5'-triphosphate disodium salt, **b)** methyleneadenosine 5'-triphosphate lithium salt (α,β–methylene ATP), Note the -CH_2- group between first and second phosphate groups in α,β–methylene ATP.

In the present study, optimum conditions to completely inhibit the platelet P2Y1 receptor with the P2Y1 specific antagonist MRS 2179 (2'-Deoxy-N6-methyladenosine 3',5'-bisphosphate) have been investigated. Also, an enzymatic system that consumes ADP and transforms it in to ATP has been employed to further eliminate the responses from ADP (apyrase used in the platelet suspension to keep the platelet P2X1 receptor in the sensitized state, also hydrolyzes ATP in to ADP initiating the ADP-P2Y signal transduction pathway). This situation eliminates the effects of ATP-P2X1 association in the ADP mediated $Ca^{2+}i$ increase in the platelet cytosol.

3.3 EXPERIMENTAL

Activation of both P2X and P2Y type purinoreceptors increase platelet $Ca^{2+}i$. Increased $Ca^{2+}i$ can induce platelet NO production by activating eNOS. $Ca^{2+}i$ can also activate glycoprotein IIb/IIIa, which is able to induce platelet shape change and aggregation. In this study, specific biological and analytical experimental platforms have been used to investigate the differential regulation of platelet $Ca^{2+}i$, NO and platelet aggregation induced by P2X1 and P2Y1 receptors. Analyses were performed immediately after platelet rich plasma (PRP) separation or platelet isolation in order to keep the maximum platelet responsiveness. Washed platelets were kept in 0.5 U/ml apyrase, which prevents rapidly desensitizing P2X1-type receptors in the sensitized state. Platelet responsiveness is measured in each experimental protocol by acquiring the platelet response for ADP.

3.3.1 Measurement of P2X1 and P2Y1 induced platelet cytosolic Ca^{2+} ($Ca^{2+}i$) increase and their differential regulation with P2X1 and P2Y1 antagonists.

Binding of ATP and ADP to their corresponding platelet receptors, P2X1 and P2Y1, respectively, increases platelet $Ca^{2+}i$. Although details of the associated signal transduction mechanisms are described in chapter 2, in brief, binding ATP to P2X1 opens the channel and allows the extracellular Ca^{2+} flux in to the cytosol. On the other hand, binding ADP to P2Y1 initiates signal transductions cascades that results in the granulated Ca^{2+} in platelets to be released into the cytosol. When two different mechanisms govern the same secondary messenger, $Ca^{2+}i$ concentration, it is challenging to determine the differential regulation by individual agonists. The goal of this particular series of $Ca^{2+}i$ determinations was to investigate the individual agonist responses with the use of optimized concentrations of P2X1 and P2Y1 antagonists.

A 2.28 mM DMSO solution of fluo-4 AM (Molecular Probes, Eugene, OR, F14201) was prepared by adding 20 µl of anhydrous DMSO in to a vial containing 50 µg of fluo-4 AM (Molecular probes). Just before the preparation of buffered fluo-4, equal volumes of fluo-4 were mixed with 200 mg/ml pluronic F-127 surfactant (molecular probes) in anhydrous DMSO to enhance the probe penetration capacity. Platelets were suspended in Ca^{2+} free modified tyrode buffer (CaFMTB) containing 0.5 U/ml apyrase, 10 % ACD (acid citrate dextrose buffer, a platelet antagonist) and 5 µM fluo-4 AM earlier mixed with the pluronic F-127. After one hour incubation at room temperature (all the incubations containing fluo-4 AM have been conducted in light excluding containers), platelets were centrifuged and washed two times with CaFMTB

containing 0.5 U/ml apyrase and 10 % ACD. The final platelet suspension was prepared in CaFMTB containing 0.5 U/ml apyrase to have a final platelet concentration of 10^{10} platelets/ml that was is kept on ice in a dark box until the analyses were performed.

Next, 20 µl of platelet suspension was added to a 21N-Q-10 far UV quartz cuvette containing 1960 µl of modified tyrode buffer with 2 mM Ca^{2+} (CaMTB) kept at 37 °C. The cuvette was placed in its cuvette holder in a Fluoromax-4 spectrofluorometer (HORIBA Jobin Yvon, Edison, NJ) with a stirrer. Fluorescence baseline emission of the sample was measured at 516 nm (with 496 excitation) with stirring for 10 seconds. A 20 µl portion of the agonist or the buffer was then added at 10 seconds and the fluorescence scan was continued for up to 300 seconds. Agonists ADP and ATP, inhibitors MRS 2179 (MRS) and NF 449, phosphoenolpyruvate and pyruvate kinase enzyme mix (En-mix) were prepared in CaFMTB. Whenever applicable, inhibitors and/or the En-mix were added to the CaMTB in the cuvette and the total volume kept constant (2 ml) by adjusting with CaMTB.

3.3.2 PRP aggregation measurements

Agonist binding, platelet $Ca^{2+}i$ increase and the Ca^{2+}-CAM mediated platelet shape change and the aggregation are the steps of ATP and ADP induced platelet activation. Although the separation of $Ca^{2+}i$ responses by individual agonists is critical, $Ca^{2+}i$ not only induces platelet aggregation, but also induces NO production that prevents platelet aggregation. Therefore a series of experiments were conducted to measure the aggregation of PRP with ATP, α,β–methylene ATP and ADP. The earlier described

PEP-PK enzymatic reaction which converts ADP to ATP has been employed in order to see the individual potential of ATP in PRP aggregation. Sustained aggregation was expected for both ATP and ADP, as both can increase $Ca^{2+}i$; no aggregation is expected with α,β–methylene ATP due to its inability to assist continuous P2X1 operation.

A Chrono-Log 490-2D dual channel aggregometer (Chrono-Log Corporation, Havertown, PA) was employed for all platelet aggregation measurements. For each aggregation study, two aliquots of 460 μL PRP were incubated in P/N 312 cuvettes in the aggregometer incubation wells. Platelet poor plasma (PPP) was used as the reference. Contents of the test and control cuvettes were adjusted with either inhibitors and or CaFMTB to 500 μM, constantly stirred using P/N 311 stir bars. All studies were performed at 37 °C. Data was obtained at 10 data points per second for 6 min. For studies involving P2Y1 inhibition or P2X1 inhibition 10 μl of the appropriate concentrated MRS 2179 or NF 449 were added to the PRP. In order to distinguish the effect of ATP on P2X receptor, 10 μl of phosphoenolpyruvate-pyruvate kinase enzyme system, PEP-PK (680 U/ml PK in 1.44 mM PEP) were added to PRP before the start of the aggregation.

3.3.3 ATP degradation assay with 0.5 U/ml apyrase

Enzyme apyrase is an essential component of purinergic signaling assessments due to its ability to keep purinergic receptors in their sensitized state. As described earlier, apyrase prevented the use of ATP that resembles bioavailable ATP. The objective of this experiment was to calculate the extent of the ATP hydrolyze by 0.5 U/ml apyrase (a

concentration equivalent to the concentration used to sensitize platelets in this chapter) in to ADP. This piece of data is a very important component in resolving the individual responses of ATP and ADP in platelet activation.

A calibration curve for different concentrations of ATP in modified tyrode buffer (MTB) ranging from 1 μM to 10 μM was constructed. In brief 100 μl of ATP and 100 μl luciferin/luciferase (L/L) reagent mixture were combined in a cuvette and the resultant chemiluminescence was measured exactly after 15 seconds of mixing. Each measurement was performed in triplicate. For the ATP decay study, 5 μM ATP was incubated with 0.25 U/ml apyrase, and the resultant chemiluminescence, which corresponds to the amount of ATP remaining, was measured at every 15 second intervals. For each time point, individually prepared, separate samples were measured in triplicate.

3.4 RESULTS AND DISCUSSION

In this work we described two approaches for evaluation of the effect of ATP on P2X1 function. PRP is a very complex mixture of biomolecules.[22] There are number of plasma ectonucleoside triphosphate diphosphohydrolases (E-NTPDases, plasma apyrases) that hydrolyze ATP and ADP present in the PRP to keep purinergic receptors in the sensitized state.[23-25] Therefore, the first approach consists of the assessment of PRP aggregation studies where more functional P2X1 receptors exist on platelets. The results of this experiment are shown in Figure 3.4 (representative curves) and Figure 3.5 (averaged % transmittances).When treated with ADP, PRP shows high aggregation

(~37%), which is shown in Figure 3.4a. Although ATP is not able to induce the same potency, ATP also induces significantly higher aggregation (~23%) shown in Figure 3.4b. One might argue that a greater fraction of ATP induced platelet aggregation in PRP is due to the break down of ATP by platelet and plasma E-NTPDases to ADP.[23,24] It is hypothesized that this aggregation could partially be due to ATP hydrolysis to ADP, but ATP still should contribute to a greater extent.

When PRP is treated with α,β–methylene ATP, there is no aggregation although α,β–methylene ATP induced Ca^{2+} influx and platelet shape change has been reported (Figure 3.4c).[8,19,20] Chemical structures of ATP and α,β– methylene ATP differ by one CH2- group in α,β–methylene ATP (Figure 3.3). It is possible that both ATP and α,β–methylene ATP open the P2X1 trimer. After initial desensitization E-NTPDase should be able to clean up the ATP binding pocket and sensitize the receptor for a fresh cycle. The stable ATP analog α,β–methylene ATP cannot be broken down by NTPDase and therefore, after initial binding, P2X1 receptors may permanently stay in the desensitized state. Though, the initial Ca^{2+} influx due to α,β–methyleneATP binding is enough to phosphorilate myosin light chains inducing the platelet shape change,[7,26-28] lack of continuous receptor function may have prevented the subsequent platelet aggregation.

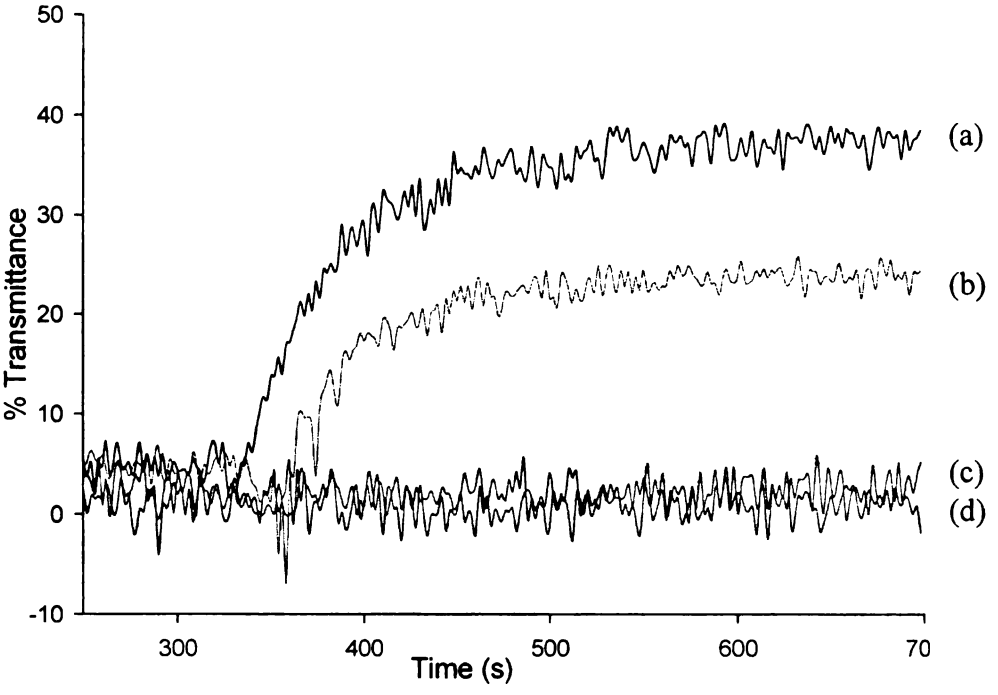

Figure 3.4 Representative aggregation curves for agonist induced PRP aggregation. Note that (d) PRP and (c) PRP treated with α,β–methyleneATP do not show any significant aggregation compared to (b) ATP and (a) ADP treated PRP.

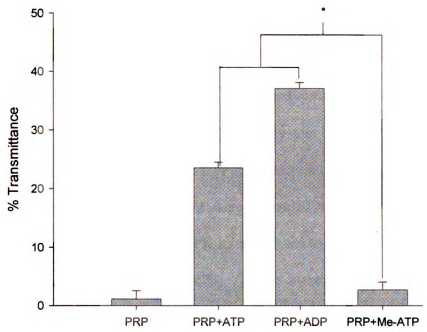

Figure 3.5 Average % transmittance propotional to the PRP aggregation. Note that PRP and PRP treated with α,β–methyleneATP do not show any significant aggregation compared to ATP and ADP treated PRP. $n=3$ *$p<0.01$

Phosphoenolpyruvate-private kinase system and P2Y1 behavior

Pyruvate kinase (PK) or pyruvate 2-O-phosphotransferase catalyses the transfer of a phosphate group from phosphoenolpyruvate (PEP) to ADP to form ATP and pyruvate in the presence of Mg^{2+} and K^{+} (Figure 3.6).[29] There is a large energy drop (ΔG = -61.9 KJ mol^{-1}) associated with the forward reaction and therefore this reaction is irreversible.[30] Application of the PEP-PK system to PRP is helpful in order to distinguish the effect of P2X1 from P2Y type receptors.

Typical aggregation curves were observed for PRP and ATP-treated PRP (Figure 3.7). The addition of PEP-PK in to PRP led to an unusual aggregation behavior in ATP induced PRP aggregation (7b). A rapid increase in aggregation followed by a 100% recovery is typical when PEP-PK is in the system. In PRP, P2X1 receptors are in the sensitized state and therefore a continuous Ca^{2+} flux is expected. ADP can aid ATP mediated cytosolic Ca^{2+} increase in two ways. ADP binding to P2Y1 activates Gαq and PLCβ pathways, ultimately mobilizing dense granular Ca^{2+}.[31] On the other hand, ADP activated P2Y12 inhibits the adenylyl cyclase catalyzed cAMP formation.[32] In this way, ADP is switching off the cAMP mediated Ca^{2+} granulation and assisting maintenance of higher concentration of $Ca^{2+}i$.[33] Furthermore, the lack of the basal ADP signaling may be accelerating the granulation of $Ca^{2+}i$, accumulated as a result of P2X1 mediated Ca2+ influx.

In the aggregation curve corresponding to the PRP-PEP-PK-ATP (7b), initial rapid Ca^{2+} influx may be substantial and the most effective towards αIIβIII activation.[34,35] ADP derived as the result of the ATP hydrolysis in this system rapidly converts back to ATP

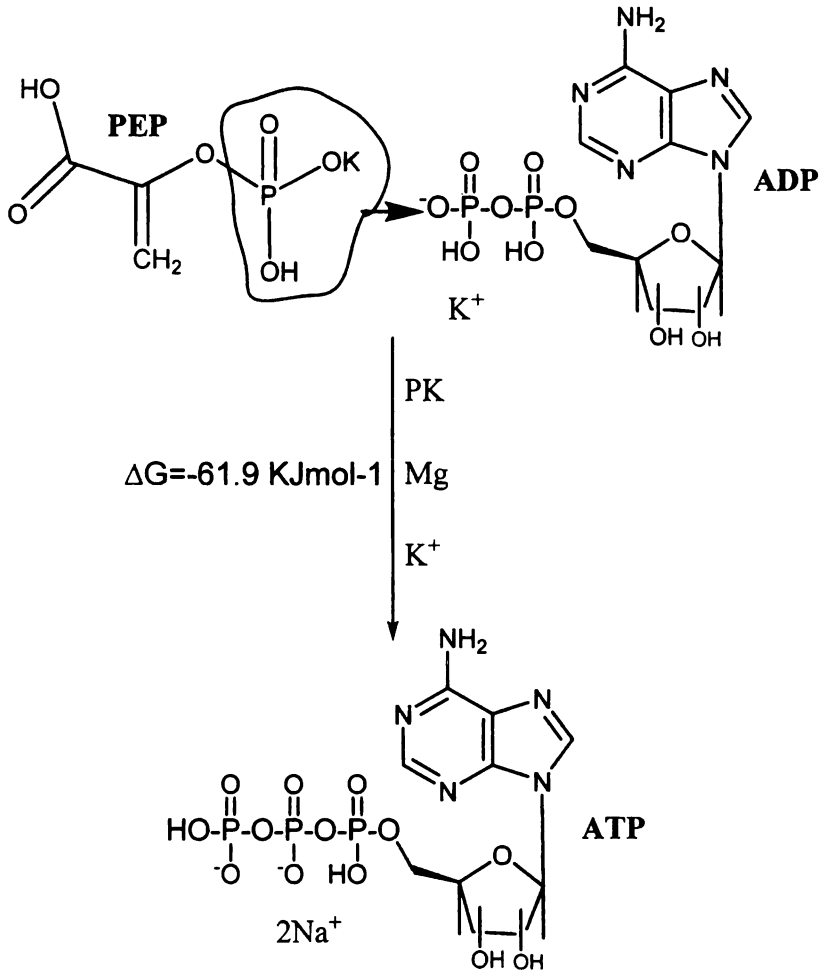

Figure 3.6 Pyruvate kinase reaction, in the presence of Mg^{2+} and K^{+}, pyruvate kinase (PK) transfers a phosphate from phosphoenolpyruvate (PEP) to ADP results in ATP production.

by PEP-PK. Under such conditions, although platelets have continuous Ca^{2+} flux, the system is lacking enough support for a high Ca^{2+} environment due to increased adenylyl cyclase activity. Despite the fact that the initial Ca^{2+} flux can induces αIIβIII activation, it seems, the system needs to have high and persistent $Ca^{2+}i$ to make it irreversible.

Regulated Ca^{2+} studies-the P2X1 response

MRS 2179 (MRS) has been used as a competitive and selective P2Y1 antagonist. Structurally MRS resembles ADP and ATP (the MRS IUPAC name is 2'-Deoxy-N6-methyladenosine 3',5'-bisphosphate). Although MRS is a P2Y1 selective inhibitor, at higher concentrations, it can also inhibit P2X1 (IC_{50} = 1.15 μM). One of the difficult issues when handling MRS as a P2Y1 antagonist is its high concentration requirement. Therefore, it is very difficult to selectively inhibit P2Y1 without inhibiting P2X1 to a certain extent. The first aim was to evaluate the minimum MRS concentration, near its IC50 value for P2X1 that can completely inhibit the cytosolic Ca^{2+} mobilizing effect of a known concentration of ADP.

Inhibition of P2Y1 with MRS 2179

In order to achieve 100% inhibition of 1.25 μM ADP induced $Ca^{2+}i$ increase, at least 2.5 μM MRS has to be used (Figure 3.8). Also it is interesting to see the inability of MRS to entirely inhibit the effect of equimolar ADP on platelet $Ca^{2+}i$ mobilizations. However, equimolar MRS is sufficient enough to inhibit approximately 97% of the ADP induced $Ca^{2+}i$ mobilizations in the presence of PEP-PK (680 U/ml PK in 1.44 mM

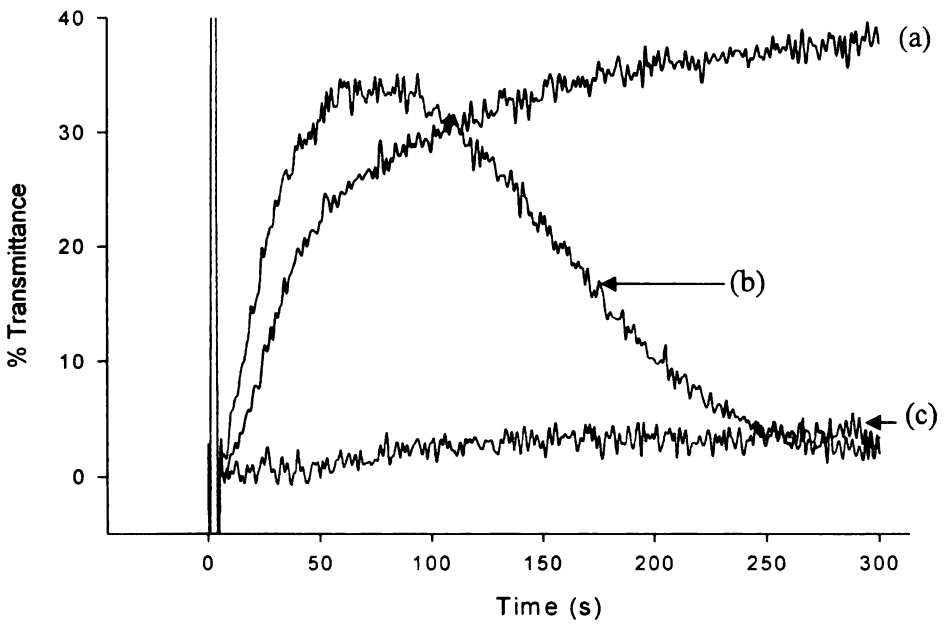

Figure 3.7 Platelet aggregation is measured in PRP, 5 μM ATP was able to induce a sustained aggregation (a) while PRP alone did not show any aggregation (c). In the presence of PEP-PK in PRP, although ATP was able induce its full aggregation potential at the beginning, it seems ATP along cannot have a sustainable aggregation, (b) without its break down product, ADP.

PEP). This trend has been shown for both 1.25 and 5 μM ADP. In general, doubling the MRS concentration compared to ADP is able to achieve 100 % inhibition of the ADP induced $Ca^{2+}i$ mobilization. To avoid deleterious effects by using higher concentrations of MRS, 2.5 μM MRS in combination with PEP-PK has been used in studies reported here. However, 2.5 μM MRS can only completely block ADP concentrations ≥1.25 μM; therefore, the maximum ADP concentration that can be generated in the system should be lower than 1.25 μM.

Determination of P2Y1 independent ATP induced P2X1 response

In the presence of 2.5 μM MRS and PEP-PK, a 100 % inhibition of 1.25 μM ADP induced $Ca^{2+}i$ increase was detected (figure 3.9 and 3.13). A considerable increase in $Ca^{2+}i$ under the same inhibitory conditions has been observed upon stimulation of platelets with 5 μM ATP (Figure 3.9c). Compared to the 100 % increase in $Ca^{2+}i$ observed with 5 μM ADP (Figure 3.9a and Figure 3.13a), 5 μM ATP has shown a 24 % increase (Figure 3.13d). This ATP associated Ca^{2+} influx is unique and only due to the P2X1 activation. However, before a conclusion can be drawn, it is necessary to validate that the amount of ADP present in the system is below the 1.25 μM concentration limit that can be completely inhibited by the 2.5 μM MRS. In the presence of 0.5 U/ml apyrase in the platelet suspension, it is possible that ATP hydrolysis generates a substantial concentration of ADP. Therefore, it is very important to evaluate this particular ADP concentration.

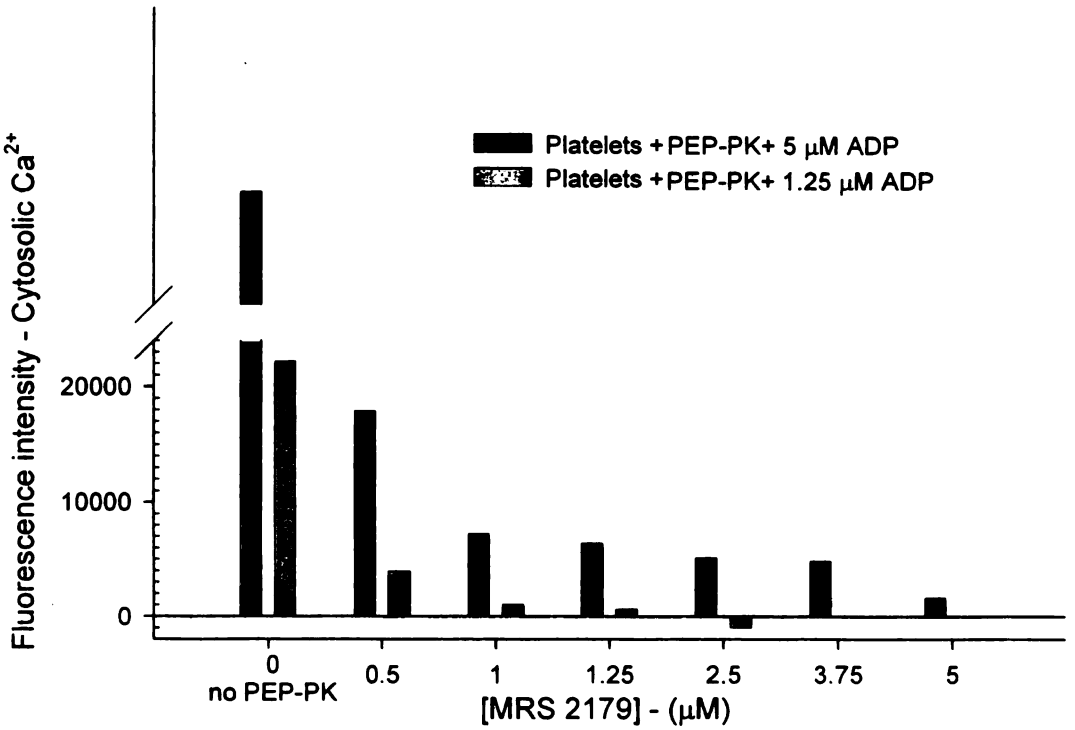

Figure 3.8 Platelet cytosolic Ca^{2+} increase was investigated upon addition of 1.25 and 5 μM ADP. Except for the first two bars, all other platelet samples contain PEP-PK and different concentrations of MRS. MRS blocks the $Ca^{2+}i$ increase in a concentration dependent manner. Comparing the $Ca^{2+}i$ concentration in the two series, it can be conclude that equimolar concentrations of MRS can block the cytosolic $Ca^{2+}i$ increase by 97%. When the MRS concentration is doubled, 100% inhibition of ADP induced $Ca^{2+}i$ increase can be achieved

ATP (5 μM) decay assay with 0.5 U/ml apyrase

Indirect determination of ADP formation as a result of the action of 0.5 U/ml on 5 μM ATP is the aim of this experiment. ATP-diphosphohydrolase, also known as apyrase, has an exceptionally high ATPase/ADPase ratio (10) and has been used to sensitize platelets.[7,36,37] Apyrase hydrolyzes nucleoside di- and triphosphates to monophosphate esters and inorganic phosphate in the presence of a divalent cation like Ca^{2+} and Mg^{2+}.[38]

$$ATP \rightarrow ADP+P_i \rightarrow AMP+2P_i$$[39]

A time dependent ATP decay profile in the presence of apyrase was generated by employing a luciferin/ luciferase bioluminescence assay. In order to maintain the exact same conditions in Figure 3.9c, a solution of 5 μM ATP with 0.5 U/ml apyrase has been used. Due to the temporal resolution of the method, a fitted exponential decay curve (Figure 3.10) has been used to calculate the remaining ATP concentration in the system within the first 20 seconds, the critical time period for ATP induced Ca^{2+} influx after the addition of ATP in to the platelet suspension shown in Figure 3.9c.

Figure 3.11 represents the projected data from Figure 3.10. The concentration of degraded ATP after 10 seconds is 0.9937 μM. Even if all the degraded ATP remained as ADP, it is still well below the concentration needed to overcome the P2Y1 inhibitory conditions elicited by 2.5 μM MRS shown in Figure 3.9c. Furthermore, apyrase used in this experiment is able to break down 1 ADP molecule for every 10 ATP molecules. In addition to that, PEP-PK enzyme system used in the platelet suspension used evaluate

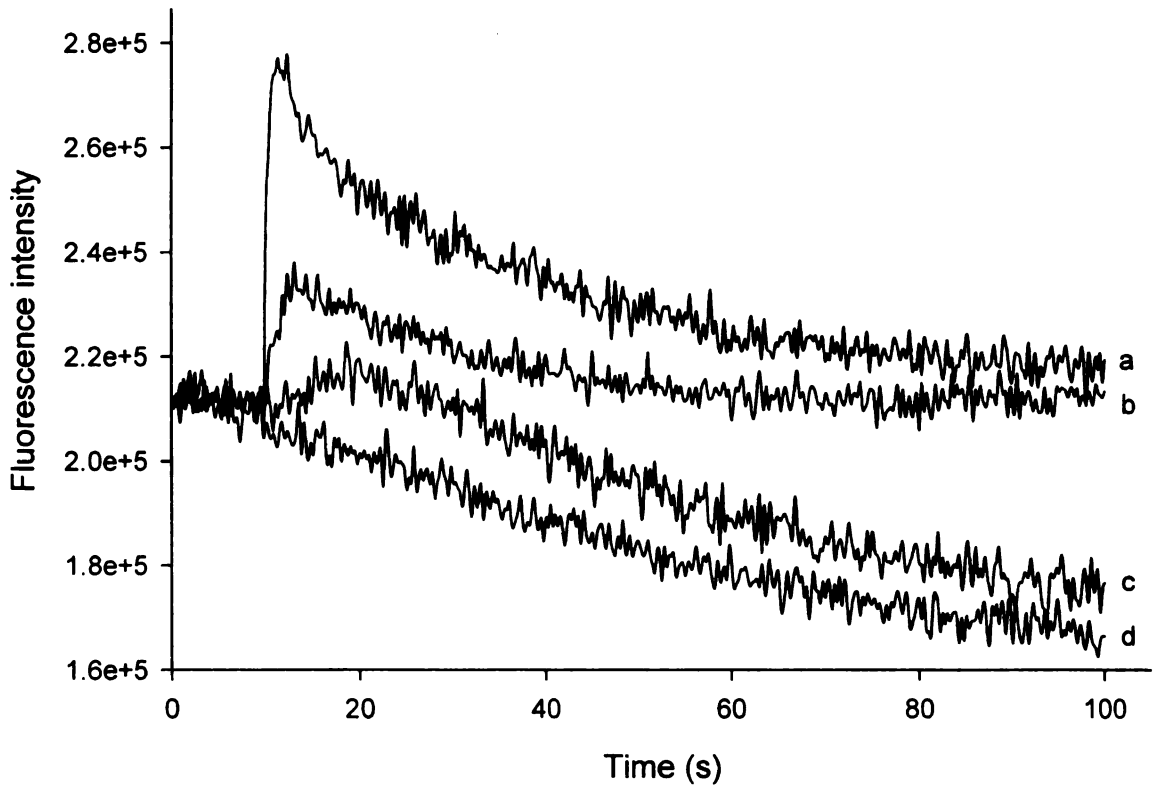

Figure 3.9 Platelet cytosolic Ca^{2+} increase upon addition of ADP and ATP. a) platelets with 5 μM ADP, b) platelets with 1.25 μM ADP, c) platelet initially incubated with 2.5 μM MRS and PEP-PK then with 5 μM ATP. d) platelet initially incubated with 2.5 μM MRS and then with 1.25 μM ADP.

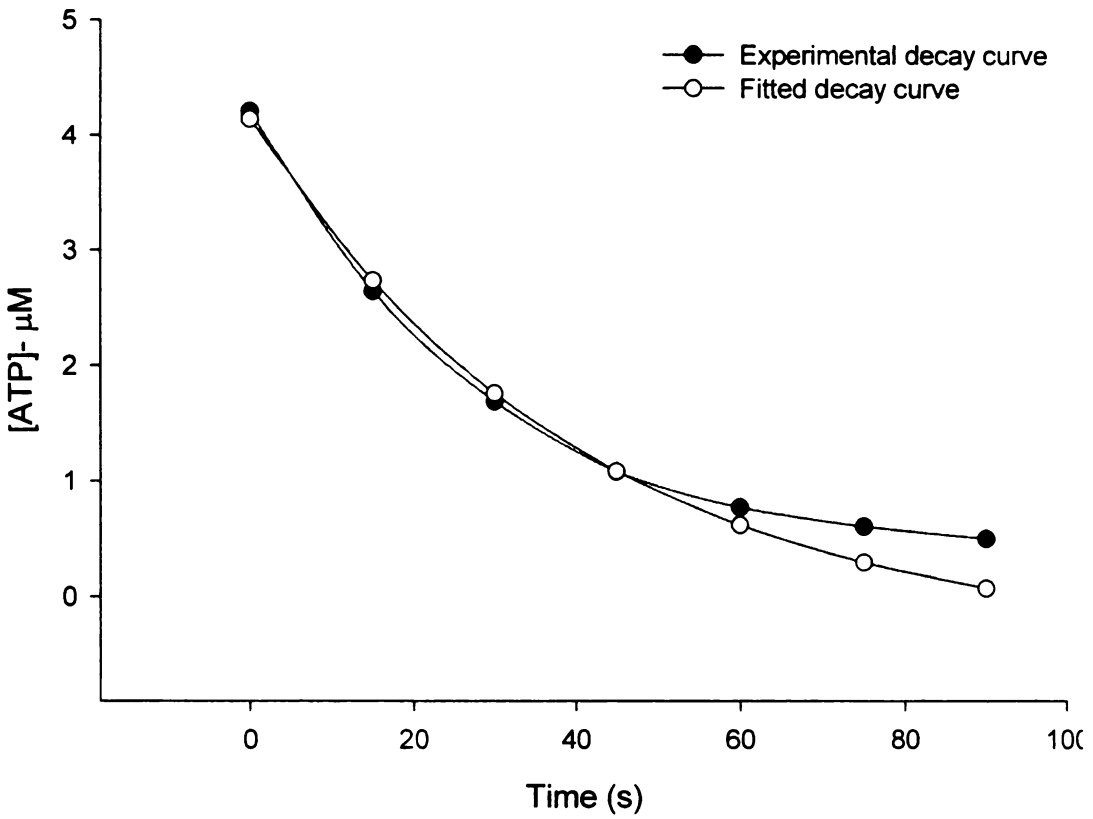

Figure 3.10 ATP breakdown profile of 5 µM ATP treated with 0.5 U/ml apyrase. Curve has been extrapolated to find the ATP decay within the first 20 seconds. The first data point at time zero is the chemiluminescence signal of 5 µM ATP without apyrase. A theoretical exponential decay curve (white circles) has been constructed using MATLAB®, which describes the decay behavior within first 20 seconds and the curve equation is f(t)=1.476e(-0.0245t).

the Ca^{2+} influx should convert the majority of ADP formed back to ATP. Therefore the actual ADP concentration in the platelet suspension should be substantially lower than 0.9937 μM. With these evidences, we can strongly conclude that the 24% increase in platelet $Ca^{2+}i$ compared to the 5 μM ADP induced $Ca^{2+}i$ increase (Figure 3.9a and 3.13a) is solely due to ATP gated P2X1 Ca^{2+} influx (Figure 3.9c and 3.13c).

In this particular experiment, the MRS concentration is 2.17 times higher than its IC50 value for P2X1, which is 1.15 μM. Therefore, the 24 % ATP induced Ca^{2+} influx should be due to the activation of less than 50% of the P2X1 receptors. Therefore, 5 μM ATP should be able to induce a Ca^{2+} influx greater than the 1.25 μM ADP induced $Ca^{2+}i$ increase. There is a ~42 % increase in $Ca^{2+}i$ corresponding to 1.25 μM ADP without inhibitors. If we assume that, due to MRS inhibition, only 50% of the P2X1 receptor function was lost, then the Ca^{2+} influx due to ATP should be ~ 48% compared to the 5 μM ADP induced $Ca^{2+}i$ increase. We have shown that 1.25 μM ADP is sufficient enough to induce a sustained aggregation in PRP (Figure 3.4). Therefore, under P2X1 functioning conditions, extracellular ATP along can induce platelet aggregation through P2X1 Ca^{2+} channel gating.

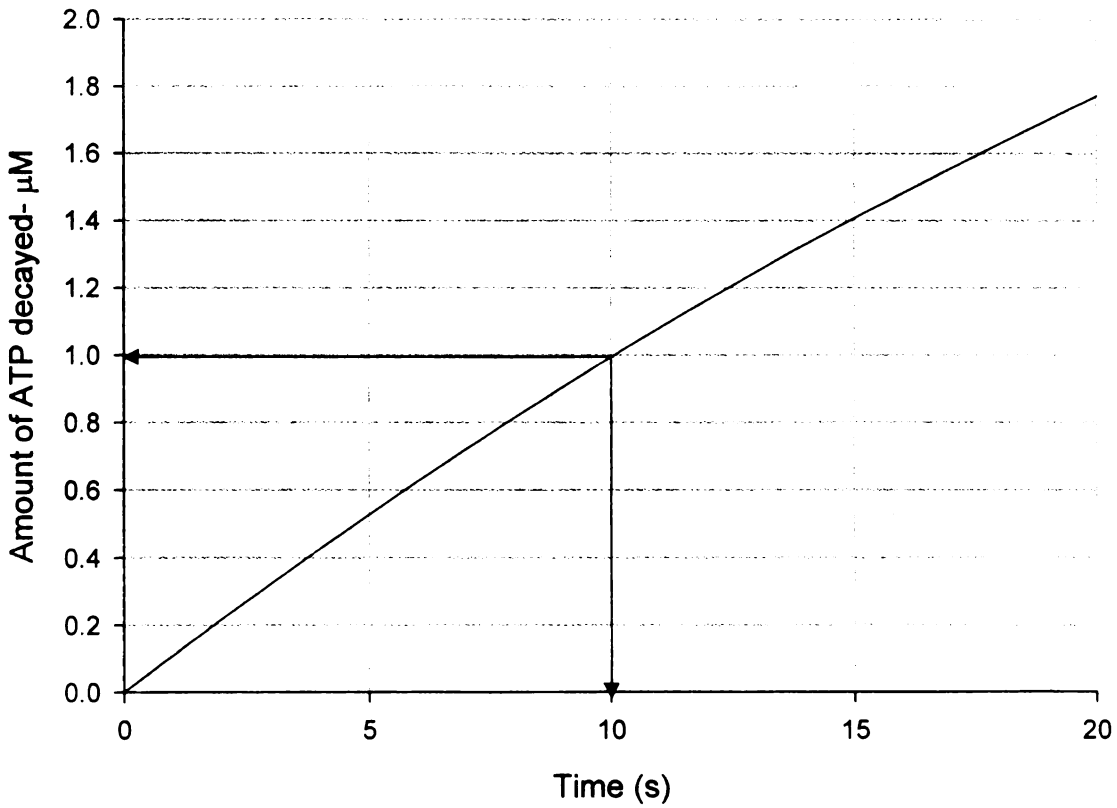

Figure 3.11 ATP breakdown profile of 5 µM ATP treated with 0.5 U/ml apyrase within first 20 seconds. The curve has been constructed using the fitted decay curve described in Figure 3.9. Note the 0.99 µM ATP decay (arrows) at 10 seconds.

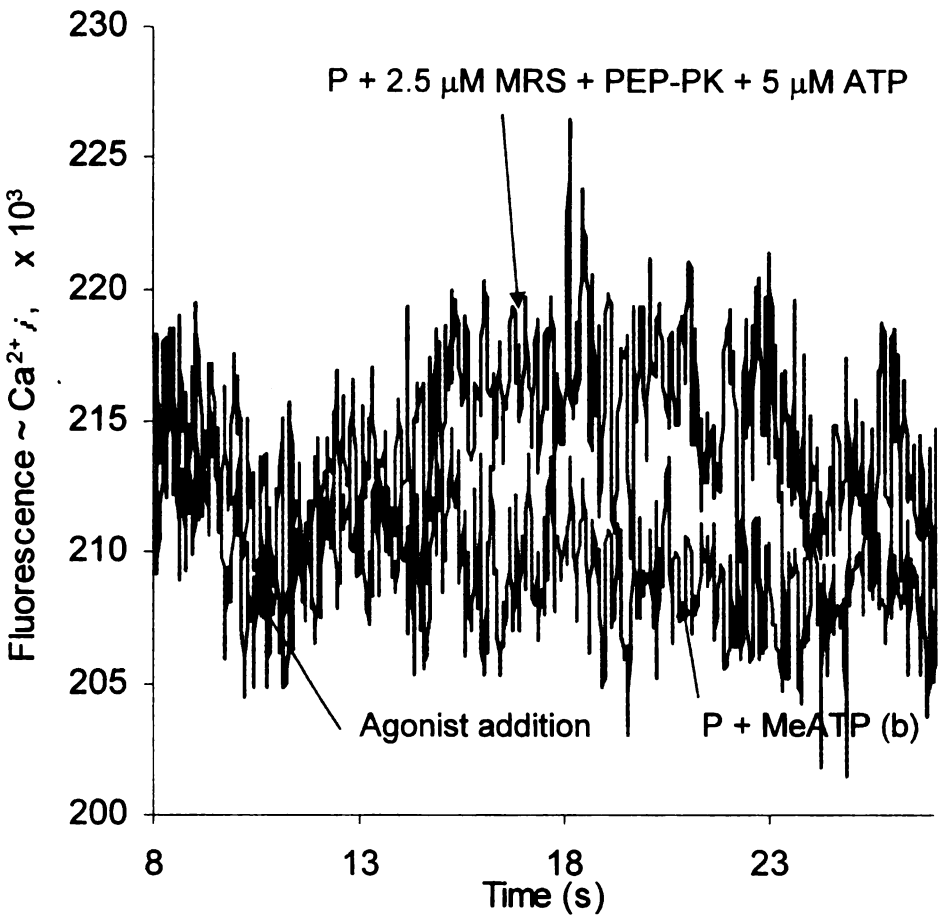

Figure 3.12 Differentiation of the magnitude of ATP and α,β–methyleneATP induced, P2X1 mediated cytosolic Ca^{2+} influx. Both platelets suspensions contained 0.5 U/ml apyrase. ATP treated sample additionally contained 2.5 μM MRS and PEP-PK. Note the 3.5 times higher Ca^{2+} influx in ATP induced platelet sample. Curve **a)** is the smoothed data and the curve **b)** the raw data of the experiment

Reasons behind the underestimation of the role of extracellular ATP and P2X1

Continuous function of P2X1 receptor is a result of the ectoapyrase's ability to recycle and sensitize the receptor after ATP binding. Apyrases and ATPases both are ATP hydrolyzing enzymes. ATPases are identified as substrate specific enzymes while apyrases can hydrolyze both tri and diphospho nucleotides.[40] Thus, it is important to be aware of the possible ways by which apyrases sensitize P2X1 receptors.

P2X1 receptors are rapidly desensitized immediately after ligand binding.[14,15] Addition of apyrase in washed platelets with desensitized P2X1 receptors (no Ca^{2+} influx response is observed for α,β–methylene ATP under this condition) brings them back to the sensitized state. It seems apyrase is able to cleave off phosphate group(s) of ATP bound to the binding pocket of the extracellular domain of P2X1. Some reports suggest that ADP binding to P2X1 can desensitize the receptor.[24,41] It is not clear whether or not initial ATP binding is associated with a cleavage of a phosphate group from ATP due to the P2X1 activity leaving ADP in the P2X1 binding pocket, which makes the receptor desensitized. If this is true, desensitized receptor nucleotide binding site should be occupied by ADP. The assumption about ADP mediated P2X1 desensitization could be a result of this behavior. Under these assumptions, we can now explain a possible way of apyrase mediated P2X1 sensitization.

When an apyrase interacts with ADP bound P2X1 receptor it would cleave off a phosphate group which may help the remaining AMP to leave the binding pocket. This may bring the receptor to the sensitized state which is ready for new ATP binding cycle. When α,β–methylene ATP, a non hydrolysable analog of ATP, is used to activate P2X1,

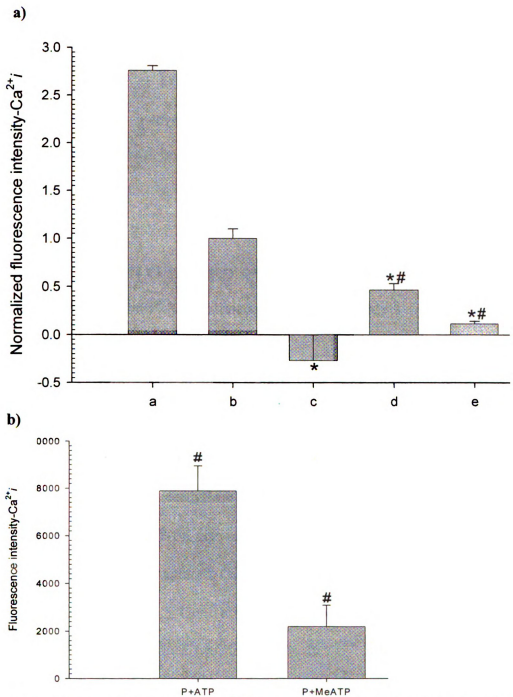

Figure 2.13 a) Averaged normalized $Ca^{2+}i$ increase in ADP, ATP and α,β-MeATP treated platelets in the presence of 0.5 U/ml apyrase. a-platelets with 5 μM ADP, b-platelets with 1.25 μM ADP, c-platelet initially incubated with 2.5 μM MRS and then with 1.25 μM ADP, d-platelet initially incubated with 2.5 μM MRS and PEP-PK then with 5 μM ATP, e-platelets with α,β-MeATP, **b)** Raw data indicating the $Ca^{2+}i$ increase in ATP and α,β-MeATP treated platelets, n=3 rabbits, $P<0.01$

the initial receptor opening and the desensitization would not be a matter (refer the structures of α,β–methylene ATP and ATP in Figure 3.3. However when it comes to the sensitization, apyrase or NTPDases, would not be able to cleave any more phosphate groups on α,β–methylene ATP due to its structure. Therefore, it is not surprising to see a Ca^{2+} influx which barely enough to induce a platelet shape change with α,β–methylene ATP.

Utilization of optimized P2Y1 inhibitory conditions to compare the effect of ATP and α,β–methylene ATP on P2X1 mediated platelet Ca^{2+} influx

Previously described platelet aggregation studies, performed with PRP reveals the inability of α,β–methylene ATP to induce any aggregation (Figure 3.4), which is consistent with the literature. Figure 3.12 and 3.13 **b)** a shows the relative magnitudes of 5 μM ATP and 5 μM α,β– methylene ATP induced Ca^{2+} influxes. Here we have employed 2.5 μM MRS and PEP-PK enzyme substrate system only with ATP study, but not with α,β–methylene ATP induced Ca^{2+} influx study. During these experiments, platelets were suspended in 0.5 U/ml apyrase. Even with greater than 50% P2X1 inhibition, ATP was able to induce two times as much Ca^{2+} influx as α,β–methylene ATP.

3.5 CONCLUSIONS AND OTHER CONSIDERATIONS

In this work, we hypothesized that the ATP receptor, P2X1 plays a critical role in platelet function. Based on our previous investigations in chapter 2 on ATP behavior as an agonist and antagonist, depending on the concentration, we predicted that previous findings in the literature conducted with α,β–methylene ATP have underestimated the P2X1 function and the potency of extracellular bioavailable ATP.

Primarily, we have demonstrated that α,β–methylene ATP cannot induce platelet activation and subsequent platelet aggregation. However, this is not due to the lack of P2X1 receptor potential, but rather the unusual behavior of α,β–methylene ATP in the receptor gating. We initially demonstrated that α,β–methyleneATP cannot induce platelet aggregation in PRP, which is consistent with the literature. Although a similar study with ATP demonstrated a substantial and sustained platelet aggregation, it was challenging to distinguish this behavior from ADP, which is the immediate hydrolyzed product of ATP due to plasma or membrane apyrases. This goal was successfully achieved by inhibiting P2Y1 receptor function with MRS while preventing ADP accumulation in the system with the use of PEP-PK enzyme substrate system. Although 1.25 μM ADP was unable to induce any $Ca^{2+}i$ increase under this conditions, 5 μM ATP was able to induce a considerable $Ca^{2+}i$ increase which is purely due to the P2X1 mediated Ca^{2+} influx. It is very important to mention that, the P2Y1 inhibitory condition we used here was two times higher than the IC50 value of the inhibitor for P2X1. Therefore, this considerable $Ca^{2+}i$ increase is a result of the function of less than the 50 % P2X1 receptor population.

The P2Y1 inhibitory conditions applied here was enough to inhibit the effect of ADP concentrations less than 1.25 μM. To ensure that, the application of 5 μM ATP, in the presence of 0.5 U/ml apyrase, would not generate ADP concentration over 1.25 μM, we have conducted a kinetic study to assess the ATP degradation under same experimental conditions. Results from this experiment suggest that, under these experimental conditions, the ADP accumulation in the system should be lower than the 1.25 μM limit.

Similar studies conducted with α,β–methylene ATP (without P2Y1 inhibition or PEP-PK ADP degrading reaction) has demonstrated that it can only induce a P2X1 mediated Ca^{2+} influx that has a half magnitude of the parallel ATP stimulation conducted with over 50% P2X1 inhibitory conditions. Although α,β–methylene ATP has a higher binding affinity towards P2X1 than ATP[42], this binding is not reflected in its capacity for overall P2X1 function regulation.

Therefore we conclude that the ATP gated P2X1 receptor is capable of inducing platelet aggregation depending on the extracellular ATP concentration. This also explains the platelet hyperactivity in disorders where RBCs can release unusually high amounts of ATP into the circulation and conditions where RBCs are more prone to lysis and create localized high concentration ATP environments in the circulation.[43-45] In this study, it has also be shown that the use α,β–methylene ATP to demonstrate the P2X1 receptor function in the literature has underestimated the P2X1 receptor potential and the significance of bioavailable ATP in platelet hyperactivity and aggregation.

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CHAPTER 4

4.1 THE EFFECTS OF CFTR DYSFUNCTION IN ALVOLAR MACROPHAGES ON CYSTIC FIBROSIS LUNG DISEASE

CF is a genetic disorder mainly characterized by chronic lung infections and deteriorating lung function caused by the mutations in CFTR gene and its product CFTR, which functions as a chloride channel in epithelial membranes.[1-3] Some of the proposed mechanisms related to the mutated CFTR focus on its chloride conductance function such as an altered airway surface liquid (ASL) composition and volume, decreased lysosomal acidification in phagocytes, defective airway sub-mucosal gland function and abnormal immune cell response are prominent as described in section 1.4.1.[3-7] Although these mechanisms provide some explanation for CF pathogenesis, the major unanswered question in CF biology, with considerable importance in development of new therapies, is how CFTR mutations cause lung disease.[8]

As a result of decreased mucus clearance, CF airways become fertile ground for bacteria and viruses.[6] These conditions have shown to inactivate the pulmonary epithelial defensins limiting the bacteria identification and destruction.[9] Defensins are small cysteine-rich cationic peptides on cell membranes specified for pathogen sensing.[10] An accelerated bacterial colonization can be expected when defensins are defective in CF airways.[11] These infections are the prime cause of death of people with CF, although advancements in CF therapy has significantly increased the life span of the patients from 15-20 years to 35-40 years.[12]

Numerous CFTR mutations are associated with CF. Efforts to relate the mutated CFTR phenotype with the severity of the CF lung disease have not found much success.[13] The most widely exhibited phenotype is caused by deletion of phenylalanine at position 508 commonly abbreviated as ΔF508.[14] CFTR mutations can be broadly categorized in six classes.[2] (1) defective CFTR synthesis, (2) defective processing, (3) malfunction in the channel regulation, (4) interferences with channel conductance, (5) partially defective CFTR production or processing, (6) defective regulation of other channels. CFTR functionality is determined by evaluating the mean channel open time (MCOT) and the closed time (MCCT). Wild type and ΔF508 mutant both show a MCOT of 220 ms but the MCCT showed a variation of 230 ms from wild type to 7.8 ms in ΔF508[15]. There are several different ways that CFTR may elicit its effects on cellular functions. Being a chloride channel, it has direct regulatory role on transmembrane pH gradient and associated mechanisms [16,17]. CFTR is also involved in the regulation of growing list of many other cellular transport processes as summarized in Table 1CFTR.[18,19]

When investigating the mutated CFTR on CF lung disease, a considerable attention has to be given to the lung defense system. Although the ASL secretion, composition, and the mucus clearance in the lung epithelia play a role in lung protection, ingestion of pathogens by alveolar macrophages is the one of the foundation mechanisms of pulmonary innate immunity against pathogen invasion. Therefore it is very important to find the role of CFTR on macrophages function. Despite the fact that CFTR has been characterized as a receptor for *P. aeruginosa*, allowing for recognition,

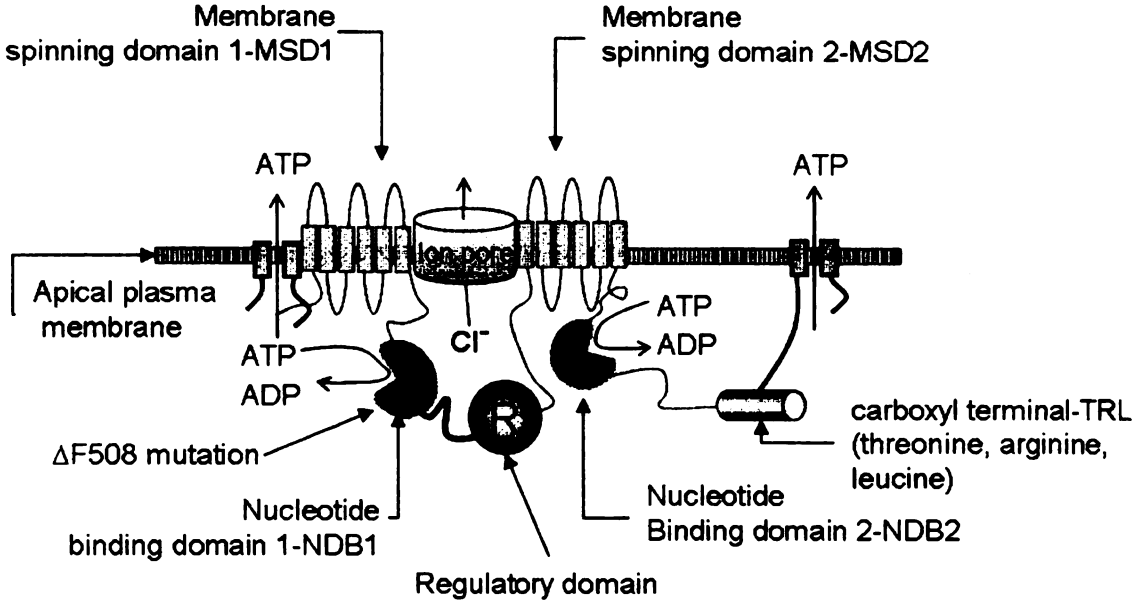

Figure 4.1 Proposed structure of CFTR protein which contains 1480 amino acids. CFTR activation depends on the phosphorylation of regulatory (R) domain mainly through protein kinase A (PKA) but other kinases like protein kinase C (PKC) are also involve in the R domain phosphorylation. The channel pore which conducts chloride ions is controlled by the conformation changes of the two nucleotide-binding domains (NDB) linked to the two membrane-spanning domains (MSD) contains six membrane-spanning alpha helixes. Although it is not certain the carboxyl terminal consisting of threonine, arginine, and leucine (TRL) of CFTR is proposed to involve in the regulation of other channels, signal transduction, and localization at the apical plasma membrane. Each, portions of which form a chloride-conductance pore. The common ΔF508 mutation in CF occurs on the surface of NDB1.

internalization and death of the pathogen,[20] evidence in the literature is conflicting about the effect of CFTR on macrophage bacterial killing ability and no information available about its phagocytosis. For example, one such study showed the requirement of CFTR for phagosomal acidification and subsequent bacterial killing,[16] while opposing studies performed later denied the involvement of CFTR in macrophage phagosomal associated bacterial killing.[17,18] In summary, the role of defective CFTR in alveolar macrophages on their function as well as the effect of such macrophage dysfunction on CF pathogenesis have not been thoroughly investigated.

4.1.1 Macrophages in brief

Macrophages are the major differentiated cells in the mononuclear phagocyte system as shown in Figure 4.2. They are widely distributed in the body and characterized by great structural and functional heterogeneity.[19] Differentiated macrophages are involved in numerous functions other than the phagocytosis such as bactericidal activity, antigen presentation, tumor cytotoxicity, removal of aged or damaged cells, injured tissue repairing, bone resorption, and special lipid metabolism.[20] Macrophages originate in the bone marrow where the resident macrophages co-exist with their monocytic precursors such as monocytes, promonocyte, and monoblasts. Monocytes spend less than 24 in bone marrow hours before entering the circulation. Once monocytes undergo extravasations (leaving the circulation), they never return, instead remain in the tissues as macrophages.[19,21,22] As 1.5×10^6 monocytes are

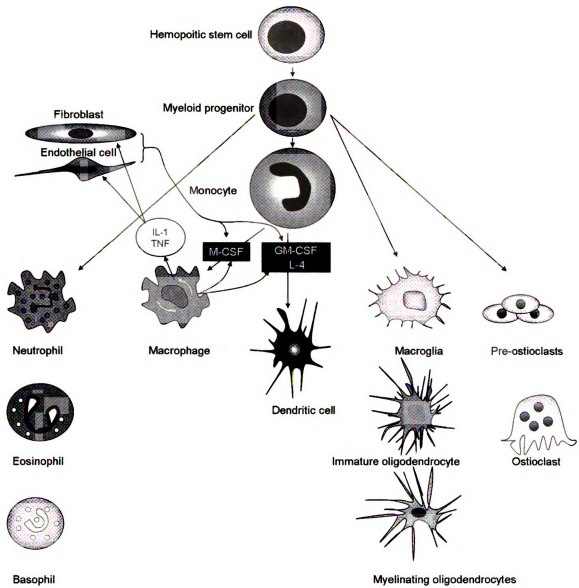

Figure 4.2. Differentiation of myeloid progenitor cells into different mononuclear cell types. GM-CSF, granulocyte–macrophage colony-stimulating factor; IL-4, interleukin-4; MCSF, macrophage colony-stimulating factor; TREM, triggering receptors expressed by myeloid cells.

produced in mouse bone marrow per day, it is predicted that there should be substantial macrophage consumption in order to protect the body from infections.[22]The discovery of macrophage specific growth factors, mostly secreted by macrophages such as macrophage colony stimulating factor (M-CSF) and granulocyte macrophage colony stimulating factor (GM-CSF) resolved the mystery of mononuclear phagocyte such as macrophages, differentiation from monocytes.[23] Macrophage exposure to external stimuli such as phagocytosable particles and endotoxins stimulates the synthesis and the secretion of M-CSF and GM-CSF.[24,25] Also macrophage derived cytokines such as interleukin-1 (IL-1) and tumor necrosis factor (TNF) have been shown to induce the production of M-CSF and GM-CSF in fibroblasts (cells that synthesize extracellular matrix) and in endothelial cells.[26-28] At the same time GM-CSF can stimulate macrophage TNF synthesis,[29] explaining the substantial production of macrophages during infections.

Macrophages are one of the longest lived cells in the body derived from the circulation. Earlier it was reported that macrophages have a half life of two weeks to one month,[30-32] but recent reports suggested a half life up to four months.[33]Although they are derived from a common bone marrow progenitor, macrophages display a remarkable heterogeneity depending on their ultimate habitat.[34,35] Depending on the tissue micro environment, macrophages are further transformed into subpopulations. Different phenotypes of macrophages with regard to their morphology, cell surface antigen expression, and function are differentiated from monocytes depending on the physio-chemical environment of the tissue. Macrophages are named on the basis of their habitat: alveolar macrophages in the lungs, peritoneal macrophages in the

Name of the channel	Function
ENaC sodium channel	Na^+ absorption
KVLQT1	K^+ channel
$HCO3^-/Cl^-$ exchanger (pancreas)	Gap-junction channels
ICOR Cl^-channels	Mucus secretion
Ca^{2+} and swelling-activated Cl^- channels	ATP-transport
ROMK2, Kir6.1 (K channel)	Glutathione transport
KCNN4 (SK4, ik)	K channel
AQP3,7	water clearance

Table 4.1 CFTR regulated the key transport processes [18,19,36]

peritoneum, Kupffer cells in the liver, microglia cells in the brain, and osteoclasts in the bones.

4.1.2 Pulmonary alveolar macrophages

Lungs have two distinct phenotypes of macrophages: alveolar macrophages (AM) and interstitial macrophages (IM).[37] AMs are frequently found protruding from alveolar epithelia to the alveolar lumen. The habitat of interstitial macrophages is the interstitial space between alveoli, much closer to the circulation. Because of this micro environment, interstitial macrophages play a significant role in specific immune responses, regulatory and proliferative functions while alveolar macrophages are critical in primary defense against inhaled particulate matter, environmental toxins and more importantly, microorganisms.[6,35,38] It has been shown that alveolar macrophage damage result in enhanced host susceptibility to microbial infection and chemotoxins.[37] Investigations described in this chapter are focused on CFTR dysfunction in pulmonary alveolar macrophages on their function.

4.2 ALVEOLAR MACROPHAGE FUNCTION, ENERGY METABOLISM AND CFTR

Macrophage glucose transport is mainly occurs through facilitative glucose transporter GLUT1, in addition, it has been shown that *P.aeruginosa* ingestion by AM

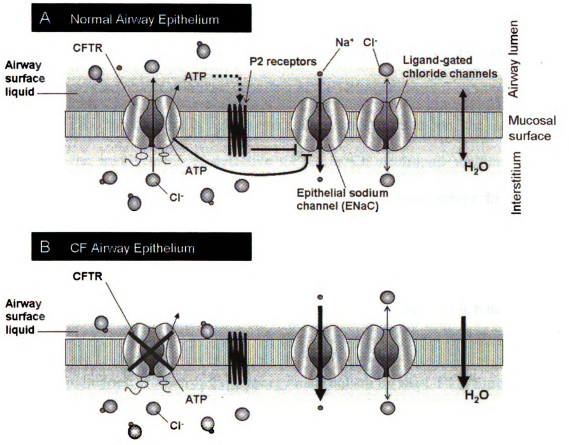

Figure 4.3. CFTR involved Airway surface liquid (ASL) volume regulation in airway epithelium. **(A)** In healthy airway epithelia, CFTR mediated airway epithelial cells release of ATP onto the airway surface and hydrolyzed products of ATP induced purinergic receptor activation, retards the epithelial sodium ion channel, ENaC. Moreover, CFTR directly inhibits ENaC and regulate Na^+ absorption. Normal airway epithelia balance Na^+ absorption against Cl^- secretion to maintain ASL homeostasis, through CFTR mediated regulatory effects. **(B)** CF airway epithelia do not express functional CFTR protein in the apical membrane. However, P2 receptor signaling regulates the ENaC up to certain extent. Therefore CF airway epithelia have a tendency to absorb more salt and water than normal because of the absence of CFTR inhibition of ENaC and CFTR Cl^- secretion. This phenomenon results in unusually dehydrated airway epithelia with decreased ASL volume. The scenario is further worsening by elevated activity of mucus glands.[3,4,39,40]

is glucose dependent.[41,42] Although there is no available direct evidence for defective macrophage phagocytosis in CF, a study on CF pulmonary neutrophils showed that investigators were unable correlate the effect of CFTR mutation as they were unable to detect CFTR on neutrophils.[43] However, later investigations on the same issue suggested the involvement of defective CFTR in CF neutrophil malfunction.[44] In general, it seems that defective CFTR plays a role in CF macrophage function. An active phagocytic cell such as the macrophage should have intensive energy consumption when activated and therefore it is likely to have a direct correlation between macrophage phagocytosis and its energy metabolism and CFTR function.

4.2.1 Dependency of Macrophage energy metabolism on the CFTR function

Energy generation pathways for different macrophages are unique. For example, the energy metabolism of alveolar macrophages relies primarily on aerobic respiration; whereas peritoneal macrophages are generally dependent on glycolysis, though they have mitochondria. This may be a result of the lungs being an oxygen rich organ and the requirement for an intense energy source to permit instantaneous phagocytosis, as the lungs are continually exposed to infectious materials in the air.[20] Away from abundant energy sources like plasma, macrophages are able to produce ATP and function under adverse conditions by utilizing a number of energy sources such as lipids other than the main energy source, glucose.[45,46] Energy intensive activities such as, phagocytosis and phagosomal bacterial killings require macrophages to mostly depend on energy from glucose whether conditions are aerobic or anaerobic.[47]

If conditions are aerobic, macrophages generate most of their energy by metabolizing glucose through glycolysis.[48,49] An increased macrophage half life has been achieved by increasing macrophage glucose uptake through a phosphoinositide 3-kinase (PI3K) and phospholiphase C (PLC) mediated mechanism (Figure 4.5).[50-52] Despite the fact that the exact mechanism is unknown, the PI3K pathway has emerged as a key regulator for both glucose transporter expression and trafficking glucose in response to extrinsic signals.[51,53,54] There are studies which demonstrate an important link between energy metabolism and the macrophage nitric oxide production.[55,56] Also, increased glucose uptake is associated with increased NADPH generation, an energy source for superoxide and other reactive oxygen species generation, which is required for the bactericidal role of macrophages. Moreover, there is an evidence for the insulin independent glucose transporter, GLUT1 to be the basic glucose transporter expressed in activated macrophages.[57] In order to attenuate macrophage oxidative stress and related disorders associated with enhanced glucose uptake in diabetes, measures to decrease the glucose uptake have been suggested.[58]

Depletion of cellular ATP triggers the up-regulation of GLUT1 and this process has been shown to regulate the glucose uptake ability of endothelial cells.[59,60] The inhibition of GLUT1 by cytoplasmic ATP accumulation has been reported,[61] suggesting a concentration dependent regulation of GLUT1 by ATP. Before this very interesting finding, ATP driven GLUT1 glucose transport inhibition triggered by AMP and ADP was reported.[62] In other words, ATP breakdown products stimulate glucose uptake. It has also been suggested that the similar mechanisms may govern the glucose uptake process in cell types where GLUT1 is the major glucose transporter.[61] Therefore

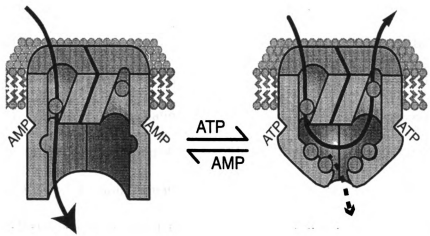

Figure 4.4[62] Carruthers model of ATP controlled GLUT1 modulation in RBCs. The figure to the left shows a section through the catalytic region of one dimer of tetrameric GluT1 embedded in a lipid bilayer and viewed from the plane of the bilayer. In the absence of ATP or when AMP is bound to GluT1, tetrameric GluT1 contains only one sugar binding site per monomer and newly imported sugar has easy access to bulk cytosolic water. Upon ATP binding (right), a conformational change occurs that restricts diffusion of sugar to and from the cytosol (a water-filled cage is formed) and exposes another sugar binding site per monomer. Under these conditions, a newly imported sugar has high probability of being exported back out of the cell or reacting with the sugar binding site. When tetrameric GluT1 is reduced to dimeric GluT1, the ATP binding site is lost, the cage relaxes, and a sugar binding site is lost.[62]

macrophage glucose transport appears to be an ATP sensitive mechanism. Depending on the ratios of AMP: ATP and ADP: ATP, the macrophage may sense the energy demand and determine their energy requirements, subsequently controlling glucose transport.

CFTR assisted conductive ATP transport from the cytosol to the extracellular matrix is well established.[63-68] Correlation between increased glucose uptake and higher cytosolic ATP concentration is also profound.[69-71] Higher ATP concentrations in the cytosol result in higher cAMP concentrations, leading to increased PKA activation.[72-74] Subsequent R domain phosphorylation and ATP hydrolysis in CFTR opens the chloride channel activating CFTR (Figure 4.1). CFTR assisted conductive ATP release may induce more ATP production, demanding more glucose uptake. The resulting increased rate of glycolysis generates higher cytosolic concentration of glucose-6-phosphate for the citric acid cycle and the pentose phosphate pathway.[48] Also extracellular ATP stimulated purinergic activation of glucose transport has been shown for other cell types.[75] Therefore increased glucose uptakes in macrophages generate more energy as well as reducing power which are essential for active phagocytosis and bactericidal activities.[37]

Considering this, it would be valuable to analyze the behavior of CFTR and determine the abundance of the mutation ΔF508 in CFTR on the apical membrane compared to the wild type. This will give us the opportunity to rule out the contribution from abnormal CFTR residence time on the apical membrane and the contribution from defective performance of the each CFTR for the overall CFTR functionality. Wild type CFTR is docked in posts Golgi -membrane in close proximity to the apical membrane

and has an average life of 16-24 hour depending on the cell type. It has been shown that ΔF508 CFTR rapidly disappears from the apical membrane and does not reappear.[76] Overall, in cystic fibrosis, the number of CFTR on apical membrane at a given time is less compared to the wild type and the chloride channel open time is also significantly less. Therefore CFTR inhibition would be an effective way to evaluate the effect of mutated CFTR in cystic fibrosis.

In an attempt to reconcile these diverse findings and mimic the RAM CFTR dysfunction in CF lung disease, experiments were performed to investigate the effect of CFTR inhibition on RAM phagocytosis and glucose transport. We have previously shown that the Zn^{2+} activated C-peptide can enhance the RBC glucose uptake through a GLUT1 mediated mechanism and increase ATP release.[77] Therefore here we also investigated the effect of Zn activated C-peptide on RAM glucose uptake and IgG opsonized bacteria particle ingestion.

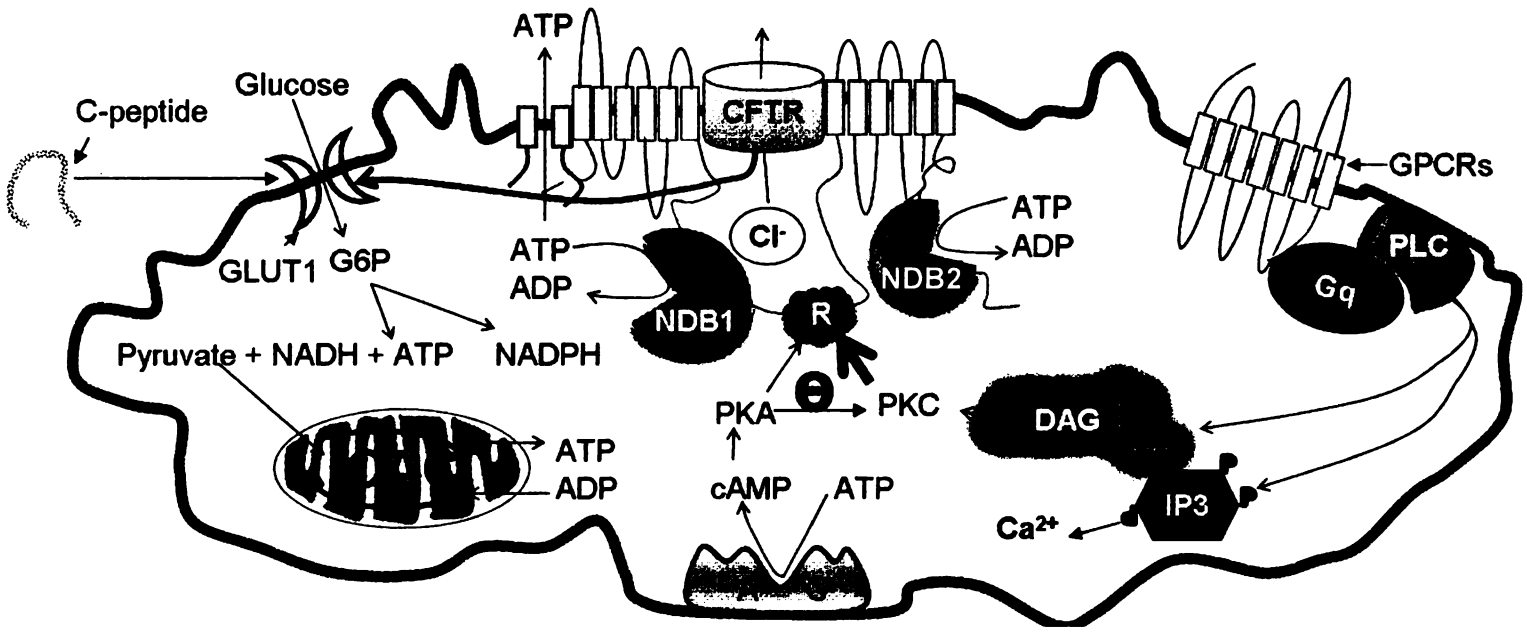

Figure 4.5 Signal transduction pathways associated with stimulated glucose transport and CFTR activation. Improved glycolysis and mitochondrial respiration generates more glucose-6-phosphate (G6P), ATP, NADH. Accumulation of G6P potentiates the pentose phosphate shunt which liberates NADPH. Cytosolic ATP buildup and cAMP generation activates CFTR and initiate conductive ATP exocytosis. Extracellular ATP instigates a series of ionotropic and metabotropic purinergic signaling leading to Ca^{2+} influx, Ca-CAM dependent and G protein coupled adenylyl cyclase (AC) activation. Extracellular ATP and its hydrolyzed products can activate GPCR signaling associated phospholiphase C activation leading to PKA activation. The same pathway is associated with IP3 activation and subsequent Ca^{2+} mobilization.

4.3 EXPERIMENTAL

4.3.1 Isolation and purification of rabbit alveolar macrophages:

Rabbits were anesthetized with ketamine (8 ml/kg, im) and xylazine (1 mg/kg, im) followed by pentobarbital sodium (15 mg/kg iv). A cannula was placed in the trachea, and the animals were ventilated with room air. A catheter was then placed into the carotid artery for administration of heparin and for phlebotomy. After heparin (500 units, iv), animals were exsanguinated and the whole blood collected for other uses. With the continuing ventilation, a sterile piece of tygon tubing is inserted 1 cm below the cannulated point in to the trachea and inserted until a resistance is felt. A 10 ml portion of sterile phosphate buffered saline (PBS) was injected in to the lungs through the tygon tubing over the period of 5 minutes and the bronchoalveolar lavarge (BAL) was retrieved by applying negative pressure. This procedure was repeated until 100 ml of BAL is collected. BAL fluid was centrifuged at 300g for 10 minutes at 6°C. The combined cell pellets were resuspended in RPMI-1640 contained L-glutamine and sodium bicarbonate (Sigma, St. Louis, MO) supplemented with 1% fetal calf serum and antibiotics (penicillin-100 U/ml, streptomycin-100 μg/ml, gentamycin- 4 μg/ml, amphotericin- 25 μg/ml). All studies involving animal use were approved by the Animal Investigation Committee at Michigan State University.

4.3.2 Liquid scintillation determination of macrophage ^{14}C labeled glucose uptake

Whether any correlation exists between macrophage glucose uptake and CFTR inhibition is critical for understanding our hypothesis. Incubation of macrophages with or without glybenclamide, a selective CFTR inhibitor and then with the β emitter, ^{14}C tagged glucose, would allow the determination of the dynamic flow of glucose in to the macrophages. It was essential here to avoid the use of 2-deoxy-D-glucose, a glucose analogue that cannot undergo further glycolysis. Although 2-deoxy-D-glucose facilitates the glucose accumulation inside the cytosol enhancing sensitivity, it may prevent or disturb the metabolic energy flow which is one of the criteria hypothesized here to govern the glucose uptake.

Aliquots of 500 μl RAM suspension were incubated in a 24-well plate with a flat bottom lid for 24 hours at 37°C with 5% CO_2. Incubation was continued another hour after addition of 40 μL buffer or buffered glybenclamide (100 μM). Cells were exposed to FITC tagged *E. Coli* bacteria particles opsonized with rabbit polyclonal antibodies (ratio of macrophage to bacteria particle~100) and further incubated for 30 minutes. Next, macrophages were treated with 5 μl of 1 μM ^{14}C labeled glucose and the incubation was continued for three more hours at 37°C with 5% CO_2. Cells were detached from the wells using a rubber policeman and centrifuged at 500g for 10 min and washed three times to remove excess bacteria particles and ^{14}C labeled glucose. Supernatant of the third washing was analyzed with the scintillation counter to ensure

the complete removal of the extracellular radiolabeled glucose. The washed RAMs were resuspended in 70 μl of HBSS. A 10 μl aliquot of this suspension is counted with the hemocytometer. Finally 15 μl of AM suspension in HBSS was mixed with 185 μl of cell lysis solution and 100 μl of scintillation cocktail in the wells of scintillation microplates. ^{14}C radioactivity of the lysed samples was measured in a Wallac 1450 PLUS microbeta liquid scintillation counter and counts per minute (CPM) were normalized to the corresponding concentration of macrophages.

4.3.3 Fluorescence spectroscopy determination of opsonized bacteria particle ingestion

Establishing the relationship between CFTR inhibition and the changes in macrophage opsonized bacteria particle ingestion is the second half of the hypothesis to link CFTR in regulating macrophage function through the regulation of glucose uptake. RAMs (500 μl) were pre-incubated for 24 hours in 24-well plate with a flat bottom at 37°C with 5% CO2. Incubation was continued another an hour after addition of 40 μL buffer or buffered glybenclamide (100 μM). Cells were exposed to FITC tagged *E. Coli* opsonized with rabbit polyclonal antibodies (ratio of macrophage to bacteria particle concentration was 100) and further incubated for 30 minutes. Cells were washed two times to remove free bacteria particles and immersed in 100 μl HBSS and cells were detached with rubber policeman. Fluorescence from any remaining extracellular bacteria particles were quenched by incubating 5 min with 60 μl of 0.08% tryphan blue.

Each suspension was further diluted up to 500 µl with HBSS and the fluorescence emission of ingested FITC tagged bacteria was detected at 518 nm with 494 nm excitation with a Fluoromax-4 spectrofluorometer (HORIBA Jobin Yvon, Edison, NJ).

4.3.4 Single cell fluorescence imaging for RAM phagocytosis:

As macrophages are large cells with a diameter ranging from 15-20 µM, confocal fluorescence imaging with a 100X oil immersion objective can be performed. This investigation is conducted in order to visualize the ingested bacterial particles and to verify that the fluorescence emission is exclusively coming from bacterial particle inside the macrophage. Cells were prepared in the same manner as the fluorescence experiment in the section 4.2.3 and a drop of the each suspension allowed settling down on cover glasses coated with fibronectin for 20 minutes. Macrophages were carefully observed with Olympus confocal fluorescence microscope under oil immersion objectives and fluorescence imaging of ingested bacterial particle was conducted.

4.3.5 Macro fluorescence microscopy evaluation of C-peptide mediated recovery of the inhibitory effect of glybenclamide on RAM phagocytosis

Expansion of limited but interesting information gathered from fluorescence imaging with limited number of macrophages to a large macrophage population was the

primary goal of this experiment. Additionally, errors associated with imaging single cells such as incubation timing, photo bleaching, macrophage size distribution and issues related to the precision also encourage this particular experiment using macro fluorescence microscopy.

Initial suspensions of RAM in RPMI-1640 with antibiotics were stimulated with 100 ng/ml lipopolysacharade (LPS-a macrophage activator), 300μl aliquots of this suspension were further incubated in a 96-well plate for 18 hours at 37°C with 5% CO_2. Incubation was continued another an hour after addition of 40 μL buffer or buffered glybenclamide (100 μM). After the incubation, 100 μl Zn^{2+} activated C-peptide (prepared according to reference 86 in HBSS) or 100 μl of HBSS was added to the each well. Following a 30 minute incubation, cells were exposed to FITC tagged *E. Coli* opsonized with rabbit polyclonal antibodies (ratio of macrophage to bacteria particle~100) and further incubated for 3 hours at 37°C with 5% CO_2. Cells were fixed with 1% glutaraldehyde and washed twice with 4°C HBSS. Just before the analysis, buffer was removed from each well and exposed to 100μl of 0.4% tryphan blue in normal saline for one minute to quench the fluorescence from all the remaining extracellular bacteria particles. Tryphan blue was removed by aspiration. Fluorescence images of ingested bacteria particles in the entire well were observed with an Olympus Macro-Fluorescence Microscope equipped with FITC filter cube (Chroma technology Corp. exciter HQ481/40, emitter HQ535/50).

Figure 4.6 Effect of CFTR inhibition on RAM glucose uptake. RAM treated with FITC tagged *E. coli* bacteria particles opsonized with rabbit polyclonal antibodies were investigated for ^{14}C labeled glucose uptake. As expected, the basal glucose uptake significantly decreased by 31.7 % in the presence of glybenclamide ($P < 0.0005$). Error bars are standard error of the mean for $n = 3$ rabbits.

4.4 RESULTS AND DISCUSSION

The information about the effects of mutated ΔF508 CFTR in CF macrophages are limited to a couple of controversial studies related to the macrophage phagosome acidification.[16-18] As hypothesized, if there is any relationship exists between CFTR function and macrophage glucose uptake, it should reflect in the glucose uptake measurements. Based on Carruthers' group's finding on cytoplasmic ATP mediated inhibition of GLUT1,[61] Speert's group's demonstration about the dependency in phagocytosis of *Pseudomonas aeruginosa* on facilitative glucose transport,[42] and CFTR being a conductive ATP transporter,[63,65,66,78] a reduced glucose uptake in macrophages incubated with glybenclamide was expected.

Data in Figure 4.6 represents the involvement of CFTR in rabbit alveolar macrophage (RAM) glucose uptake as measure of ^{14}C labeled glucose intake, in the presence and absence of glybenclamide evaluated with liquid scintillation counting. The data is normalized with respect to the basal glucose uptake of macrophages without any CFTR inhibition. The percentage decrease over basal glucose uptake in CFTR inhibited RAM is 33.3 ± 3.8 %, a value representing a significant decrease in of glucose uptake after inhibiting CFTR ($p < 0.0005$). However, the up taken glucose by macrophages are continually metabolized and the therefore this difference in glucose uptake may be greater, if the glucose consumption could be abolished.

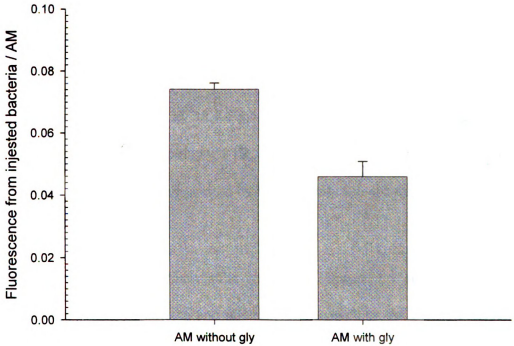

Figure 4.7 Influence of CFTR inhibition on opsonized bacteria particle phagocytosis. RAM treated with FITC tagged *E. coli* bacteria particles opsonized with rabbit polyclonal antibodies were investigated for bacteria particle uptake with and without CFTR inhibition. After washing to remove the excess bacteria and quenching the fluorescence from un-ingested bacteria with tryphan blue, RAM were resuspended investigated for the fluorescence from ingested bacteria. Inhibition of CFTR significantly reduced bacteria particle uptake ($P < 0.005$). . Error bars are standard error of the mean for $n = 3$ rabbits.

Macrophages may use the metabolic energy from glucose to compensate the intense energy requirement of the phagocytosis as well as to generate the reducing power in the form of NADPH for bactericidal activities. Results shown in Figure 4.6 clearly demonstrate that the macrophage glucose uptake mechanism either directly or indirectly depends on CFTR function. Therefore experiments were performed to examine the effect of CFTR inhibition on opsonized bacterial particle phagocytosis by RAM. The data in the Figure 4.7 demonstrate the ingestion of FITC tagged *E. coli* bacteria particles by RAM in the presence and the absence of glybenclamide. The phagocytosis is decreased by 38.1 ± 8.5 % in CFTR inhibited macrophages compared to macrophages with functioning CFTR. This reduction is not only proportional to the reduction in glucose uptake elicited by CFTR inhibition shown in Figure 4.6 but also in the same percentage.

An appraisal of the results shown in Figures 4.6 and 4.7 demonstrate the correlation of glucose intake and phagocytosis as well as the possible effect of CFTR inhibition on CF macrophage functionality. By analyzing the overall possible outcome of ΔF508, the most prominent CFTR mutation in CF, explained in detail in the introduction of this chapter, mutated CFTR gene results in overall decrease in entire CFTR function.[2] Therefore, the inhibition of CFTR is a reasonable way to mimic the clinical situation exists in the CF macrophages. Another way to conduct this experiment is to use $CFTR^{-/-}$ macrophages. However, the deletion CFTR entirely eliminates the CFTR function which cannot be expected in CF. Therefore glybenclamide mediated CFTR inhibition would be a better way to mimic mutated CFTR in CF.

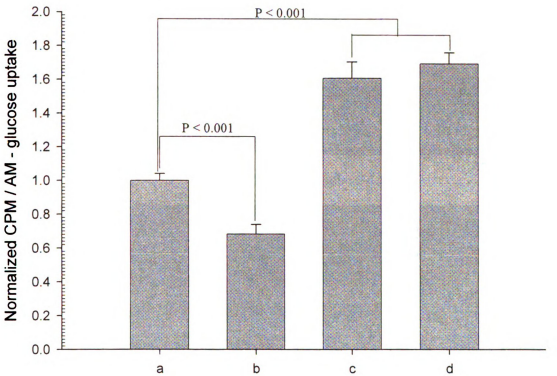

Figure 4.8 Effect of CFTR inhibition on RAM glucose uptake and the enhancement of glucose transport by Zn activated c-peptide. RAM treated with FITC tagged *E. coli* bacteria particles opsonized with rabbit polyclonal antibodies were investigated for ^{14}C labeled glucose uptake. As expected, the basal glucose uptake (a), was significantly decreased in the presence of glybenclamide (b). Incubation of CFTR inhibited RAM with Zn activated C-peptide was not only able to completely overcome, but also enhance the glucose uptake (c), the effect of Zn activated C-peptide alone on RAM (d) is not statistically different from (c) ($P < 0.001$). Error bars are standard error of the mean for n = 3 rabbits.

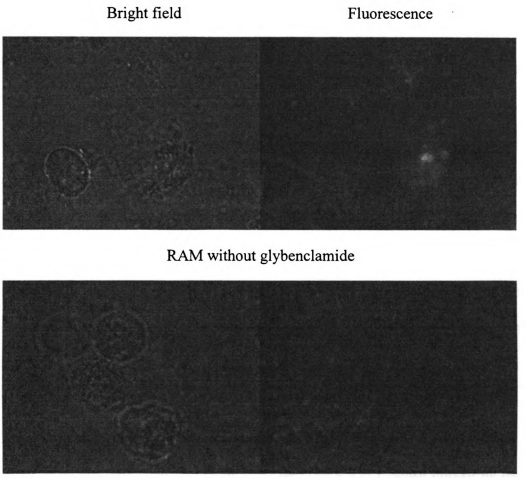

Figure 4.9 Confocal fluorescence microscopy images captured with 100X oil immersion objectives. A dramatic reduction in phagocytosis was observed after CFTR inhibition

In order to determine whether there are ways to reverse the CFTR inhibition and/or the ways to improve the glucose transport, Zn^{2+} activated C-peptide, a known red blood cell glucose transport enhancer[77] was evaluated with RAM in terms of glucose uptake and phagocytosis. We have previously shown that Zn^{2+} activated C-peptide has the ability increase the glucose uptake in to RBCs through GLUT1 and promote ATP release from RBCs. However, at this point, it is not clear whether C-peptide improve glucose transport directly through a GLUT1 or a CFTR mediated mechanism, or both. Similar to the determination of the effect of CFTR inhibition on RAM glucose uptake, the effect of Zn^{2+} activated C-peptide, in the presence and absence of glybenclamide, on RAM glucose uptake was determined and is shown in Figure 4.8. Incubation of glybenclamide treated RAM with Zn^{2+} activated C-peptide increased the RAM glucose uptake by 60.8 ± 6.8 % compared to the normal RAM glucose uptake, and a 1.35 fold increase when compared to the glybenclamide treated RAM. Interestingly RAM incubated with Zn^{2+} activated C-peptide increased the RAM glucose uptake by 69.3 ± 5.3 % compared to the RAM alone. Therefore, while the process through which Zn^{2+} activated C-peptide acts on RAM GLUT1 up-regulation may be independent of CFTR, it can compensate the CFTR mediated inhibitory effect of glucose uptake.

The next step of this analytical process was linking the Zn^{2+} activated RAM glucose uptake with its phagocytosis. FITC tagged opsonized bacteria particle ingestion by RAM in the presence and absence of glybenclamide and the effect of Zn^{2+} activated

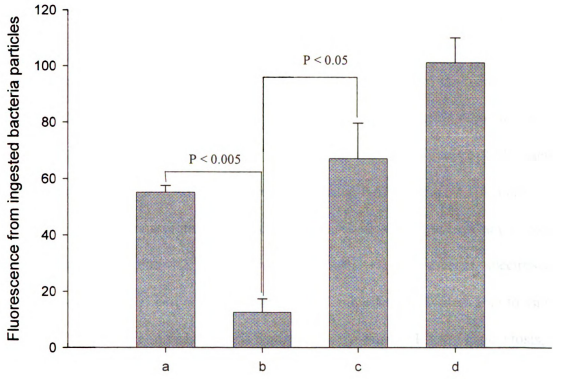

Figure 4.10 Confocal macro fluorescence microscopy determination of RAM-opsonized bacteria particle intake. Mean gray values were calculated from the fluorescence images of RAM plated 96 well plate after the background subtraction, a) and (b) are opsonized bacteria particle treated RAM without and with CFTR inhibition respectively, $P < 0.005$. (c) Incubation of CFTR inhibited RAM with Zn activated C-peptide was able to entirely overcome the inhibitory effect of glybenclamide on CFTR, $P < 0.05$. Treating RAM with Zn activated C-peptide may or may not enhance the opsonized bacteria particle intake (d) as it is statistically not different from (c). Error bars are standard error of the mean for $n = 3$ rabbits.

C-peptide on this process has been evaluated using macro fluorescence microscopy. The experiment has been conducted using 24 hour cultured RAM on 96 well plates. An advantage of using the Olympus macro fluorescence imaging system with Olympus MicroSuite software is its capability of not only providing qualitative bright field and fluorescence images but also the quantitative data calculated from the mean gray value intensities. Data obtained from this experiment is shown Figure 4.10 and in this experiment glybenclamide induced CFTR inhibition was able to reduce RAM bacteria particle uptake by 77.2 ± 3.6 % compared to untreated RAM (Figure 4.10a and b). This reduction is two times higher than the reduction observed in glybenclamide treated macrophages in section 4.2.3 which was determined via fluorescence spectroscopy. When treated with Zn activated C-peptide, CFTR inhibited RAM was able to show a 21.8 ± 7.5 % increase in phagocytosis compared to the normal RAM Phagocytosis, and five fold increase when compared to the glybenclamide treated RAM. Although RAM incubated with Zn activated C-peptide increased the RAM phagocytosis by 83.9 ± 5.7 % (Figure 4.10c) compared to the RAM alone (Figure 4.10a), this value is not statistically different (P=0.4) from RAM with C-peptide and glybenclamide together.

4.5 CONCLUSIONS AND OTHER CONSIDERATIONS

The work shown here demonstrates that the RAM glucose transport is highly dependent upon the proper function of CFTR. When CFTR is blocked with glybenclamide, a significant decrease in glucose intake is measured. This is the same relationship observed between CFTR inhibition and RAM phagocytosis. However,

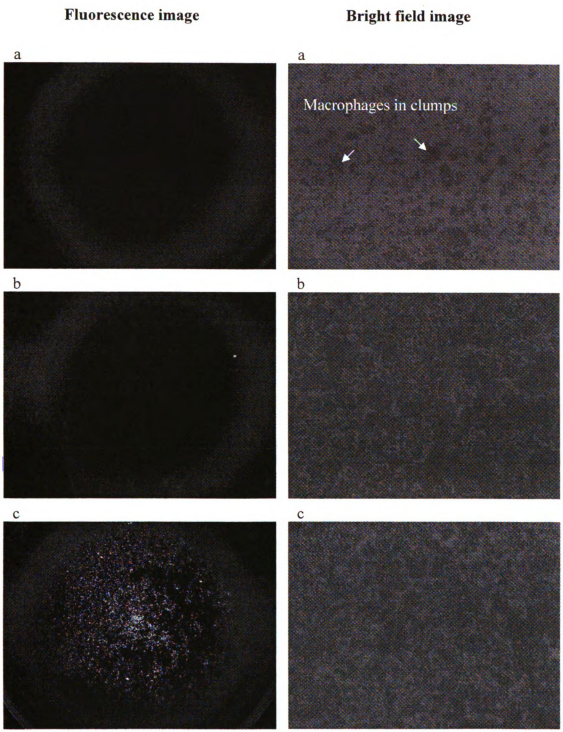

Figure 4.11 Fluorescence imaging for the evaluations of RAM phagocytosis, (Look at next page for detailed figure caption)

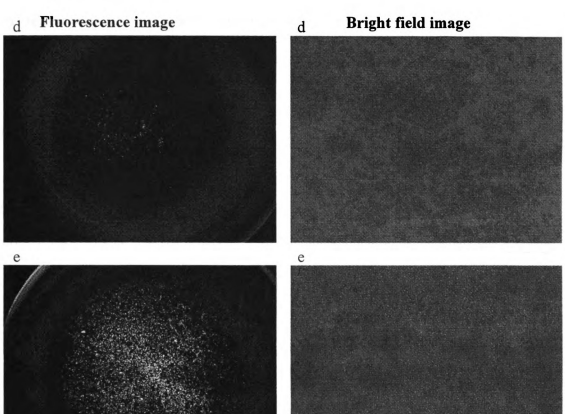

Figure 4.12 Fluorescence imaging evaluations of RAM phagocytosis. Phagocytosis of IgG-opsonized bacterial particles was diminished by pre-incubation with glybenclamide and recovered by incubation with Zn activated C-peptide. Macrophages (2 x10^5 cells/ well) were incubated in HEPES-supplemented RPMI 1640 medium 17 hours at 37°C. Then, cells were incubated with FITC-conjugated IgG-opsonized *E. coli* for 3 hours at 37°C. Cells were washed and incubated with tryphan blue to quench any extracellular fluorescence. The internalized fluorescence signal was determined with macro fluorescence microscope and normalized by the number of viable cells in the well as determined with the use of cell tracker red fluorescence probes. Fluorescence images are at the right hand side of the figure and the bright field images are on the left. a: RAM without IgG-opsonized bacterial particles, b: Heat killed RAM with IgG-opsonized bacterial particles, c: RAM with IgG-opsonized bacterial particles, d: RAM treated with glybenclamide and then with IgG-opsonized bacterial particles, e: RAM treated with glybenclamide, incubated Zn activated C-peptide and then with IgG-opsonized bacterial particles.

when treated with Zn^{2+} activated C-peptide, even in the presence of glybenclamide, RAM phagocytosis not only completely recovered but increased to a level that is statistically equivalent to that of the C-peptide treated RAM. In summary, CFTR inhibition resulted in 33.3 ± 3.8% ($p<0.001$) reduction in glucose transport compared to control RAM. Fluorescence intensity of ingested FITC tagged IgG opsonized bacterial particles decreased by 38.1 ± 8.5 % ($p<0.005$) in CFTR inhibited RAM. Incubation of CFTR inhibited RAM with C-peptide resulted in 60.8 ± 6.8 % increase in ^{14}C labeled glucose uptake and 77.2 ± 3.6 % increase in opsonized bacteria particles ingestion, compared to the control RAM.

Based on these results, we conclude that the CFTR function is required for alveolar macrophage glucose uptake and subsequent phagocytosis. Zn^{2+} activated C-peptide can enhance alveolar macrophage phagocytic function by up-regulating the macrophage glucose uptake. Therefore, the macrophage glucose uptake seems to be regulated indirectly by CFTR, through an ATP mediated mechanism. As we discussed under section 4.2.1, CFTR inhibition can increase the accumulation of ATP in the macrophage cytosol. As shown in the signal transduction pathways in Figure 4.5, increased cytosolic ATP can inhibit the GLUT1 activity while acting as a negative feedback for glycolysis. Therefore, CFTR inhibition (or dysfunction) can hinder the macrophage glucose uptake. Reduced glucose uptake results in decreased NADPH production and reactive oxygen species generation, reduces macrophage phagocytosis and bactericidal activities. As suggested previously, if the application of Zn^{2+} activated C-peptide can increase GLUT1 activity,[77] that would clearly demonstrate the

mechanism of Zn^{2+} activated C-peptide mediated improvement of RAM phagocytic activity.

This investigation not only reveals the key role of CFTR in RAM glucose transport and associated phagocytosis but also the avenues to manipulation of alveolar macrophage glucose transport as a therapeutic strategy in CF lung pathogenesis. From a therapeutic point of view, stimulation of alveolar macrophage glucose transport would be an effective way in treating CF lung disease. With further investigations and clinical trials, Zn^{2+}-activated C-peptide may be able to be used as an aerosol to treat patients with cystic fibrosis.

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CHAPTER 5

5.1 OVERALL CONCLUSIONS FROM PLATELET STUDIES

Platelets are the main components of arterial thrombus formation. They are also involved in all phases of atherosclerosis and when activated, platelets express mediators of inflammation and smooth-muscle-cell proliferation. Platelets play a central role in the pathogenesis of cardiovascular diseases mainly by contributing to atherothrombosis, a condition where sudden disruption of an atherosclerotic plaque leads to platelet activation and thrombus formation.[1] Risk factors associated with life style such as high calorie diet, unhealthy eating habits, mental stress, and lack of exercise substantially increase the risk of cardiovascular diseases and diabetes. An important feature of platelets in both of these conditions is their enhanced sensitivity to the activation stimuli. Platelet hyperactivity has been explained by a gradual build up of platelet activating factors in the cardiovascular system as a result of atherosclerotic plaque formation and progression. Although platelet activating factors account for the larger fraction of systemic thrombus formation, they do not explain the entire spectrum of platelet hyperactivity.

Therefore, here we have investigated the function of platelets beyond the expression of activating factors. Recent findings by our group concerning RBC-derived ATP release into the circulation unveiled the importance of the RBCs physiological behavior on the proper function of other cell types and tissues. For example, in a condition such as diabetes, the less deformable RBCs release less ATP while traversing through the microcirculation and therefore, result in decreased production of endothelial

NO, which is essential for vasodilation. In turn, decreased NO in the circulation, results in increased blood pressure as well as platelet activation. On the other hand, hyperactive platelets have also been found in the painful crisis of sickle cell disease where RBCs are subjected to lysis and release large amounts of ATP into the circulaton.[2,3]

Therefore, it was predicted that platelet hyperactivity may be related to the physiological behavior of RBCs and their ATP release. Furthermore, the potential of ATP and its primary receptor in platelets, P2X1 has been underestimated as a result of the lack of available analytical tools and methodologies. Due to the high propensity of P2X1 receptor for desensitization, it is necessary to use the enzyme, apyrase in platelet investigations to keep the P2X1 receptor sensitive to ATP. Therefore, the evaluations of the effect of ATP on platelet function described in the literature have been conducted using a non hydrolysable ATP analogue, α,β-methylene ATP, based on its resistance to apyrase mediated hydrolysis and high affinity to the P2X1 receptor.

By correlating the conditions where platelets are hyperactive with the physiological behavior of RBCs, we hypothesized that bioavailable ATP may act as a concentration dependent agonist and antagonist for platelet function. Also, by carefully analyzing the literature and the molecular kinetics of ATP and α,β-methylene ATP, we found that the high affinity of α,β-methylene ATP can possibly open the P2X1 channel one time and allows Ca^{2+} influx into the cytosol, but prevents the receptor recycling and continuous operation.

The work shown here demonstrates that ATP mediated activation of P2X1 receptor affects both the NO production and aggregation of platelets, but in a concentration-dependent manner. When ATP is added to platelets, a significant increase

in NO production is observed. However when the P2X1 receptor is blocked with NF 449, the NO production is decreased to a level equivalent to the un-activated platelets. In these studies, apyrase was also employed; however, after the addition of apyrase, platelets were washed twice in apyrase-free buffer. This washing step was performed in order to guarantee that any authentic ATP added to the platelets would have an opportunity to bind to the P2X1 receptor before being converted to ADP or other nucleotides form by the apyrase.

The outcomes of these conducted investigations suggest that the effect of ATP on platelet behavior is concentration dependent and, moreover, zero to low levels of ATP result in platelet aggregation. The incremental increase of ATP concentrations reduces platelet aggregation initially, only to begin increasing with continued increments in ATP. The essential Ca^{2+} influx into the cytosol may be mediated by ATP and therefore very low concentrations of ATP may result in decreased production of the Ca^{2+}-calmodulin complex and lower production of NO. It seems that a basal level of NO is required to prevent platelet from activating and aggregating. Our data can most accurately explain the reduction of platelet aggregation with increasing concentrations of ATP. In one of our previous publications, we have shown that, at a certain concentration of ATP, platelet NO production became constant, indicating saturation of eNOS activity with adequate amounts of Ca^{2+}-calmodulin.[4] Beyond this amount of added ATP, the NO production remained constant.

Collectively, it may be possible that levels of RBC-derived ATP that are abnormally low (resulting in platelet low Ca^{2+} influx) may subsequently lead to insufficient eNOS activation and insufficient production of NO. When the concentration

of ATP exceeds the amount required for maximum NO production through Ca^{2+} influx, the continuing Ca^{2+}-calmodulin formation could activate the myosin light chains and GPIIb/IIIa integrin leading to platelet shape change and aggregation. Conversely, the exposure of the platelets to large increments of ATP, such as hemolysis observed in sickle cell disease, could increase platelet aggregation. Interestingly, another investigator in the group was able to verify this dual effect of ATP on platelet aggregation by evaluating platelet adhesion to an immobilized endothelium in a microfluidic device. Importantly, the effect of ATP on both aggregation and adhesion studies are strikingly similar and both show a bi-phasic effect of ATP on platelet function.

Our findings on dual role of ATP as a platelet inhibitor and platelet activator are in good agreement with established findings involving hyperactive platelets and clinical outcomes in certain types of disease. For example, people with diabetes, cystic fibrosis, and primary pulmonary hypertension have RBCs that release less ATP than healthy controls.[5,6] Furthermore, all these patient groups have platelets that are more hyperactive than platelets obtained from controls. Also, the increase in platelet adhesion due to inhibition of RBC-derived ATP does provide a model of "low" ATP release as would occur with the RBCs from people with diabetes, cystic fibrosis, or primary pulmonary hypertension. Interestingly, there are other patient groups with hyperactive platelets who may have excessive extracellular ATP levels. For example, people with sickle cell disease whose cells are prone to hemolysis, are known to suffer from complications (e.g., stroke) associated with hyperactive platelets

In the subsequent studies, the hypothesis that the ATP receptor, P2X1, plays a critical role in platelet function has been investigated and the attention has been directed

towards the understanding of the true potential of ATP mediated P2X1 function in platelet cytosolic Ca^{2+} increase and aggregation. Primarily, we have demonstrated that α,β–methylene ATP cannot induce platelet activation and subsequent platelet aggregation, not due to the lack of P2X1 receptor potential, but due to the unusual behavior of α,β–methylene ATP in the receptor gating. We initially demonstrated that α,β–methylene ATP cannot induce platelet aggregation in PRP which is consistent with the literature. Although a similar study with ATP demonstrated a substantial and sustained platelet aggregation, it was challenging to distinguish this behavior from ADP, which is the immediate hydrolyzed product of ATP due to plasma or membrane apyrases. This goal was achieved by successfully inhibiting P2Y1 receptor and preventing ADP accumulation in the system.

Similar studies conducted with α,β–methylene ATP have demonstrated that it can only induce a P2X1 mediated Ca^{2+} influx that has only half the magnitude of the parallel ATP stimulated Ca^{2+} influx. However, the condition applied in this experiment has inhibited over 50% of P2X1 population while inhibiting P2Y1 completely. Therefore, ATP mediated Ca^{2+} influx would have been several orders of magnitudes larger than α,β–methylene ATP induced Ca^{2+} influx. In addition, consideration of the extent of ADP induced PRP aggregation with the ATP induced Ca^{2+} influx further suggests that ATP and its receptor, P2X1 can induce platelet aggregation at higher ATP concentrations. Therefore, the higher binding affinity of α,β–methylene ATP towards P2X1, which is greater than that of ATP[7], would not essentially reflect the α,β–methylene ATP ability to govern the P2X1 function. Here we have found strong evidences to conclude the ability of ATP gated P2X1 receptor to induce platelet aggregation depending on the extracellular

ATP concentration. In this study, it has also been shown that the use of α,β–methylene ATP to demonstrate the P2X1 receptor function described in the literature has underestimated the P2X1 potential in platelet aggregation.

5.2 FUTURE DIRECTIONS FOR PLATELET FUNCTION INVESTIGATIONS

The ultimate goal of the present work is to provide complete understanding of the ATP gated P2X1 receptor in platelet function regulation and the importance of bioavailable ATP on platelet activity. The investigation described in chapters 2 and 3 of this dissertation provides a semi-qualitative description of this receptor function. Here the authors were able to optimize the methods to prevent interferences from ADP and its receptor, P2Y1 and evaluate the absolute potential of P2X1 in terms of platelet Ca^{2+} influx and aggregation. Although the P2Y1 interference is avoided, the full P2X1 potential has be extrapolated as the conditions applied in the investigations are partially inhibitory to the P2X1 receptor too.

Although inhibitors such as NF 449 and MRS 2179 used in this experiments are selective, they are competitive inhibitors. Therefore, higher inhibitor concentrations have to be used in order to achieve complete inhibition. Higher concentrations of the inhibitor usually exceed the IC50 value of that particular inhibitor for the other receptor (P2X1 or P2Y1). Moreover, receptors cross inhibition complicates the measurements and disturbs the true value measurements. Therefore, it would be worth to seek synthetic approaches to develop non-competitive and selective inhibitors for these receptors.

Another way to avoid P2Y1 interferences is to use $P2Y1^{-/-}$ platelets. The lack of P2Y1 may be able to provide absolute P2X1 mediated Ca^{2+} influx information. The disadvantage of P2Y1 knockout platelets is the lack of receptor crosstalk, which may play an important role in the individual receptor function as well. Differential analysis of P2X1 function with and without P2Y1 would be very useful for the understanding of receptor crosstalk. Genetically modified f P2Y1 or Gαq protein would be another way to isolate the P2X1 mediated Ca^{2+} currents from P2Y1. The advantage of this method would be the possibility to preserve receptor cross talks to a certain extent. Differential analysis of P2X1 mediated Ca^{2+} currents after inhibiting phospoliphase C β (PLCβ), could be another useful way to determine the P2X1 function, as ADP elicits its Ca^{2+} de-granulation effect through PLCβ.

The activation of GPIIb/IIIa upon ATP binding to P2X1 is another way to measure platelet activation. However, the authors were unable to obtain conclusive data using flow cytometric analysis for P2X1 activation in terms of GPIIb/IIIa activation. This was mainly due to the lack of sensitivity of the probe used towards activated GPIIb/IIIa. Although increased GPIIb/IIIa activity was observed upon ATP mediated P2X1 activation, due to the lack of precision, the data has not been included in this dissertation. Therefore, in the future it would be beneficial to conduct a flow cytometric analysis of ATP activated platelets with a sensitive probe towards the activated GPIIb/IIIa.

5.3 OVERALL CONCLUSIONS FROM MACROPHAGE STUDIES

When addressing the RBC deformability in several disease states with its ATP release, special attention has been paid to the mechanism involved in such conductive ATP transport. At this date, there is no definite channel/s identified for ATP transport, however ATP binding cassette family transporters (ABC), especially cystic fibrosis transmembrane conductance regulator (CFTR) have been shown to participate in this process.[8] Inhibition of CFTR results in the reduction of RBC derived ATP release.[9-11]

Cystic fibrosis (CF) is a genetic disorder associated with the mutations in CFTR gene and its protein product, CFTR. Although CF is characterized by the unique CF lung disease with chronic pulmonary infections and inflammations, the available molecular level understanding of this disease is not able to explain the failures in the lung defense against the pathogen invasion, which is the leading cause of the CF lung disease.

Alveolar macrophages are described as the front line defense in the lungs against particulate matter and pathogen invasion. The primary goal of these investigations is finding out the relationship between the CFTR dysfunction in alveolar macrophages with the pathogenesis of CF lung disease. We found that inhibition of CFTR on alveolar macrophages results in reduced glucose uptake. This was expected due to CFTR involvement in ATP conductive transport. Under inhibitory conditions, ATP may accumulate in the cytosol where it has been shown to inhibit GLUT1 which is the primary glucose transporter in macrophages. Similarly, CFTR inhibition has resulted in substantial reduction in opsonized bacterial particle phagocytosis. Here we conclude that the dysfunction of CFTR prevents macrophage activation, phagocytosis and bacterial

killing by reducing the required intense energy production through abolishing the glucose uptake. Therefore, the investigations described in this dissertation not only unveil the contribution of the defective alveolar macrophage function in the CF lung disease, but also provides the molecular basis of this dysfunction to the disease pathogenesis.

We further elaborated our studies to find out whether there are ways to reverse the effects of CFTR inhibition. Our strategy here was to investigate the effect of RBC glucose uptake enhancer; Zn activated C-peptide, on CFTR inhibited macrophages. It has been shown that Zn activated C-peptide enhanced the RBC glucose uptake through a GLUT1 mediated mechanism.[12] As RBCs shares GLUT1 glucose transporter with macrophages, it was expected that Zn activated C-peptide may be able to enhance the macrophage glucose uptake too.

We found that Zn activated C-peptide has been able not only to completely reverse the reduced glucose uptake due to the CFTR inhibition;, it further enhanced the uptake to a level even grater than macrophages without any CFTR inhibition. Same relationship has been observed in terms of opsonized bacterial particle phagocytosis. Zn activated C-peptide completely overcomes the effect of CFTR inhibition on macrophage phagocytosis. However, at this point it is not conclusive whether Zn activated C-peptide drives the glucose uptake through a CFTR mediated mechanism or it is completely independent of the CFTR.

The investigations described in chapter 4 of this dissertation provides a clear and more meaningful description for CF lung disease from the standpoint of the failures in alveolar macrophage function as a result of mutated CFTR. CF lung disease has never

before explained as a disorder related to the macrophage dysfunction even though alveolar macrophages are considered as the front line defense in the lungs. Our investigations further elaborate the mechanistic detail of CFTR dysfunction and its correlation with glucose uptake which eventually regulates the phagocytosis and the bactericidal activities. From a therapeutic point of view, the ability of Zn activated C-peptide to overcome the CFTR inhibition or enhance the macrophage glucose uptake would be very important. Furthermore, C-peptide being a natural product produced by the β cells in the islets of langerhan in the pancreas, it would be an added advantage if activated C-peptide could improve the CF lung defense due its minimal side effects.

5.4 FUTURE DIRECTIONS FOR MACROPHAGE FUNCTION INVESTIGATIONS

All investigations described in chapter 4 were conducted using rabbit alveolar macrophages (RAM) and the defective CFTR was mimicked by inhibition of CFTR with a selective inhibitor. It is highly advisable to repeat these investigations with real CF macrophages. Although some investigators prefer $CFTR^{-/-}$ macrophages, in CF, CFTR is present and partially functional. Therefore, ΔF508 mutation transfected macrophages would be a good choice. Also, correlation of the defective CFTR and alveolar macrophages glucose uptake and subsequent phagocytosis would have to be evaluated with human CF macrophages before any trials with activated C-peptide.

Application of radiolabeled 2-deoxy glucose, instead of ^{14}C glucose would be useful for a more quantitative evaluation of the changes in macrophage glucose uptake.

Single cell analysis with trapped individual macrophages in a microfluidic device may be useful for evaluation of the changes at a single macrophage level. In this way, subtle changes in the macrophage behavior with the inhibition of CFTR and the effect of activated C-peptide could be easily obtained. This type of analysis would further assist to understand the underlying detailed mechanisms, by probing the components of the macrophage energy metabolism pathways such as NADPH, glucose-6-phosphate and superoxide. Additionally, the components associated with CFTR signal transduction pathway such as PKA, PKC, cAMP and adenylyl cyclase could also be evaluated. Monitoring the up regulation of GLUT1 will be useful for the evaluation of Zn^{2+} activation C-peptide mechanism. It may be an increased expression of GLUT1 on the cell membrane of activation of existing GLUT or both.

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