Critical Reviews in Biochemistry and Molecular Biology, 3(6):437–502 (1997)

ADP-Induced Platelet Activation

Rajinder N. Puri and Robert W. Colman*

The Sol Sherry Thrombosis Research Center, Temple University School of Medicine, 3400 N. Broad Street, Philadelphia, PA 19140

* To whom correspondence should be addressed. Rajinder N. Puri, Ph.D., The Sol Sherry Thrombosis Research Center, Temple University School of Medicine, 3400 N. Broad Street, Philadelphia, PA 19140.

Referee: Dr. Michael A. Liberman, University of Cinncinnati, College of Medicine, Department of Molecular Genetics, Biochemistry and Molecular Biology, 231 Bethesda Ave., Cinncinnati, OH 45267-0524

ABSTRACT: Platelet activation is central to the pathogenesis of hemostasis and arterial thrombosis. Platelet aggregation plays a major role in acute coronary artery diseases, myocardial infarction, unstable angina, and stroke. ADP is the first known and an important agonist for platelet aggregation. ADP not only causes primary aggregation of platelets but is also responsible for the secondary aggregation induced by ADP and other agonists. ADP also induces platelet shape change, secretion from storage granules, influx and intracellular mobilization of Ca^{2+}, and inhibition of stimulated adenylyl cyclase activity. The ADP-receptor protein mediating ADP-induced platelet responses has neither been purified nor cloned. Therefore, signal transduction mechanisms underlying ADP-induced platelet responses either remain uncertain or less well understood. Recent contributions from chemists, biochemists, cell biologists, pharmacologists, molecular biologists, and clinical investigators have added considerably to and enhanced our knowledge of ADP-induced platelet responses. Although considerable efforts have been directed toward identifying and cloning the ADP-receptor, these have not been completely successful or without controversy. Considerable progress has been made toward understanding the mechanisms of ADP-induced platelet responses but disagreements persist. New drugs that do not mimic ADP have been found to inhibit fairly selectively ADP-induced platelet activation *ex vivo*. Drugs that mimic ADP and selectively act at the platelet ADP-receptor have been designed, synthesized, and evaluated for their therapeutic efficacy to block selectively ADP-induced platelet responses. This review examines in detail the developments that have taken place to identify the ADP-receptor protein and to better understand mechanisms underlying ADP-induced platelet responses to develop strategies for designing innovative drugs that block ADP-induced platelet responses by acting selectively at the ADP-receptor and/or by selectively interfering with components of ADP-induced platelet activation mechanisms.

KEY WORDS: Purinergic Receptors, ADP-receptor, aggregin, identification and cloning, ADP-induced platelet activation mechanisms, drugs inhibiting ADP-induced platelet aggregation.

1040-9238/97/$.50

ABBREVIATIONS: CP, creatine phosphate; **CPK**, creatinephosphokinase; **PEP**, phosphoeno;pyruvate; **PK**, pyruvate kinase; **FSBA,** 5′*p*-fluorosulfonylbenzoyladenosine; **FSBG**, 5′p-fluorosulfonylbenzoylguanosine; ***o*ADP**, adenosine 5′-diphosphate 2′,3′-dialdehyde; ***ro*ADP**, adenosine 5′-diphosphate 2′,3′-dialchol; **2-MeS-ADP**, 2-methylthio-ADP; **2-N3-ADP**, 2-azido-Adenosine 5′-diphosphate; **2-AzPET-ADP**, 2-(*p*-azidophenyl)ethylthioadenosine 5′-diphosphate; **ATP-α-S**, adenosine 5′-*O*-(-2-thiophosphate); **AMPCCl$_2$P**, α,β-dichloromethylene-adenosine 5′-diphosphate; **AMPCF$_2$P**, α,β-difluoromethylene-adenosine 5′-diphosphate; **AMPNP**, α,β-imido-adenosine 5′-diphosphate; **AMPPCP**, β,γ-methlyene-adenosine; **Ap$_n$A**, α,ω-diadenosine polyphosphate; **WR-K**, Woodward's Reagent-K, *N*-ethyl-5-phenylisooxazolium-3′-sulfonate; **NaBH$_4$**, sodium borohydride; **2-BOP-TADP**, 2-(3-bromo-2-oxopropylthio)adenosine-5′-diphosphate; **2-BDB-TADP**, 2-(4-bromo-2,3-dioxobutylthio)adenosine-5′-diphosphate; **6-BDB-TADP**, 6-(4-bromo-2,3-dioxobutylthio)adenosine-5′-diphosphate; **8-BDB-TADP**, 8-(4-bromo-2,3-dioxobutylthio)adenosine-5′-diphosphate; **pCMBS**, *p*-chloromercuribenzenesulfonate; **NBD-Cl**, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole; **A23187**, a calcium ionophore; **U46619**, 9,11-dideoxy-9α,11α -methanepoxy prostaglandin F2α; **vWF**, von Willebrand Factor; **PAF**, platelet activating factor; **PMA**, phorbol 12-myristate 13-acetate; **[Ca^{2+}]$_i$**, intracellular calcium ion concentration; **AA**, arachidonic acid; **PIP$_2$**, phosphatidylinositol-4,5-bisphosphate; **IP$_3$**, inositol-1,4,5-triphosphate; **IP$_4$**, inositol-1,3,4,5-tetrakisphosphate; **DAG**, diacylglycerol; **PKC**, protein kinase C; **PLC**, phospholipase C; **PLA$_2$**, phospholipase A$_2$; **PDGF**, platelet-derived growth factor; **δ-SPD**, storage pool deficiency due to defects in dense granule secretion; **CFV**, cyclic blood flow variation; **SDS-PAGE**, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; **PRP**, platelet-rich plasma; **GP**, glycoprotein; **TM**, transmembrane.

I. INTRODUCTION

Nucleotides have long been known to act as stores of chemical energy that are used in metabolic processes and repositories of structural information that are used in the assembly of DNA and RNA. Szent-Gyorgyi first brought the awareness to the scientific community that extracellular nucleotides such as ATP also exert effects on the cardiovascular physiology.[1] Although unnoticed for almost 20 years, this discovery led to an enormous surge of interest in the extracellular effects of ATP on a wide variety of cellular responses.[2–12] More recently, the role of the extracellular ATP in modulating autoimmune functions[13,14] and apoptosis in thymocytes, macrophages, and other cells has come to light.[15,16]

ADP was not only the earliest but the first low-molecular-weight compound identified as a platelet agonist. Hellem demonstrated that a low-molecular-weight compound derived from red cells induced adhesion of the cells to glass;[17] the same compound was later found to aggregate platelets[18] and identified as ADP.[19] Born first showed that ADP induced platelet aggregation *in vitro*.[20] Because platelet aggregation induced by ADP was antagonized by adenosine and compounds structurally similar to ADP, it was thought that ADP-induced platelet aggregation played a central role in aggregation induced by other agonists.[21,22] Later on it was demonstrated that ADP was not responsible for the primary wave of aggregation observed with several platelet agonists.[23,24] ADP is also released from intracellular stores and the released ADP is responsible for the secondary wave

of aggregation seen during platelet stimulation by other agonists. ADP stimulates primary aggregation of platelets and the ADP secreted by the storage granules produces secondary aggregation. Understanding of the biochemical and cellular mechanisms underlying ADP-induced physiological responses has required participation of several disciplines, including cell biology, pharmacology, and, more recently, molecular biology.

II. ADP-INDUCED PLATELET AGGREGATION AND CLINICAL IMPORTANCE

Why has ADP-induced platelet aggregation been singled out for extensive and intensive investigation? Platelet aggregation is central to the pathogenesis of hemostasis and arterial thrombosis. Platelet aggregation plays a major role in acute coronary artery diseases, myocardial infarction, unstable angina, and stroke.[25-30] The deposition of platelets on an injured vessel wall is also important in hemostatic plugs as well as thrombus formation and depends on the rheology of blood flow, blood components, and properties of the vessel wall.[31,32] Adherence of platelets to injured vessel wall is also facilitated by the release of the contents of their storage granules into the surrounding medium. Low-molecular-mass substances such as ADP, ATP, serotonin, and, Ca^{2+} are components of the platelet dense granules and dense tubular system, respectively; high-molecular-mass substances such as adhesive, coagulant, and mitogenic proteins are components of α-granules. The interaction of components released from the storage granules of platelets exposed to stimuli leads to their interaction with binding sites for these components on platelet surface leading to platelet activation or with other components on the surface of blood or vascular cells leading to cell-cell communication. ADP-induced platelet activation *in vivo* promotes platelet adhesion to exposed surface of the vessel wall at the site of injury and is mediated by glycoprotein-Ib (GPIb) complex and von Willebrand factor (vWF)[33,34] followed by platelet-platelet bridge formation (aggregation) that is mediated by the adhesive proteins and conformationally competent GPIIb-IIIa complex, a receptor for fibrinogen.[35,36]

ADP-removing enzyme systems creatine phosphate (CP) with creatine phospho kinase (CPK) and phosphoenolpyruvate (PEP) with pyruvate kinase (PK) when injected into the superior mesenteric artery have been shown to increase bleeding times significantly in rats and rabbits.[37] CPK/CP and PEP/PK have been shown to inhibit significantly deposition of platelets to type I collagen, suggesting a role of ADP in mediating platelet recruitment in thrombus formation. Clopidogrel,[38] a drug that inhibits ADP-induced activation of platelets *in vivo*, has been shown to completely abolish cyclic blood flow variations (CFVs) in stenosed and endothelium-injured coronary arteries, suggesting that ADP antagonists may provide significant protection against platelet aggregation leading to pathophysiological states in clinical settings.[39] Ticlopidine[40] and PCR 4099,[41] like clopidogrel, block ADP-induced platelet activation *in vivo* and were found to completely inhibit ADP-induced platelet aggregation *ex vivo* in rats and greatly prolonged rat tail transection bleeding time, suggesting that ADP plays a key role in thrombogenesis.[42]

A congenital defect of platelet functions in a human patient due to severe impairment of the primary wave of platelet aggregation induced by ADP and other agonists that release ADP from intracellular stores has been described.[43] Impairment of platelet aggregation due to defective interaction between ADP and its platelet receptor or the defect in the receptor protein itself has been

linked to a spasmodic history of brief episodes of excessive bleeding in another patient.[44] Storage pool disease (δ-SPD) due to defects in the storage of nucleotides and/or their secretion from dense granules has been shown to lead to impairment of platelet aggregation in response to collagen and other stimuli due to absence of the potentiating effects of ADP released during secretion.[45–47] ADP-induced primary aggregation of platelets is related to increasing age regardless of sex.[48] The rate of primary wave of aggregation increased with an increase in age (up to 50 years). The same pattern was observed with the secondary wave of aggregation, but only with males.[48]

ADP-induced platelet activation, particularly the ADP-induced platelet aggregation, plays a key role in maintaining normal hemostasis and thrombosis. Defects in the binding of ADP to its receptor or the abnormalities in the ADP-receptor protein could have pathophysiological consequences. Therefore, characterization of the platelet ADP-receptor protein and availability of monoclonal antibodies to the receptor could pave the way to elucidate signal transduction mechanisms underlying ADP-induced platelet activation and help design specific antithrombotic drugs that might prove useful in clinical settings.

III. PURINORECEPTORS

The concept that nucleotides mediate physiological responses by binding to specific binding sites on cell surfaces was put forward by Burnstock.[49] Purinergic receptors have been classified based on the relative potencies of agonists and antagonists for these receptors. More recently, it has been suggested that classification of receptors should also include not only pharmacological but also biochemical information.[50] Such a description of purinoreceptors is helpful in understanding the place of an ADP-receptor and ADP-induced platelet activation in the broader context of cell responses mediated by purinoreceptors.

A. P1-Purinergic Receptors

Purinoreceptors were initially divided into two broad catagories: P1-purinergic receptors that followed the agonist potency order of adenosine > AMP > ADP > ATP and P2-purinoreceptors that followed the agonist potency order of ATP > ADP > AMP > adenosine.[51] Subsequently, another criteria of biochemical responses was added and led to the proposal for subclassification of purinergic receptors. The P1-purinergic receptors for adenosine were divided into A_1- and A_2-receptors[52] or R_1- and R_2-receptors.[53] A_1-and A_2-receptors appeared to be similar to R_1- and R_2-receptors.[54] This classification has been amended to accommodate additional pharmacological responses mediated by adenosine receptors[55] and places them in the seven transmembrane (TM) superfamily[56] of which crystalline bacteriorhodopsin is structurally archetypical.

B. P2-Purinergic Receptors

P2-purinoreceptors were initially divided into P2X and P2Y categories.[57] Since then the enormous amount of literature in the area of purinoreceptors made it necessary to include additionally P2X-, P2Z-, P2U-, P2T-, P2D (P3)-, and P4-type purinergic receptors in the parent category of P2-purinergic receptors.[56–61]

1. P2X-Purinergic Receptors

P2X type purinergic receptors are ligand-gated ion (Na^+, K^+, and Ca^{2+})-channels[8,62,63] (alternatively named as ionotropic ATP receptors)[64] for which the agonist potency order is α,β-methylene ATP (α,β-Me-ATP) > β,γ-Me-ATP > ATP = 2-methylthio-ATP (2-MeS-ATP).[60] β,γ-Me-L-ATP (L-AMP-PCP) is not only the most potent agonist acting at P2X receptors but also the most selective because it displays no agonist or antagonist activities at any of the known P2-receptors.[59] Pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) is a very potent inhibitor of P2X purinoreceptor-mediated cellular responses.[65] GTP, CTP, UTP, and ADP are as active as ATP at P2X receptors.[59] P2X receptors are widely distributed and evoke responses in various areas of brain as well as contraction of cardiac and smooth muscle. Recently, the presence of P2X Receptors in differentiated HL-60 cells has been reported.[66] P2X receptors in NGF-differentiated PC-12 cells[64] and vas deferens cells[67] have been cloned using *Xenopus* oocyte expression cloning system. A characteristic structural feature of the cloned P2X receptors is that they exhibit only two hydrophobic domains,[64,67] a structural feature that distinguishes them from other ligand-gated ion channels that have three to five hydrophobic domains.[63]

2. P2Y-Purinergic Receptor

ATP-binding P2Y receptors are G-protein coupled receptors and act via stimulation of phospholipase C (PLC) leading to the formation of inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) as well as an increase in intracellular levels of Ca^{2+} $[Ca^{2+}]_i$.[68–70] P2Y receptors also have been shown to modulate intracellular levels of cAMP[69,70] and arachidonic acid (AA) metabolism.[71] P2Y-purinergic receptors follow the agonist potency order 2-MeS-ATP >> ATP > α,β-Me-ATP > β,γ-Me-ATP. GTP, CTP, UTP, and ADP are as potent in inducing relaxation of *taenia coli*.[59] Suramin is a nonspecific antagonist of P2Y receptors;[72] reactive blue has been shown to be a somewhat more selective antagonist of cellular responses mediated by P2Y receptors.[60] The metabotropic P2Y-purinergic receptors are 7-TM G-protein coupled receptors, but the mode of binding of the adenine ring differs from that of G-protein coupled 7 TM adenosine receptors.[56] The $P2Y_1$ (human),[73] $P2Y_2$ (human),[74] $P2Y_3$ (chicken),[75] $P2Y_4$ (human),[76,77] $P2Y_5$ (chicken),[78] $P2Y_6$ (rat),[79] and $P2Y_7$ (human)[80] receptors have been cloned. The numerical order of the P2Y receptors corresponds to the order in which they have been cloned. There are some noteworthy structural features among the P2Y receptors that are present in the N-terminus of the extracellular domain as well as in TM1 through TM7[56] regions that are also present in the sequences of members of the G-protein coupled receptor family.[81] It has been suggested that P2Y receptors should be numerically numbered according to the common structural features inherited by them instead of the order in which they have been cloned.[56] The P2Y receptors are widely distributed and are present in brain neurons, astrocytes, osteoblasts, endothelial and epithelial cells, pituitary and pancreatic β-cells, and hepatocytes.

3. P2Z-Purinergic Receptors

P2Z-purinergic receptors are unique and are activated only by ATP^{4-}.[82] When ATP is chelated with Mg^{2+} or Ca^{2+}, it is rendered

ineffective in stimulating cellular responses mediated by P2Z receptor. Binding of ATP^{4-} renders the cells nonselectively permeable to Na^+, K^+ and Ca^{2+} and allows the flux of substances up to 900 Da. GTP, CTP, UTP, ADP, and AMP are inactive at P2Z. Another peculiar characteristic of P2Z Receptors is that they do not distinguish between S_p and R_p stereoisomers of phosphothioate analogs. No selective antagonists of this receptor are known. However, 10-fold molar excess of 2-MeS-L-ATP added simultaneously with ATP inhibited by 50% permeabilization of rat mast cells.[83] P2Z-purinergic receptors are present in inflammatory cells, such as macrophages and mast cells.[58]

4. P2U-Purinergic Receptors

P2U-Purinergic Receptors are activated by UTP as well as ATP.[68,74] P2U receptors are also metabotropic and coupled to G-proteins. Stimulation of PLC leads to phosphoinositide metabolism and an increase in $[Ca^{2+}]_i$.[74] The P2U receptor cloned from human sources is also responsive to ATP.[74] P2U receptors described by Erb et al.[84] and Lustig et al.[85] appear to have the same metabotropic characteristics and are stimulated by UTP and ATP. Photoaffinity labeling of the cloned P2U receptor 3′-*O*(4-benzoylbenzoyl)adenosine-5′-[α-^{32}P] triphosphate suggests that the binding site for ATP and UTP may be distinct. The P2U receptor cloned from chick brain by Communi et al. exhibits a preference for uridine over adenine nucleotides and therefore constitutes the first example of a pyrimidinergic receptor.[77] UTP and ATP have been shown to evoke Cl^- currents in airway epithelia that are generated by a mechanism different from the one involved with cystic fibrosis (CF) membrane conductance regulator.[86] This finding is important because it suggests that nucleotides may be therapeutically beneficial for the induction of Cl^- secretion in the airways of individuals with CF.[87–89] There are no known specific antagonists of P2U-purinergic receptors.

5. P2T-Purinergic Receptors

P2T receptors located in blood platelets are unique because ADP is an agonist and ATP an antagonist. ATP and AMP are competitive antagonists at P2T receptors.[90,91] CDP, UDP, GDP are almost inactive at P2T receptors. Binding of ADP at P2T stimulates release of Ca^{2+} from intracellular stores as well as influx of extracellular Ca^{2+} [92–96] and causes inhibition of prostaglandin-stimulated adenylyl cyclase activity.[97] The agonist potency rank order for P2T receptors is 2-MeS-ADP > ADP = 2-Cl-ADP.[90,98,99] The antagonists for the P2T receptors are discussed in detail separately later on in Section IV. For a long time the existence of P2T receptors was limited to platelets. In the last few years, the presence of P2T receptors has been demonstrated in megakaryoblastic cell line CHRF-288-11,[100] human erythroleukemia (HEL) cells,[101,102] U937 and K562 leukemia cells,[101] and megakaryocytic Dami cells,[103] all of which express platelet proteins.

6. P2D (P3) and P4-Purinergic Receptors

Diadenosine polyphosphates (Ap_nA, n = 2 – 6) are a class of nucleotides that are gaining increasing attention in the extracellular regulation of a variety of cellular systems.[61] They are found in the dense granules of platelets[104,105] and adrenal chromaffin cells.[106] Binding of adenosine polyphosphate to platelets can cause platelet aggregation[107]

and inhibition of ADP-induced platelet aggregation;[105,108,109] binding to chromaffin cells causes release of catecholamines.[110] Available evidence suggests that they also regulate intracellular mobilization and uptake of Ca^{2+} in chromaffin cells.[61] Although they appear to show similarities with P2Y receptors in this respect, a separate classification, P2D or P3, has been suggested for receptors that bind with high affinity to diadenosine polyphosphates.[61] Recently, different binding sites for ATP and Ap_nA on rat brain synaptic terminals have been detected; those binding to Ap_nA have been termed as P4-purinergic receptors.[61]

IV. REVERSIBLE AGONISTS AND ANTAGONISTS AT P2T-RECEPTOR

Chemical modification of ADP can be carried out in the adenine ring, ribose moiety, and diphosphate moiety. Substitution at the C_8 position results in lowering or complete loss of agonist potency. 8-Br-ADP[111] is a weak reversible inhibitor and 8-(4-bromo-2,3-dioxybutylthio)adenosine-5′-diphosphate [8-BDB-TADP][112] is an irreversible (covalent) inhibitor of ADP-induced platelet activation. Substitution at C_2 produces ADP derivatives that are either as potent as ADP or more potent than ADP in inducing platelet activation. 2-Cl-ADP, 2-N_3-ADP and 2-MeS-ADP are more potent than ADP as platelet agonists [90, 98,113] and their actions are completely antagonized by ATP.[98] 2-MeS-ADP, 2-ethylthio-ADP (2-EtS-ADP), and 2-butylthio-ADP (2-BuS-ADP) have all been shown to activate platelets.[113] 2-(4-bromo-2,3-dioxobutylthio)adenosine-5′-diphosphate [2-BDB-TADP] (Figure 1c) at short time intervals has been shown to be half as potent as ADP in eliciting platelet responses.[114] The effect of substitution at C_2 on the ability of ADP analogs to activate platelets is discussed in more detail in the following section in this review. Gough et al. demonstrated that small functional groups at N^6 reduced and large substitution completely abolished the ability of ADP-analogs to aggregate platelets.[113] Investigations carried out in our laboratory showed that 5′-*p*-fluorosulfonylbenzoylguanosine (FSBG) (Figure 2c), unlike 5′-*p*-fluorosulfonylbenzoyladenosine (FSBA) (Figure 2b), was completely ineffective in inhibiting platelet aggregation by agonists that depend on binding of ADP to its receptor.[115] 6-(4-bromo-2,3-dioxobutylthio)-adenosine-5′-diphosphate [6-BDB-TADP] (Figure 1b) was a reversible inhibitor of ADP-induced platelet activation.[114]

Chemical modification of ribose moiety in ADP results in loss of ADP-induced platelet functions. Adenosine-5′-diphosphate 2′,3′-dialdehyde (*o*ADP) (Figure 3b) and adenosine-5′-diphosphate 2′,3′-dialcohol (r*o*ADP) (Figure 3c) only induce platelet shape change.[116,117] They are completely ineffective in eliciting other platelet responses, such as aggregation,[116,117] and intracellular mobilization of Ca^{2+}.[117] When platelets are exposed to *o*ADP for longer periods of time, it inhibits ADP-induced platelet aggregation,[116,117] secretion,[117] and mobilization of $[Ca^{2+}]_i$.[117] Adeninearabinosyl 5′-diphosphate did not elicit shape change or aggregation but inhibited ADP-induced platelet shape change.[117] Work from our laboratory demonstrated that *o*ADP and r*o*ADP can interact with P2T receptor but fail to activate signal transduction machinery necessary for platelet activation.[117]

Chemical modifications in the diphosphate moiety have a varying degree of influence on the ability of ADP-analogs to modulate platelet functions. Adenosine 5′-*O*-(-2-thiophosphate) [ADP-β-S] can act as a partial agonist, whereas ADP-α-S is completely ineffective; instead, it is a competi-

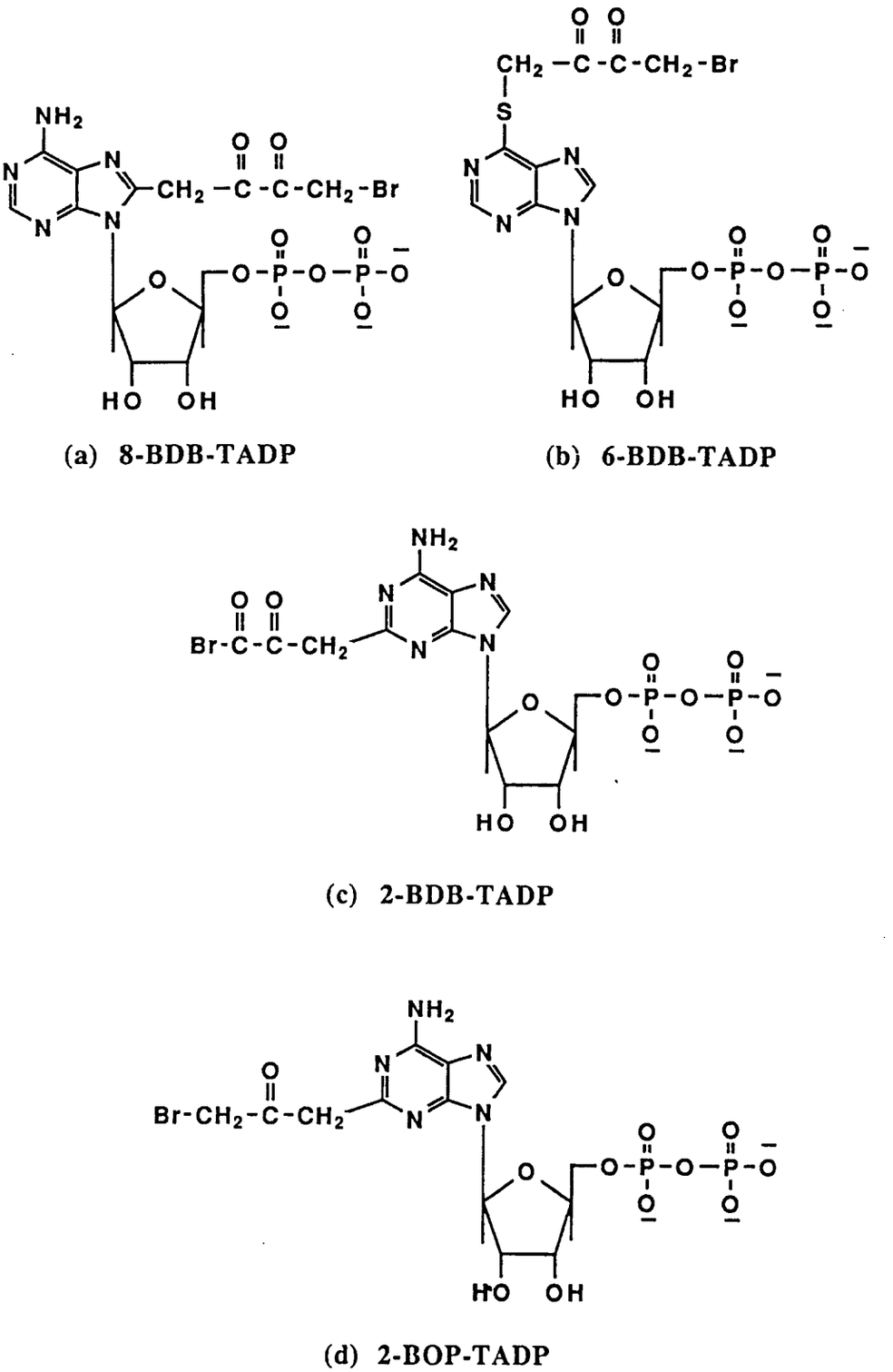
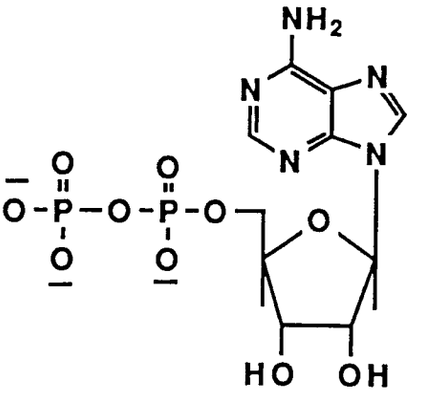
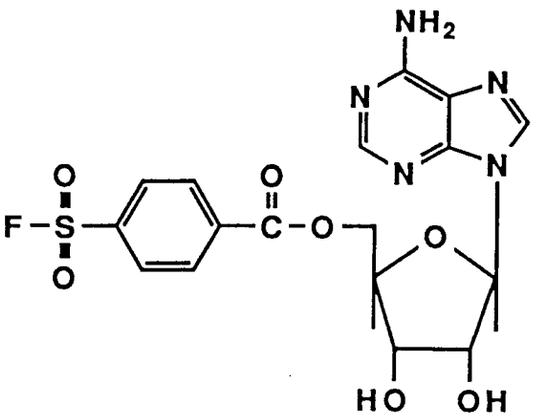


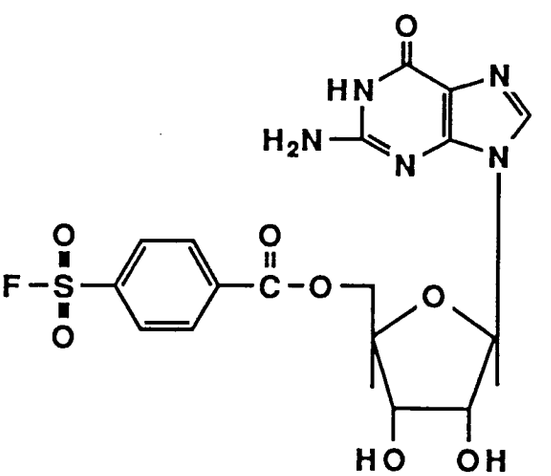
FIGURE 1. Chemical representations of chemically reactive ADP-affinity analogs. 8-BDB-TADP (a),[112] 6-BDB-TADP (b),[114] 2-BDB-TADP (c),[114] and 2-BOP-TADP (d).[128] For complete name of the nucleotide analogs, please refer to abbreviations.



(a) ADP



(b) FSBA



(c) FSBG

FIGURE 2. Chemical representations of nucleotides modified at 5′-position. FSBA (b)[158] and FSBG (c).[115] ADP (a) is included for comparison.

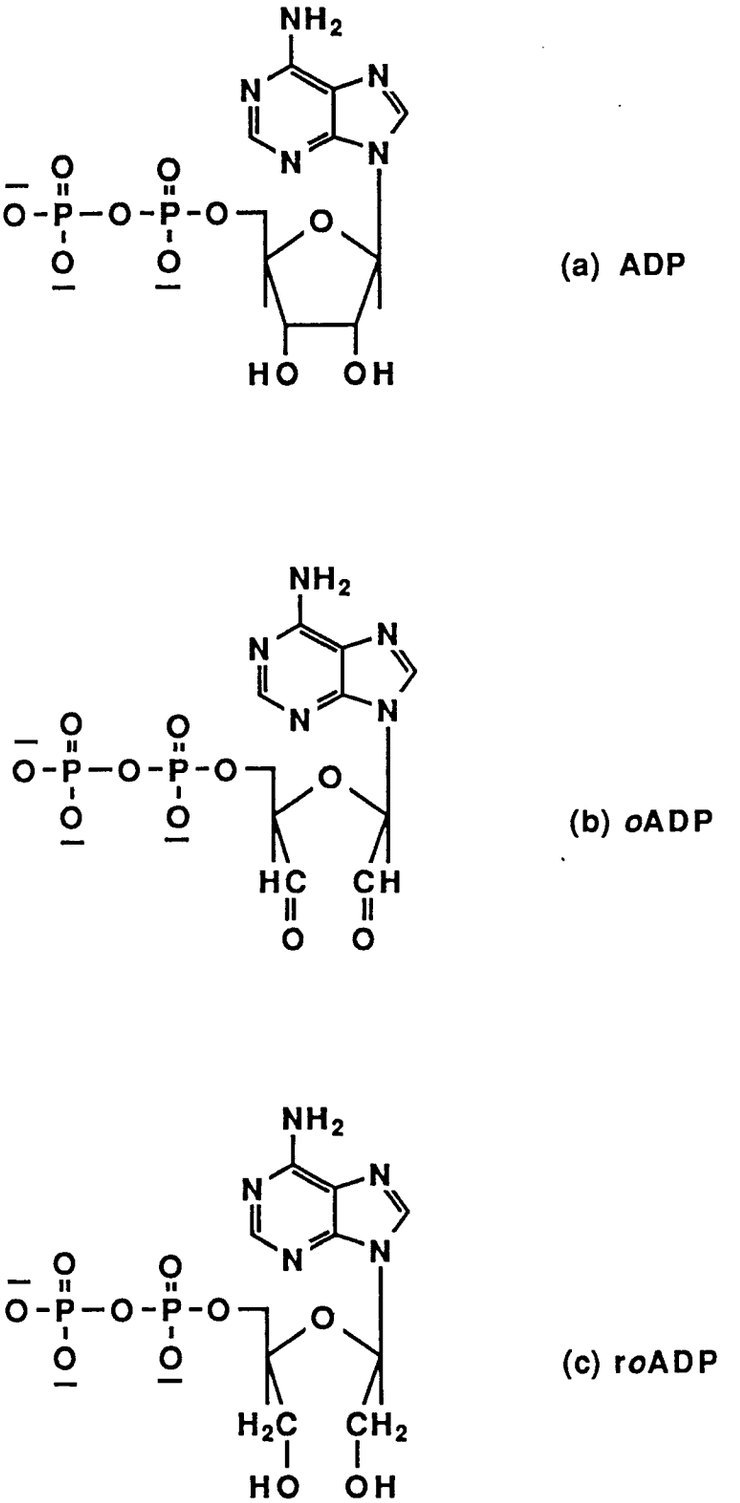


FIGURE 3. Chemical representations of ADP-affinity analogs modified in the ribose moiety. *o*ADP (b)[116,117] and *ro*ADP (c).[116,117] ADP (a) is included for comparison.

tive antagonist of ADP-induced inhibition of stimulated adenylate cyclase activity.[118,119] α,β-Dichloromethylene-ADP [AMPCCl$_2$P] is equipotent and α,β-difluoromethylene-ADP [AMPCF$_2$P] is more potent than α,β-Me ADP as antagonist of ADP-induced platelet aggregation and inhibition of stimulated adenylyl cyclase activity.[120] AMP and ATP are both competitive inhibitors at P2T receptor.[90,91] α,β-Imido-ADP (AMPNP) is a poor agonist at the P2T receptor.[120] 2-Cl-ATP, 2-chloroadenosine-5′-monophosphorothioate [2-Cl-AMPSP], β,γ-methylene-ATP (AMPPCP), Sp- and Rp-ATP-α-S and γ-fluoro-ATP have been shown to be competitive antagonists at P2T receptor.[90] 2-Alkylthio analogs of ATP were found to be non-competitive inhibitors at P2T receptor.[121,122]

α,ω-Diadenosine polyphosphates (Ap$_n$A) have been shown to be antagonists of ADP-induced platelet aggregation.[108] Ap$_4$A has been shown to be the most potent of all the Ap$_n$A analogs (n = 2 – 6), as an inhibitor of ADP-induced platelet aggregation.[108] Due to the presence of an axis of symmetry in the structure of Ap$_4$A, it was thought that it might act competitively with ADP at P2T receptor.[108] However, one- and two-dimensional proton magnetic resonance investigations of Ap$_n$A (n = 2 – 6) showed that base stacking was minimum in Ap$_2$A and Ap$_3$A.[109] It remains to be determined that it is also true of the platelet bound Ap$_2$A and Ap$_3$A. Therefore, the molecular basis for the strong inhibitory action of Ap$_4$A on ADP-induced platelet aggregation remains to be ascertained.

The P2T receptor exhibits stereoselectivity toward ligands that act as agonists and antagonists of the receptor: Rp-ADP-α-S is five times more stereoselective than the Sp-ADP-α-S at the P2T receptor. Similarly, Rp-ATP-α-S is almost twice as potent as Sp-ATP-α-S in inhibiting ADP-induced platelet aggregation.[90] L-ADP, 2-N$_3$-L-ADP and 2-Cl-L-ADP are completely inactive as agonists or antagonists at P2T receptor.[123] In marked contrast, 2-N$_3$-L-ATP, 2-Cl-L-ATP and 2-MeS-L-ATP are 25- to 125-fold more potent than their enantiomeric counterparts as agonists at P2Y receptor.[124]

V. 2-SUBSTITUTED ADP ANALOGS

Substitution, large or small, at C$_2$ in ADP produces compounds that have similar apparent affinities at P2T receptor.[58] 2-N$_3$-ADP[124,125] (Figure 4a) was found to be 5-fold more potent, and 2-Cl-ADP and 2-MeS-ADP[124,126] were 10-fold more potent than ADP in inducing platelet aggregation. 2-AzPET-ADP[126] (Figure 4b), 2-aminopropyl-thioadenosine-5′-diphosphate,[127] and 2-BDB-TADP[128] were found to be less potent than ADP in inducing platelet aggregation. Furthermore, at the same concentration the potency of ADP analogs to induce platelet aggregation is different from the potency to cause inhibition of intracellular cAMP formation. What is the molecular basis for comparing the relative potency of ADP analogs? 2-Cl-ADP, 2-N$_3$-ADP, and 2-MeS-ADP not only contain substituents at C$_2$ that have different sizes but also are covalently bound to C$_2$ by a hetero atom that has different ionic radius and carries different number of lone pairs of electrons in their outermost electronic shell. Electronic interactions with neighboring atoms are the result of the number of lone pairs of electrons and configuration of orbitals (s,p,d,f) that hold these electrons. Size of an atom, therefore, manifests itself in positioning a hetero atom in relation to other atoms in its neighborhood. Inspection of the structure of a C$_2$-substituted ADP analog does not permit predictions about its potency as a platelet aggregating agent and inhibitor of stimulated adenylyl cyclase activity. It is not possible to predict the relative potency

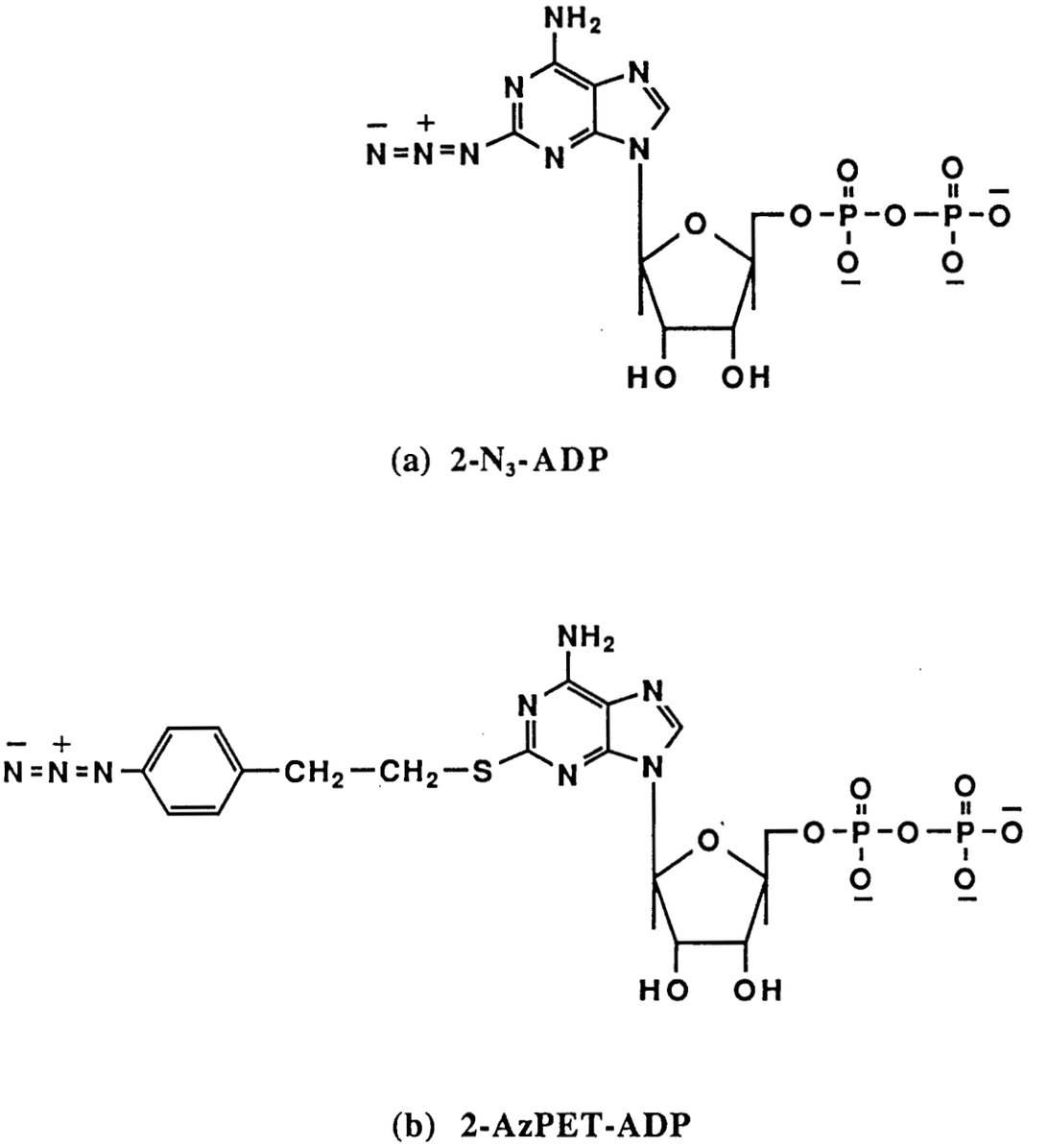


FIGURE 4. Chemical representations of ADP-affinity analogs containing the azido group. 2-N3-ADP (a)[124,125] and (b) 2-AzPET-ADP.[126]

of compounds such as 2-N_3-ADP[124] (Figure 4a) and 2-AzPET-ADP[126] (Figure 4b), although they carry the same reactive functionality, the azido group, but are bonded to C_2 in ADP by nitrogen and sulfur, respectively. Recent investigations from our laboratory showed that 2-BDB-TADP [2-(4-bromo-2,3-dioxobutylthio)adenosine 5′-diphosphate] (Figure 1c) was half as potent as ADP in inducing platelet shape change, aggregation, exposure of fibrinogen binding sites, serotonin from dense granules and mobilization of $[Ca^{2+}]_i$.[114] In order to examine the effect of the size of the substitution at C_2 in ADP,

we synthesized 2-BOP-TADP [2-(3-bromo-2-oxopropylthio)adenosin-5′-diphosphate][128] (Figure 1d) and investigated and compared its potency with ADP in inducing various platelet responses. 2-BOP-TADP is one carbon atom lower homolog of 2-BDB-TADP, but the two compounds contain the same chemical functionality and are bound to C_2 in ADP by a carbon atom. It was found that at equimolar concentration, 2-BOP-TADP was as potent as ADP in eliciting platelet responses.[128] We suggest that the size of the substituent at C_2 in ADP has a bearing on the potency of an ADP analog to elicit platelet responses. Moreover, the size of a substituent at C_2 in AMP has a bearing on its potency to inhibit ADP-induced platelet responses. For example, the rank order of potency for inhibiting ADP-induced platelet aggregation by 2-alkylthio analogs of AMP was found to be 2-ethylthio-AMP > 2-(pentan-1-yl)-AMP > 2-methylthio-AMP.[122] We believe this to be true for ATP derivatives as well.

VI. IDENTIFICATION OF THE PLATELET ADP-RECEPTOR

A. Photoaffinity Labeling

Agonists and antagonists that bind reversibly at P2T receptor, by definition are unsuitable for covalent labeling with radioactive compounds necessary for the identification of the ADP-receptor protein. 2-N_3-ADP (Figure 4a) has been shown to aggregate platelets and inhibit stimulated adenylate cyclase activity.[125] 2-N_3-ADP has also been used to label the active-site of adenosine deaminase.[129] Attempts to identify the platelet ADP-receptor protein by photoaffinity labeling with 2-N_3-[β-^{32}P]-ADP, however, were unsuccessful.[130] Photoaffinity labeling of platelets by 2-AzPET-[β-^{32}P]-ADP {2-(*p*-azidophenyl)ethylthioadenosine [β-^{32}P]-5′-diphosphate} (Figure 4b), an ADP analog that also aggregated platelets and inhibited intracellular formation of cAMP resulted in the labeling of several proteins in the range of 20 to 200 kDa; incorporation of the label into a 43-kDa protein was inhibited by 0.8 m*M* ADP and ATP.[126] Other prominent bands of radioactivity in the autoradiogram of platelets labeled with 2-AzPET-[β-^{32}P]-ADP correspond to proteins of 68 and 100 kDa.[126] The band of radioactivity at 68 kDa has been ascribed to radiolabeled albumin present as contaminant in the platelet washing and suspension buffer, but seems unlikely in washed platelets. Actin (43 kDa) has been shown to be radiolabeled when isolated platelet membranes, but not intact platelets, were exposed to [^{3}H]-FSBA, an ADP-affinity reagent.[131,132] Firm evidence that the 43-kDa protein labeled by 2-AzPET-[β-^{32}P]-ADP is not actin has been lacking.[126]

ATP-α-S is a potent antagonist of ADP-induced platelet responses[90] and also binds tightly to the ADP-binding sites on platelets.[133] Photoaffinity labeling of intact platelets with [^{35}S]-ATP-α-S resulted in the incorporation of the radiolabel in a 120-kDa protein identified as GPIIbα.[134] These results only suggest that there is a nucleotide binding site on GPIIb and in no way indicate that GPIIb is a candidate for the platelet ADP-receptor.[134] Platelets from individuals with Glanzmann's thrombasthenia (impairment and/or lack of GPIIb) show normal ADP-induced platelet shape change and influx of Ca^{2+},[135] but are poorly labeled with [^{35}S]-ATP-α-S.[136] Furthermore, GPIIb has been shown to be radiolabeled with [^{35}S]-GTP-α-S and [^{35}S]-UTP-α-S.[136] GPIIb has never been shown to be labeled with any ADP-affinity analog. The platelet ADP-receptor has never been shown to be labeled with a GTP or a UTP affinity-analog and

platelet responses induced by ADP are not inhibited by GTP and UTP.

Mayinger and Gawaz demonstrated that 8-N_3-[γ-^{32}P]-ATP labeled both GPIIb and GPIIIa of the GPIIb-IIIa complex (the fibrinogen receptor) in human blood platelets and suggested that extracellular nucleotides modulate platelet responses by directly regulating the fibrinogen receptor.[137] It seems that 8-N_3-[γ-^{32}P]-ATP and [^{35}S]-ATP-α-S label the same protein, GPIIb, on platelet surface, thus preventing the binding of fibrinogen to its receptor, resulting in the inhibition of ADP-induced platelet aggregation.

B. Photoaffinity Labeling: Effects of Ultra-Violet Radiation on Cellular Responses

Photoaffinity labeling requires the photolytic action of ultraviolet (UV) radiation on a photolabile group incorporated into site-specific affinity ligand. Cyclic nucleotides[138–140] and nucleotides[141,142] containing photolabile groups have been used in the photoaffinity-labeling of functional proteins for which they are competitive inhibitors. UV radiation-induced labeling of non-competitive blockers of acetylcholine receptor has been used to ascertain binding site of these blockers on the receptor.[143] Photoaffinity labeling of proteins in intact cellular systems frequently does not take into account effects of UV radiation on cellular responses before designing experiments largely because of the lack of scientific information available on the subject prior to 1990. UV radiation has now been shown to expose fibrinogen binding sites on platelets leading to platelet aggregation.[144,145] UV radiation-induced exposure of fibrinogen binding sites was correlated with PKC activation.[146] Exposure of platelets to UV radiation has been shown to cause progressive increase in the expression of activation markers P-selectin (GMP-140: CD 62) and LIMP (GP 53: CD 63) on the platelet membrane over a time in a dose-dependent manner.[147] The phenomenon of UV radiation-induced cellular activation is not limited to platelets. It has been shown that UV radiation induces tyrosine phosphorylation in lymphocytes in a dose- and wavelength-dependent manner and also induces Ca^{2+} signals in Jurkat T cells via tyrosine phosphorylation of PLCγ1 and associated proteins.[148,149] Radiation has also been shown to recruit stress-activated protein kinases (SAPKs, also called c-Jun-N-terminal kinases [JNKs]),[150,151] which are involved in specific gene expression.[152,153] These investigations demonstrate and support the conclusion that UV radiation can mimic the effect of binding of small physiological molecules (agonists) to their receptors in activating signal transducing machinery in mammalian cells. Photoaffinity labeling of intact human blood platelets carried out by using 2-AzPET-[β-^{32}P]-ADP[126] and [^{35}S]-ATP-α-S[134] did not consider these problems. It would be more appropriate to describe these investigations either as "photolabeling" or as "UV radiation-induced labeling" instead of photoaffinity labeling. These investigations failed to examine the effects of UV radiation on the ability of 2-AzPET-ADP and ATP-α-S to induce exposure of platelet fibrinogen binding sites, secretion, and mobilization of $[Ca^{2+}]_i$.[126,134] The agonist specificity of these reagents in the presence of UV radiation was never evaluated.[126,134] In one report, it has been stated that 2-AzPET-ADP was less potent than ADP in inducing platelet aggregation but was nearly as potent as ADP under the conditions of photolysis, but no data were presented to substantiate this statement.[126] If anything, 2-AzPET-ADP under the conditions of photolysis would be expected to behave as an inhibitor of ADP-induced platelet aggregation because of covalent modification of ADP-

binding sites by the nitrenes generated from the affinity label.

C. Affinity Labeling

FSBA (Figure 2b) is an ADP derivative in which the diphosphate moiety at the 5′-end has been replaced by chemically reactive but spatially equivalent *p*-fluorosulfonylbenzoyl moiety that is capable of forming a covalent bond with nucleophiles such as –SH (cysteine), –OH (tyrosine), –N = NH (histidine) and $-\epsilon_{NH_2}$ (lysine) groups (Figure 5) at the active and/or regulatory sites of purified enzymes as well as ligand binding domains of intact cellular systems.[132,154] FSBA remains the most extensively used nucleotide affinity analog to elucidate the structure-function relationship of nucleotide binding proteins.[132,154] FSBA has been demonstrated to inhibit ADP-induced platelet shape change,[131] aggregation, and exposure of fibrinogen binding sites.[155] Inhibition of ADP-induced platelet shape change correlated with covalent incorporation of FSBA into platelets in a time- and concentration-dependent manner.[156] [^{3}H]-FSBA was shown to label a single surface-membrane protein, aggregin (100 kDa)[157,158] in intact human blood platelets, although four different nucleotide binding proteins, including actin, were radiolabeled in membranes prepared from platelets.[131] FSBA is a slow-acting irreversible inhibitor of ADP-induced platelet shape change and aggregation (cf., covalent modification). FSBA has been shown to have no effect on ADP-induced inhibition of stimulated adenylate cyclase activity or the binding of 2-MeS-[β-^{32}P]-ADP to platelets.[159] At higher concentration (300 μ*M*), FSBA-induced platelet shape change and caused concentration-dependent rise in $[Ca^{2+}]_i$.[160] Platelet aggregation and exposure of fibrinogen binding sites induced by collagen[115] and U46619 (a thromboxane mimetic)[161] have been shown to be inhibited by treatment of platelets with FSBA, suggesting involvement of ADP-dependent mechanism. Investigations using unlabeled FSBA and [^{3}H]-FSBA demonstrated that epinephrine increases the avidity of ADP in potentiating ADP-induced platelet aggregation.[162] In contrast, covalent modification of platelets by FSBA had no effect on the rates of aggregation of platelets by high concentrations of thrombin[163] and plasmin,[164] suggesting that platelet aggregation induced by these two pathophysiological proteases under these conditions does not require binding of ADP to its receptor. Instead, experimental evidence suggests that thrombin- and plasmin-induced platelet aggregation involve an indirect cleavage of the ADP receptor aggregin by calpain expressed on the surface.[163,164] Either binding of ADP to its receptor or proteolytic cleavage of ADP receptor may have the same consequences of removing the latency of the platelet fibrinogen receptor, the GPIIb-IIIa complex.[158,163,164]

FSBA does not contain the negatively charged diphosphate moiety at the 5′-end and hence is insoluble in aqueous medium and its solution needs to be freshly prepared in either dimethylformamide or dimethylsulfoxide. FSBA carries an ester linkage between *p*-fluorosulfonylbenzoyl group and 5′-hydroxyl group of adenosine, which is prone to hydrolytic cleavage catalyzed by acids or bases as well as enzymatic cleavage by ectonucleotidases at the surface of cells (Figure 6). These processes lead to the formation of adenosine, which is an inhibitor of ADP-induced platelet activation. Therefore, the use of FSBA requires inclusion of adenosine demeans in the incubation mixtures.[155,159] Experimental methods available for the synthesis of [^{3}H]-FSBA produce a product of relatively low specific radioactivity, thus making the process of identifying the platelet surface radiolabeled proteins cum-

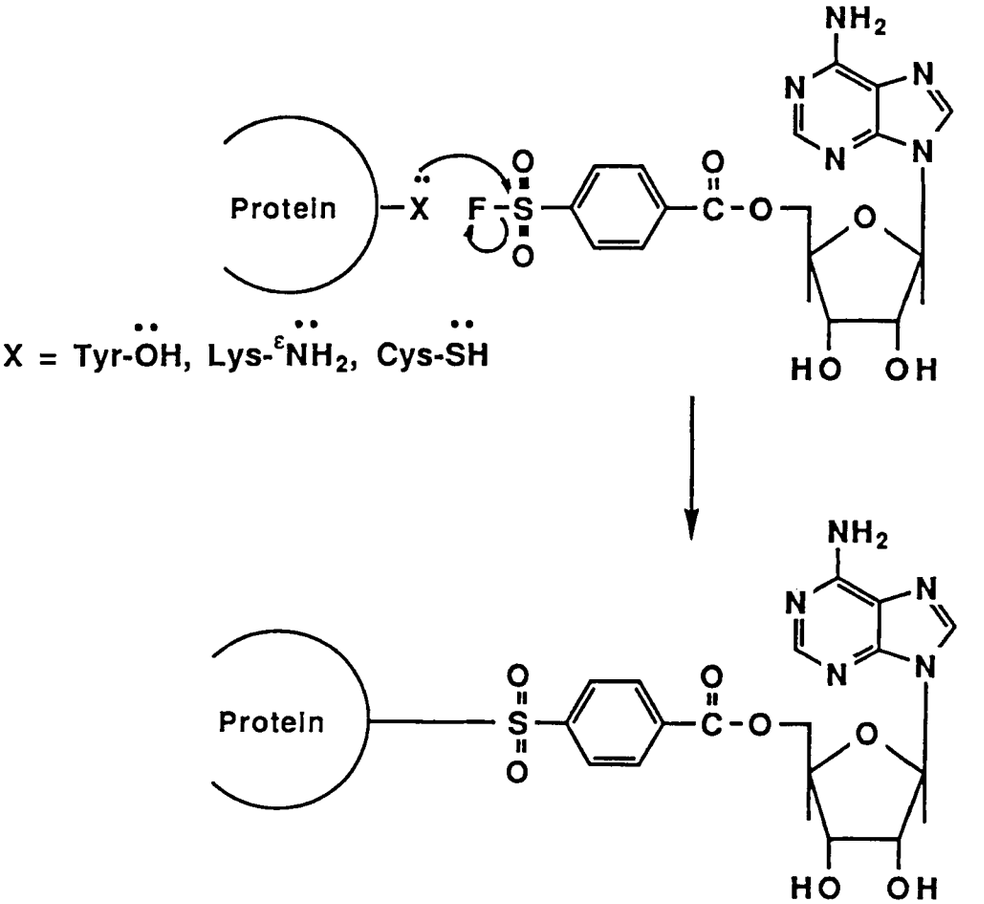


FIGURE 5. Schematic representation of covalent adduct formation between FSBA and proteins. Nucleophilic attack by the lone pair of electrons in reactive amino acids present in the functional domain(s) of proteins on the electrophilic sulfur in FSBA is accompanied by the displacement of fluoride ion leading to the formation of a covalent adduct. The negative inductive effect of the two sulfonyl groups facilitates such reaction.

bersome and time consuming.[163,164] At higher concentrations, FSBA induces platelet shape change and mobilization of $[Ca^{2+}]_i$.[160]

In view of the limitations on the use of FSBA as an ADP affinity analog to probe ADP-induced platelet functions and identify the ADP receptor protein, a new generation of nucleotide-based affinity reagents were thoroughly investigated. The ADP affinity analogs containing 4-bromo-2,3-dioxobutylthio (BDB-T) moiety at C_2, C_8, and N^6 positions (the only positions in the adenine ring in ADP available for covalent modification) (Figure 1) have been used previously by Dr. Roberta F. Colman's group to investigate structure-function relationship of nucleotide binding enzymes.[132] The BDB-TADP compounds have several advantages over FSBA. These compounds are true ADP affinity reagents and contain a negatively charged diphosphate moiety at the 5′ end, thus making them soluble in aqueous medium, and their aqueous solutions are stable over a period of months when stored at –80°C. The

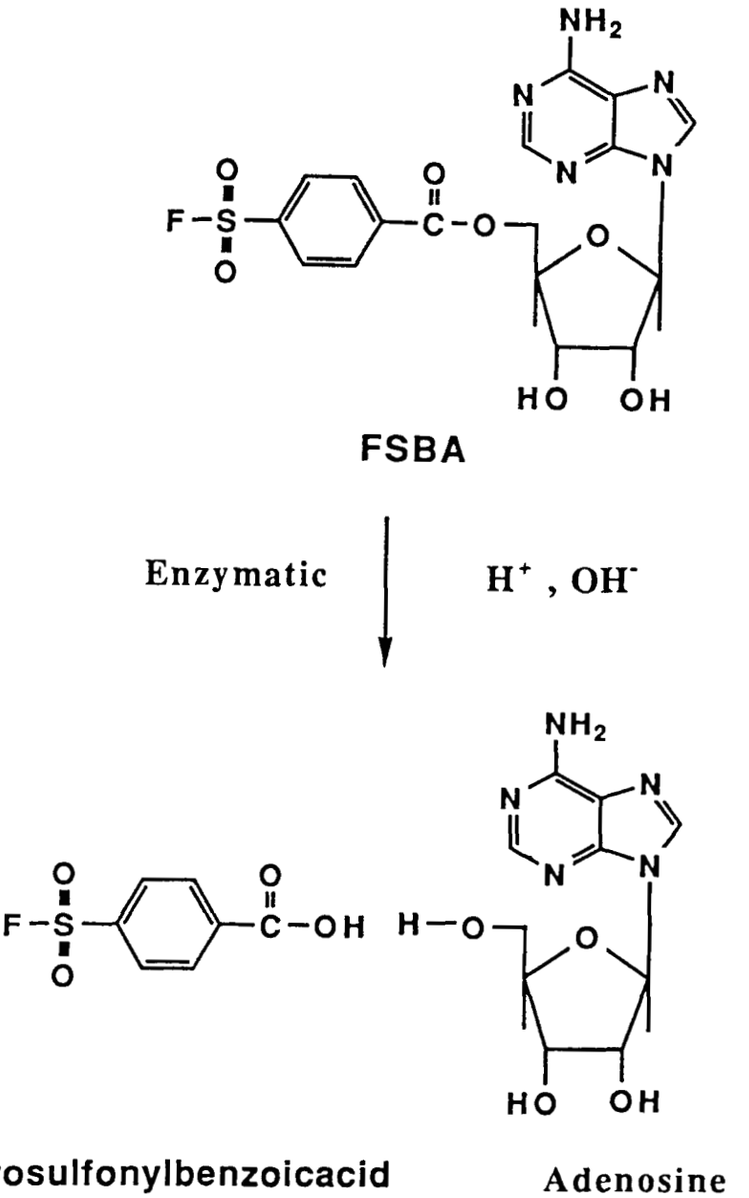
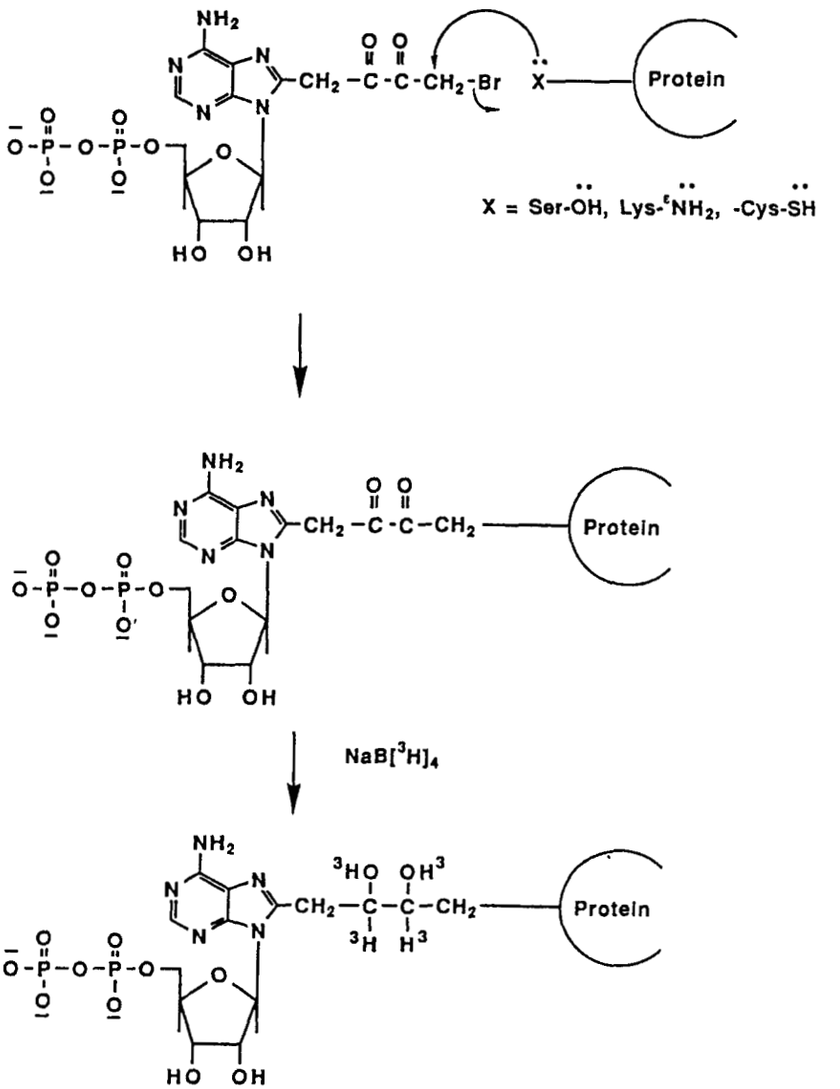


FIGURE 6. Schematic representation of the cleavage of the ester bond at the 5′-end in FSBA. Cleavage of the ester bond at 5′-end in FSBA by acids, bases, and ectonucleotidases generates adenosine and 5′-*p*-fluorosulfonylbenzoic acid. Adenosine is an antiplatelet agent.

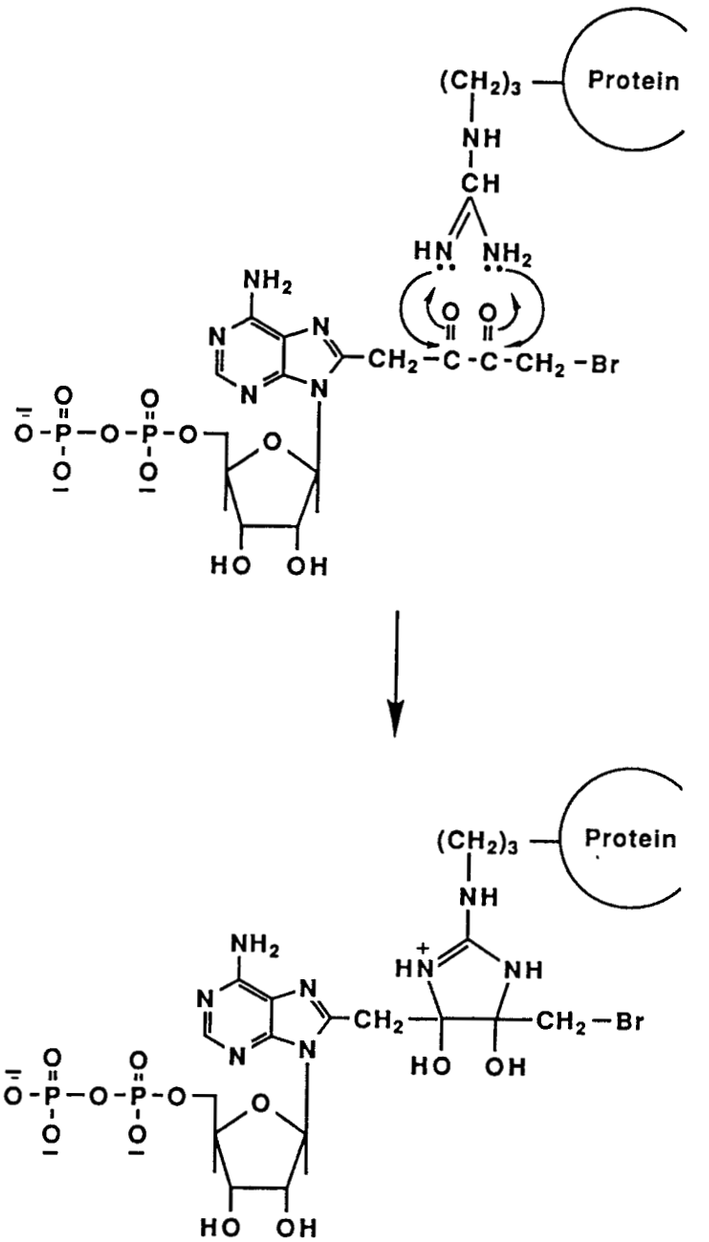
compounds can be synthesized as ^{32}P-labeled derivatives of high specific radioactivity. They can also be used as unlabeled compounds followed by reduction with commercially available $NaB[^3H]_4$ (Figure 7, Scheme 1) of very high specific radioactivity to increase incorporation of the label into compound covalently bound to the platelet receptor.



Scheme 1

FIGURE 7. Schematic representation of the possible modes of adduct formation between 8-BDB-TADP and proteins.

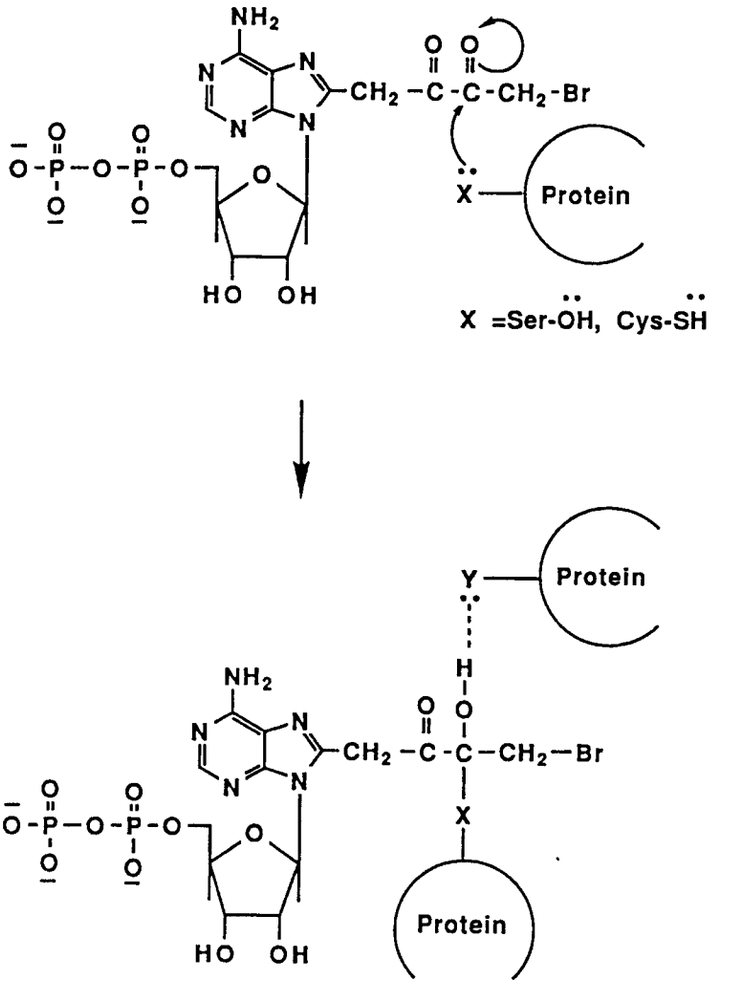
Scheme 1. Covalent adduct formation by nucleophilic displacement at alpha carbon atom in 8-BDB-TADP. Nucleophilic attack by the lone pair of electrons in reactive amino acids present in the functional domain(s) of proteins on the carbon atom alpha to the diketone function in 8-BDB-TADP, is accompanied by the displacement of bromide ion leading to the formation of a covalent adduct. The negative inductive effect of the 1,2-diketo function attached to carbon bearing bromine facilitates such a reaction. The reaction is representative of the general reaction between the BDB-TADP and BOP-TADP compounds and proteins. The adduct formed is amenable to reduction with $NaB[^3H]_4$ (bottom figure). Tritium in the vicinal diol may exchange to varying degrees depending on the solvent used and its polarity. Reduction of the adduct between 8-BDB-TADP and proteins with $NaB[^3H]_4$ provides an effective means to radiolabel proteins with affinity analogs containing the BDB-T and BOP-T groups. Alternatively, the same goal can be accomplished by using [^{32}P]-labeled BDB-TADP and BOP-TADP compounds and omitting the reductive step shown in the Figure 8a.



Scheme 2

FIGURE 7.

Scheme 2. Covalent adduct formation by nucleophilic addition at both carbonyl groups of the 1,2-diketone moiety in 8-BDB-TADP. Simultaneous nucleophilic attack by the lone pair of electrons on the two nitrogens of arginine leads to the formation of a substituted imidazole derivative that is not amenable to reduction with $NaB[^3H]_4$. Thus, the ability to detect formation of a radiolabeled protein adduct following treatment with $NaB[^3H]_4$ provides a way to distinguish between arginine and other residues being modified by the affinity analogs containing the BDB-T group.

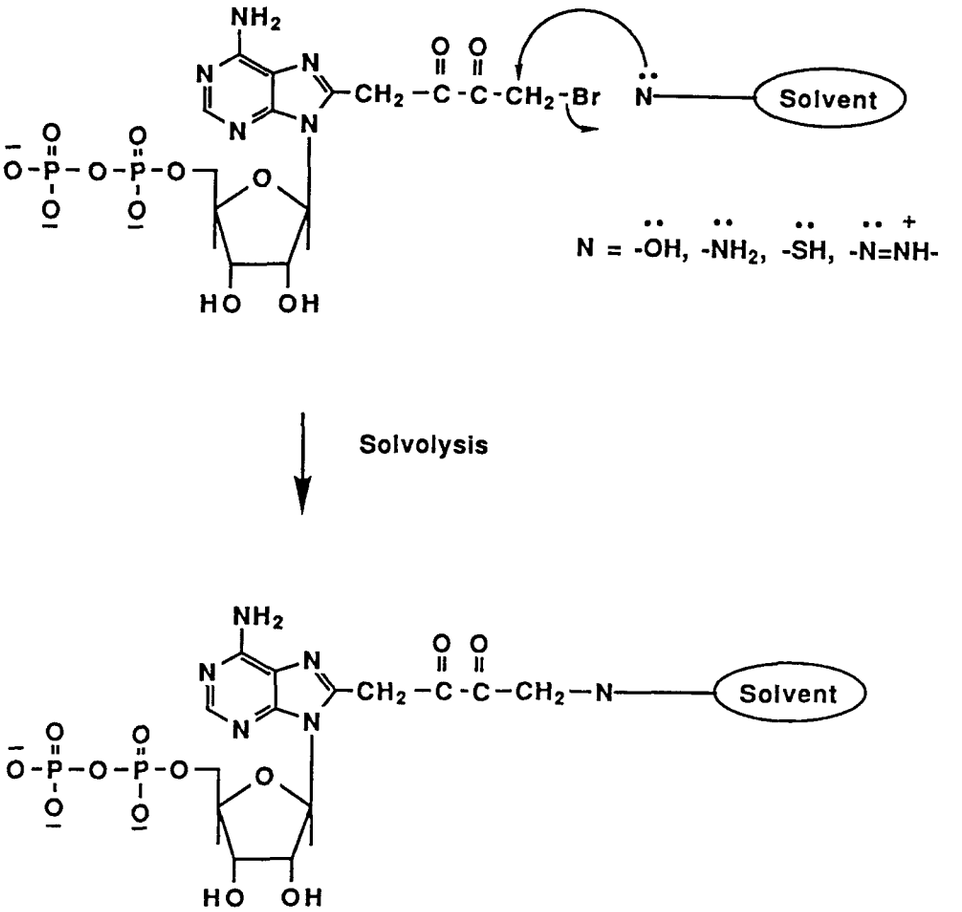


Scheme 3

FIGURE 7 (continued).

Scheme 3. Covalent adduct formation by nucleophilic addition at one carbonyl group of the 1,2-diketone function in 8-BDB-TADP. Nucleophilic attack by the lone pair of electrons on the hetero atoms in serine and cysteine in proteins on the carbonyl function of the affinity analog leads to the formation of hemiacetals and thiohemiacetals. The formation of hemiacetals and thiohemiacetals is a reversible reaction, but such adducts may be stabilized by hydrogen bond formation between the generated hydroxyl groups and hetero atoms of appropriately disposed groups in reactive amino acids in a protein (bottom figure). The products formed are not amenable to reduction with $NaB[^3H]_4$.

The compounds are susceptible to solvolysis (hydrolysis is a special case of solvolysis) (cf., α-haloketones) at high and low pH, but the hydrolysis products simply tend to lower the effective concentration of the parent compounds and do not generate adenosine, an inhibitor of platelet functions (Figure 7, Scheme 4).



Scheme 4

FIGURE 7.
Scheme 4. Schematic representation of solvolysis of 8-BDB-TADP. The BDB-TADP and BOP-TADP affinity analogs are prone to hydrolysis at moderate to high basic pH or solvolysis by the reactive functional groups in solvents. The reaction involves nucleophilic displacement of bromide ion by the elctrophilic groups present in the solvent. Such reactions simply tend to lower the effective concentration of the affinity analogs but do not generate adenosine (cf. FSBA; Figure 6), an antiplatelet compound.

Unlike the ADP-analogs used in photoaffinity labeling to identify the ADP receptor protein,[126,134] the nature of the chemical reactions of the BDB-TADP compounds are well known and the products formed by interactions with proteins have been identified.[132] There are three modes of chemical reaction between the BDB-TADP compounds and functional proteins. First, nucleophilic displacement of bromine, at carbon atom alpha to the diketone moiety, by the reactive groups (–OH, –SH, $-\varepsilon_{NH_2}$) in essential amino acids leads to formation of a covalent bond (Figure 7, Scheme 1); the product of such a reaction is amenable to reduction by $NaB[^3H]_4$ (Figure 7, Scheme 1). Second, the

essential arginine residue in proteins can react with the α-diketone function in BDB-TADP analogs to yield a cyclic adduct[165] that cannot be reduced by $NaB[^3H]_4$ (Figure 7, Scheme 2). Third, the OH (serine) and –SH (cysteine) functions can undergo nucleophilic addition with the carbonyl moiety(ies) of α-keto (cf., 2-BOP-TADP; Figure 1d) or α-diketofunction (cf., 2-BDB-TADP; Figure 1c) to form hemiacetals or thiohemiacetals (Figure 7, Scheme 3). Formation of hemiacetals and thiohemiacetals is reversible, but such chemical linkages can be stabilized by hydrogen bonding in the functional domains of proteins (Figure 7, Scheme 3) (cf., α-ketoamide inhibitors of the active-site of thrombin); however, such adducts or the enols derived from them cannot be reduced by $NaB[^3H]_4$.

8-Br-ADP has been previously shown to be an inhibitor of ADP-induced platelet aggregation.[134] Therefore, 8-BDB-TADP appeared to be the first choice of investigation for its effects on ADP-induced platelet functions and the surface proteins modified by it. 8-BDB-TADP (Figure 1a) has been used previously to investigate structure-function relationship of ADP-requiring enzymes such as pyruvate kinase[166] and glutamate dehydrogenase.[167] 8-BDB-TADP did not induce platelet shape change and aggregation or mobilization of $[Ca^{2+}]_i$.[112] 8-BDB-TADP inhibited ADP-induced platelet shape change, aggregation, exposure of fibrinogen binding sites, secretion, mobilization of $[Ca^{2+}]_i$, and formation of intracellular cAMP mediated by prostaglandins.[112] 8-BDB-TADP inhibited platelet aggregation induced by those agonists that completely (ADP)[157] or partially (collagen, U46619)[115,161] depend on the binding of ADP to its receptor. An autoradiogram of the gel obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of solubilized platelets modified by either 8-BDB-T-[β-^{32}P]-ADP or 8-BDB-TADP and $NaB[^3H]_4$ showed the presence of a single radiolabeled protein band at 100 kDa (Figure 8a). The intensity of this band was reduced when platelets were preincubated with ADP, ATP, 8-bromo-ADP, or covalently modified with *p*-chloromercuribenzenesulfonate (pCMBS) and FSBA before labeling with the above reagents (Figures 8a and 1c). These results show that 8-BDB-TADP selectively and covalently modified aggregin, a putative ADP-receptor on the platelet surface, and such modification led to the loss of ADP-induced platelet functions.[112] These results also suggest that the presence of a cysteine and/or lysine residue(s), but not an arginine residue, at or near the ADP-binding site in platelets. These results are in accord with those that showed *o*-phthalaldehyde (OPTH), a reagent that cross-links suitably disposed –SH and $-\varepsilon_{NH_2}$ groups, blocked ADP-induced platelet shape change and aggregation by covalently modifying the ADP-binding sites in platelets.[168]

We then compared 8-BDB-TADP with 2-BDB-TADP for the ability of the latter to modulate platelet responses. 2-BDB-TADP (Figure 1c) has been used previously to probe the allosteric site of pig heart NAD^+-dependent isocitrate dehydrogenase that binds to ADP.[169] ADP analogs substituted at C_2 have been shown to activate platelets.[113] 2-BDB-TADP at short times was found to induce platelet shape change, aggregation, exposure of fibrinogen binding sites, secretion, and mobilization of $[Ca^{2+}]_i$.[114] When platelets were incubated with 2-BDB-TADP for longer time periods, ADP-induced platelet responses, including ADP-induced inhibition of stimulated adenylate cyclase, were blocked. Under these experimental conditions, 2-BDB-TADP partially inhibited collagen- and U46619-induced platelet aggregation but minimally affected ADP-independent platelet aggregation induced by thrombin, A23187 (a Ca^{2+} ionophore) and phorbol 12-myristate 13-acetate (PMA). 2-BDB-TADP and $NaB[^3H]_4$ radiolabeled a single 100-kDa surface protein in platelets (Figure 9). Prior treatment with

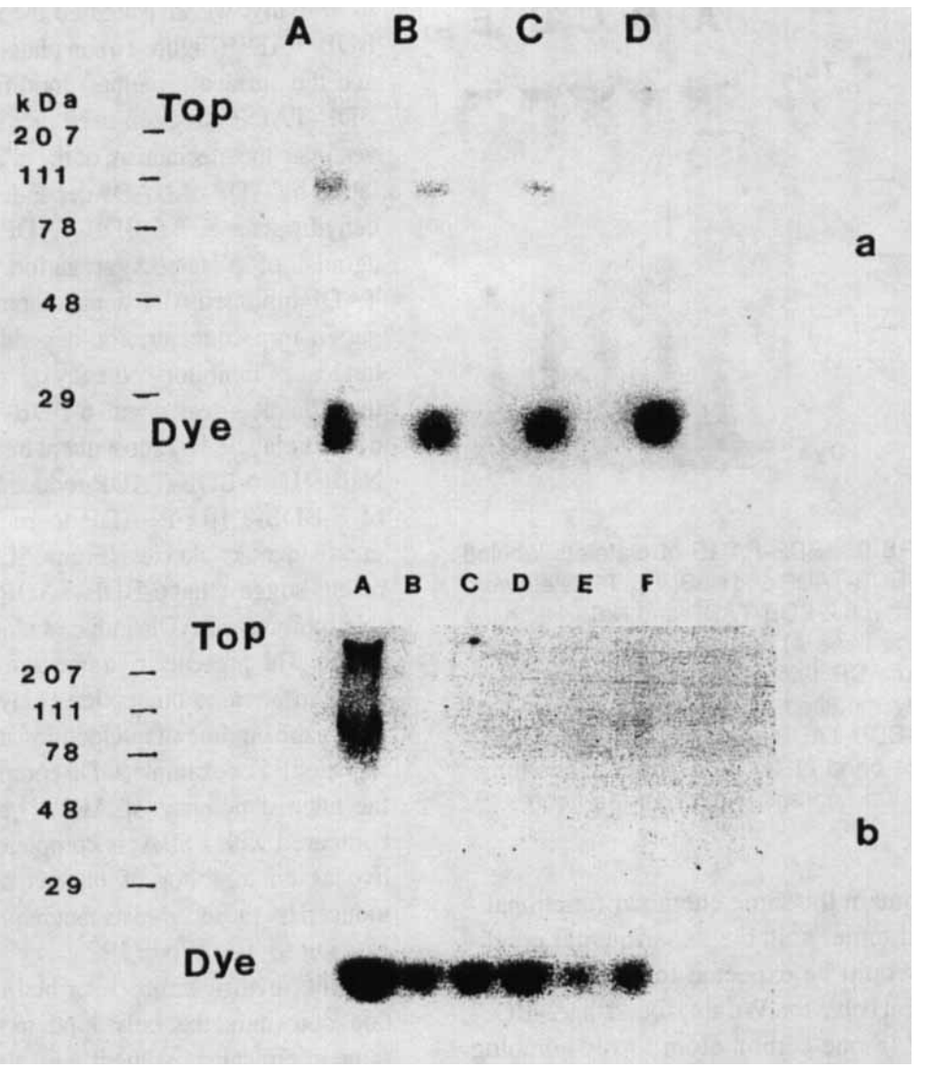


FIGURE 8. SDS-PAGE of platelets labeled with [β-^{32}P]-8-BDB-TADP. Panel a) autoradiogram of platelets labeled with [β-^{32}P]-8-BDB-TADP in the absence (lanes A through C) and in the presence of ADP (lane D).[112] Panel b platelets were labeled with [β-^{32}P]-8-BDB-TADP in the absence (lane A) and presence of ADP (lane B). ATP (lane C) and platelets were covalently modified with FSBA before modifying with [β-^{32}P]-8-BDB-TADP (lanes E and F). Lane D corresponds to an aliquote of [β -^{32}P]-8-BDB-TADP in the absence of platelets. The band at 100 kDa corresponds to radiolabeled aggregin, a putative ADP-receptor in both the figures.[112]

ADP, ATP, or covalent modification with FSBA reduced such labeling (Figure 9). Moreover, the 100-kDa protein in platelets covalently modified with 2-BDB-TADP was not radiolabeled with 8-BDB-T-[β-^{32}P]-ADP (Figure 10a). These results suggest that when platelets were exposed to 2-BDB-TADP for longer periods of time, it abrogated ADP-induced platelet responses by covalently modifying aggregin. These results are consistent with previous data because 2-BDB-TADP and 8-BDB-TADP are structurally similar

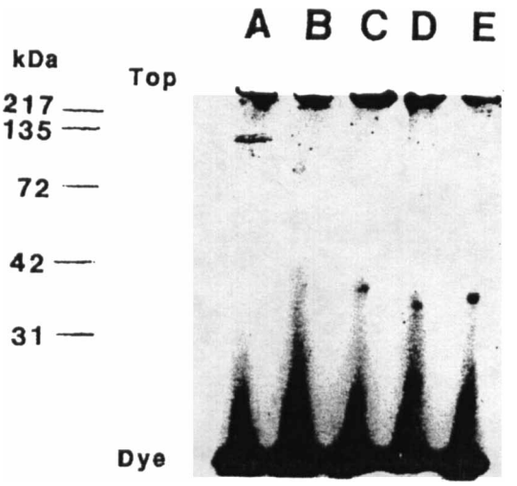


FIGURE 9. SDS-PAGE of platelets labeled with 2-BDB-TADP and NaB[^{3}H]$_4$. Platelets were labeled with 2-BDB-TADP and NaB[^{3}H]$_4$ in the absence (lane A) and presence of ATP (lane D) and ADP (lane E).[114] Platelets were covalently modified with FSBA before labeling with 2-BDB-TADP and NaB[^{3}H]$_4$ (lanes B and C). The band at 100 kDa corresponds to radiolabeled aggregin in both of the figures.[114]

and contain the same chemical functionality, and under suitable experimental conditions would be expected to exhibit similar chemical behavior. We also found that 2-BOP-TADP (a one carbon atom lower homolog of 2-BDB-TADP) behaved similarly to 2-BDB-TADP in inducing platelet responses but was twice as potent as 2-BDB-TADP[128] and equivalent to ADP. 2-BOP-TADP (Figure 1d) and NaB[^{3}H]$_4$ radiolabeled a single 100-kDa protein in platelets and the intensity of the band corresponding to the 100-kDa radiolabeled band was decreased by prior treatment of platelets by ADP, ATP, and covalent modification with FSBA (Figure 11). Platelets covalently modified by 2-BOP-TADP were not labeled by 8-BDB-T-[β-^{32}P]-ADP demonstrating that 2-BOP-TADP, under appropriate experimental conditions, blocks ADP-induced platelet responses by covalently modifying aggregin.[128]

Finally, we investigated the effects of 6-BDB-TADP (Figure 1b) on platelet responses and the surface protein(s) modified by it. 6-BDB-TADP has been used previously to investigate the mechanism of the allosteric regulation by ADP of NAD$^+$-dependent isocitrate dehydrogenase.[170] 6-BDB-TADP was not an agonist of platelet aggregation.[114] 6-BDB-TADP inhibited ADP-induced platelet aggregation in a concentration-dependent manner but lost its inhibitory potency over the next 20 min. Platelets treated with 6-BDB-TADP were not radiolabeled by subsequent treatment with NaB[^{3}H]$_4$. 6-BDB-TADP reduced the ability of 8-BDB-T-[β-^{32}P]-ADP to radiolabel aggregin in intact platelets (Figure 10b).[114] These results suggest that 6-BDB-TADP is a reversible inhibitor of ADP-induced platelet aggregation. The presence of a free amino function at N^6 in adenine nucleotides plays a critical role in the binding of nucleotides at their binding-site.[171] For example, GDP completely lacks the agonist potency of ADP,[113] and FSBG, compared with FSBA, is completely ineffective as an inhibitor of platelet aggregation induced by those agonists that require binding of ADP to its receptor.[115]

The investigations described in this section constitute the only kind in which the same chemical substituent was placed at C_2, C_8, and N^6 to systematically examine the effect of this substitution on the ability of ADP analogs to modulate platelet responses. Two important findings of these investigations are first, 2-BDB-TADP, 2-BOP-TADP, 6-BDB-TADP, and 8-BDB-TADP, like FSBA, modulate platelet responses by interacting with aggregin, a putative ADP receptor[112,114,128] and second, the size of a substituent at C_2 has a bearing on the potency of an ADP analog to function as a platelet agonist.[128] It is important to mention that aggregin (100 kDa) is a unique protein that is different from GPIIb[134] and GPIIIa.[172]

The ADP analogs modified at the ribose moiety have been shown to stimulate plate-

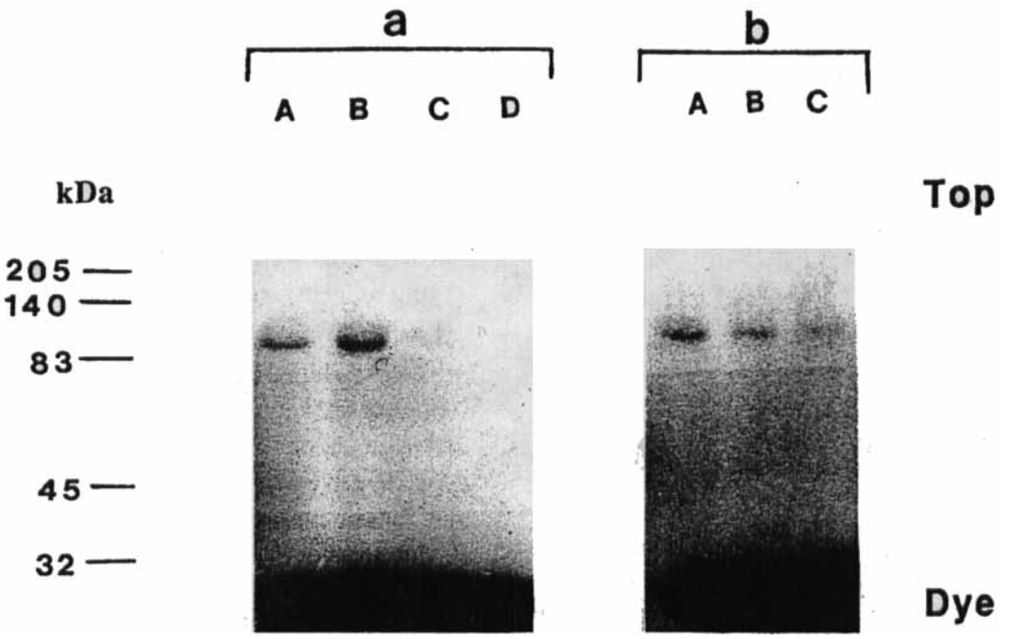


FIGURE 10. SDS-PAGE of platelets labeled with [β-^{32}P]-8-BDB-TADP in the absence and presence of 2-BDB-TADP and 6-BDB-TADP. Panel a) platelets were labeled with [β-^{32}P]-8-BDB-TADP in the absence (lanes A and B) and presence (covalently modified) of 2-BDB-TADP (lanes C and D).[114] Panel b platelets were labeled with [β-^{32}P]-8-BDB-TADP in the absence (lanes A) and presence (incubated for 10 min) of 6-BDB-TADP (lanes B and C). The band at 100 kDa corresponds to radiolabeled aggregin in both of the figures.[114]

let functions.[116] *o*ADP (Figure 3b), *o*ATP, r*o*ADP (Figure 3c) and r*o*ATP were found to induce platelet shape change but not aggregation and mobilization of $[Ca^{2+}]_i$.[116,117] *o*AMP and *o*Adn were ineffective in inducing platelet shape change. When platelets were incubated with *o*ADP for 5 min, it inhibited ADP-induced platelet shape change and aggregation. Using an immunoaffinity method (described in the next sections) it was found that *o*ADP initially interacts with aggregin to form an unstable Schiff's base and subsequent reduction with $NaBH_4$ stabilized this interaction.[117]

2-BDB-TADP, 2-BOP-TADP, and *o*ADP are some of the few examples of those affinity compounds that act both as agonists and irreversible antagonists of cellular functions. Affinity reagents are generally regarded as inhibitors of enzyme functions and antagonists of receptor-mediated cellular responses. Zabwcki et al. described affinity labels for the estrogen receptor that function as estrogen antagonists.[173] Wrzeszcynski and Colman demonstrated that adenosine 5′-*O*[S-(4-bromo-2,3-dioxobytyl)thiophosphate], an ADP-affinity analog, caused time-dependent activation (instead of inhibition) of bovine liver glutamate dehydrogenase.[174] In the case of cellular receptors, it is easier to understand that affinity reagents directed to the receptor site could first bind reversibly to transmit signals for receptor-mediated cell responses and more slowly modify these sites covalently, thus interfering with agonist-induced responses by externally added ligands.

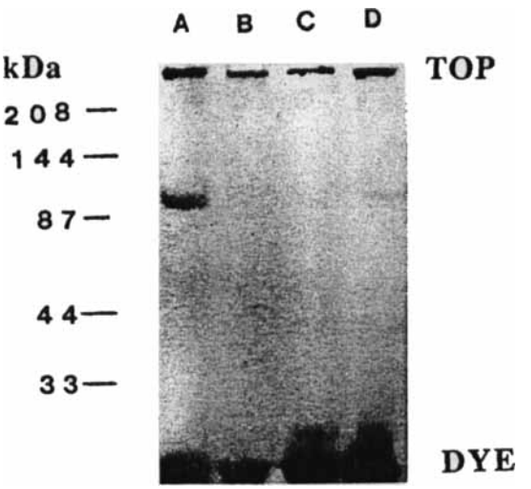
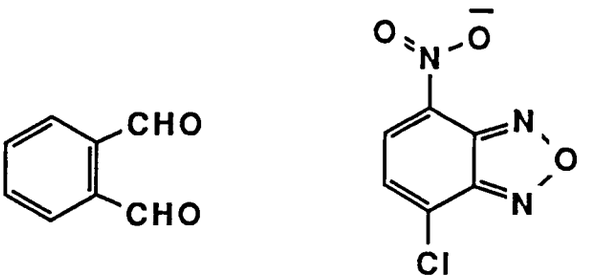


FIGURE 11. SDS-PAGE of platelets labeled with 2-BOP-TADP and NaB[^{3}H]$_4$. Platelets were labeled with 2-BDB-TADP and NaB[^{3}H]$_4$ in the absence (lane A) and presence of ADP (lane B) and ATP (lane C).[128] Platelets were covalently modified with FSBA before labeling with 2-BOP-TADP and NaB[^{3}H]$_4$ (lane D). The band at 100 kDa corresponds to radiolabeled aggregin in both of the figures.[128]

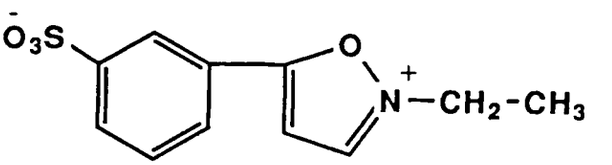
D. Labeling with Pseudo-Affinity Compounds

Hydrophobic interactions between the nucleotide binding site and the adenine ring of the nucleotides play an important role in directing these ligands to the binding site. Therefore, it has been possible to use aromatic compounds containing suitable chemically reactive groups (pseudo-affinity compounds) to probe the nucleotide binding site in enzymes. Salicylic acid and its iodinated derivatives have been demonstrated to interact with the adenine binding site of dehydrogenases and kinases. One of the examples is the inactivation of isocitrate dehydrogenase by covalent modification by 4-iodoacetamidosalicylic acid.[175] Another reagent OPTH (Figure 12a) that covalently forms an isoindole adduct with closely situated lysine and cysteine residues has been used to investigate the catalytic subunit of cAMP-dependent protein kinase[176] and catalytic and regulatory sites of cGMP-dependent protein kinase.[177] Because ADP-induced platelet aggregation is inhibited by sulfhydryl group-modifying reagents, such as *N*-ethylmaleimide (NEM)[178,179] and pCMBS, as well as by amino group-modifying reagents, such as pyridoxal phosphate (PLP).[180] We examined the effect of OPTH on ADP-induced platelet responses. OPTH inhibited ADP-induced platelet shape change and aggregation in a time- and concentration-dependent manner and the excitation and emission fluorescence spectra were consistent with the formation of an isoindole adduct between platelets and OPTH.[168] OPTH inhibited labeling of platelets by [^{3}H]-FSBA. Taken together the results suggest that covalent modification of essential cysteine and lysine residues by OPTH at or near the ADP-binding domain in aggregin led to the loss of ADP-induced platelet activation.[168]

7-Chloro-4-nitobenz-2-oxa-1,3-diazole (NBD-Cl) (Figure 12b) is a chemical modification reagent that forms fluorogenic adducts with O, S, and N amino acids.[181] The reagent has been used successfully to investigate structure-function relationships of nucleotide-binding enzymes, such as the catalytic subunit of cAMP-dependent protein kinase [182] as well as intact cellular systems, such as HL-60 cells.[183] NBD-Cl was shown to inhibit ADP-induced platelet responses by covalent modification of ADP-binding sites in platelets.[184] [^{14}C]-NBD-Cl radiolabeled a 100-kDa protein in platelets and the intensity of this radiolabeled protein band was reduced by ADP and ATP, as well as prior covalent modification of platelets by pCMBS or FSBA.[184] We also demonstrated that NBD-Cl and pCMBS prevented radiolabeling of aggregin in platelets exposed to 8-BDB-T-[β-^{32}P]-ADP.[185] Taken together, the results show that covalent modification of aggregin by NBD-Cl inhibited ADP-induced platelet activation.



(a) OPTH (b)NBD-Cl



(c) WR-K

FIGURE 12. Chemical representation of pseudo-affinity compounds. (a) OPTH ,[168] (b) NBD-Cl ,[185] and (c) WR-K.[186] For complete names of the compounds see abbreviations. The pseudo-affinity compounds were used to investigate their effect on ADP-induced platelet activation and the surface protein(s) modified by them. For details of their chemical reactions with proteins see appropriate references.

Woodward's Reagent-K (WR-K) (Figure 12c) contains an aromatic nucleus, a heterocyclic five-membered ring containing nitrogen and a negative charge, structural features present in ADP. WR-K has been used to investigate structure-function relationship of enzymes such as Na^+-mg^{2+}-ATPase[186] as well as anion-proton cotransport of human red blood cells.[187] Although the reagent was originally designed to react with essential carboxylic groups, it has been shown to react with essential sufhydryl group(s) in L-threonine dehydrogenase.[188] We demonstrated that treatment of platelets with WR-K led to loss of ADP-induced platelet functions and WR-K and $NaB[^3H]_4$ were found to radiolabel a single surface protein at 100 kDa.[185] Radiolabeling of a 100-kDa protein in intact platelets by these reagents was reduced by prior treatment with ADP and ATP or covalent modification with sulfhydryl group-modifying reagents, such as pCMBS, NBD-Cl, and OPTH. Platelets covalently modified by WR-K were not radiolabeled with 8-BDB-T-[β-^{32}P]-ADP. Together the results show that covalent modification of an ADP-re-

ceptor with WR-K led to inhibition of ADP-induced platelet responses.

The results obtained by ADP affinity analogs and pseudo-affinity compounds agree with each other and suggest that aggregin is a strong candidate for an ADP receptor in human blood platelets. Results also suggest that the ADP-binding site on platelets contain essential cysteine and/or lysine residues and covalent modification of these residues blocks the ability of ADP to stimulate platelets by inhibiting binding of ADP to its receptor.

E. Immunoaffinity Labeling

Information presented in the previous sections shows that despite the limitations on its use, FSBA served a very useful purpose in understanding details of ADP-induced platelet activation and the initial identification of the surface protein responsible for binding to ADP. We have also described several ADP affinity analogs and pseudo-affinity compounds to identify an ADP receptor protein and their effects on ADP-induced platelet functions.

The available method of synthesis of [^{3}H]-FSBA yields a product of relatively low specific activity, thus limiting its use in the identification and purification of the ADP-receptor protein. The use of 2- and 8-BDB-TADP compounds to selectively and covalently label aggregin requires synthesis of these compounds either as radioactive materials or as non-radioactive compounds that then require additional use of NaB[^{3}H]$_4$. Although these reagents provide an effective and useful means to identify aggregin by radiochemical methods, the synthesis of the ADP-affinity analogs on a large scale is laborious and time consuming.

In order to develop a rapid method to identify aggregin, we undertook to investigate the use of a commercially available affinity-purified polyclonal antibody to FSBA-labeled glutamate dehydrogenase (GDH)[189] to identify FSBA-labeled aggregin in correspondingly labeled platelets. The polyclonal antibody neither recognized GDH nor FSBA, but it did recognize FSBA-labeled proteins, such as FSBA-labeled pyruvate kinase.[190] An immunoblot of the gel obtained by SDS-PAGE of the solubilized FSBA-labeled platelets showed the presence of a 100-kDa protein band, and this band was absent in platelets either preincubated with ADP or ATP (Figure 13, left panel) or covalently modified with 2-BDB-TADP, 2-BOP-TADP, and 8-BDB-TADP (Figure 14). Corresponding gels stained with Coomassie blue showed the presence of a multitude of proteins in the range of 20 to 200 kDa (Figure 13, right panel).[190] The results show that the polyclonal antibody to FSBA-modified GDH is capable of recognizing FSBA-labeled aggregin in intact platelets and thus could potentially be used to purify aggregin by immunoaffinity column chromatography. The immunoaffinity method was found to be more sensitive than the radiochemical methods developed in our laboratory to identify the platelet ADP receptor. As FSBA is also capable of reacting with enzymes that require ATP for their catalytic function, the polyclonal antibody may be used to identify and purify other P2 type purinergic receptors that require binding of ATP to elicit platelet responses. It should be noted that in cells other than platelets this method could potentially recognize more than one nucleotide-binding receptor. However, the method is limited to the identification of those surface ADP and ATP binding proteins that react covalently with FSBA.

F. Miscellaneous

Adler and Hardin obtained a platelet soluble fraction that contained 80% of the

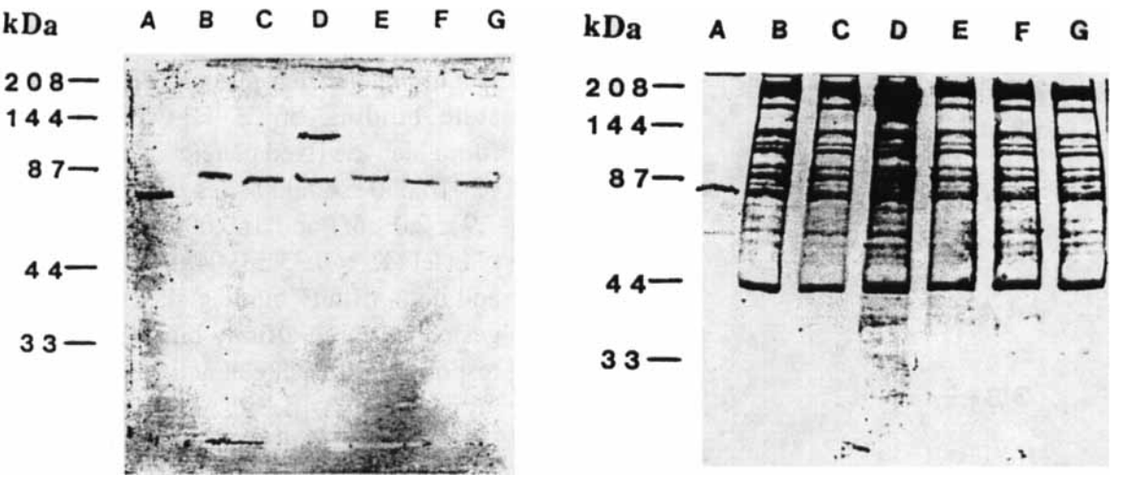


FIGURE 13. Immunoblot of platelets labeled with FSBA in the absence and presence of nucleotides. Immunoblots were prepared by SDS-PAGE of platelets, transferring the proteins from the gel onto a polyvinyledenefluoride membrane and developing the membrane with a polyclonal antibody to FSBA-labeled glutamate dehydrogenase (GDH).[189] Left panel, the lanes correspond to the following: FSBA-modified GDH (lane A); platelets were first incubated with ADP before modifying with FSBA (lane B); platelets were first incubated with ATP before modifying with FSBA (lane C); platelets modified with FSBA (lane D); platelets (lane E), platelets treated with ADP only (lane F), and platelets treated with ATP only (lane G).[190] Right panel, gel obtained by SDS-PAGE of samples described in the top panel was stained with Coomassie blue.[190] The immunoblot in the left panel shows the presence of two proteins: a 100-kDa protein corresponding to aggregin covalently modified with FSBA and a 87-kDa protein tentatively identified as glycoprotein-IIIa. The latter was accomplished in a separate experiment by using a highly purified sample of glycoprotein-IIb-IIIa complex. Polyclonal antibody to FSBA-GDH used to prepare the immunoblot is not monospecific. In addition to recognizing FSBA-modified proteins, the polyclonal antibody also recognizes an ester linkage (cf. FSBA-modified proteins contain an ester linkage at 5′-end. See Figures 2 and 4). Such ester linkages are present in platelet membrane-surface proteins like glycoprotein-IIIa, which are myristylated and/or palmitoylated.[190] In contrast, multitude of proteins are present on the surface of platelets as revealed by the Coomassie blue stained gel (right panel).

membrane ADP binding activity that corresponded to 20% of the total membrane protein (4-fold purification)[191]. The ADP binding activity was destroyed following trypsin digestion and the rank order of potency for nucleotide antagonism of [^{3}H]-ADP binding to the fraction was ATP, ADP > AMP >> adenosine. The membrane fraction was further purified by sucrose density gradient and a value of 61 kDa was established for the ADP binding protein. The authors suggested that the hydrodynamic properties (axial ratio, $f/f_0 = 1.09$) indicate that the protein is spherical but anchored to the platelet membrane by a large hydrophobic tail that is cleaved from the main protein during membrane isolation. There has been no follow-up on this investigation.

VII. ADP BINDING SITES ON PLATELETS

The first definitive attempt to estimate the number of ADP binding sites on platelets was made by Nachman and Ferris, who reported a value of 100,000 sites/platelet with an affinity constant of 6.5 μM.[192] Scatchard

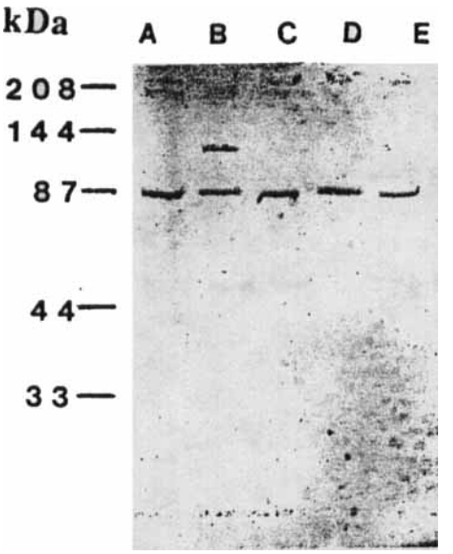


FIGURE 14. Immunoblot of platelets labeled with FSBA in the presence of chemically reactive ADP-affinity analogs. Immunoblots were prepared as described in the legend to Figure 13. The lanes correspond to the following: platelets (lane A); FSBA-modified platelets (lane B); platelets covalently modified with 8-BDB-TADP before modifying with FSBA (lane C); platelets covalently modified with 2-BDB-TADP before modifying with FSBA (lane D), and platelets covalently modified with 2-BOP-TADP before modifying with FSBA (lane E).[190] The results show that the protein band at 100 kDA recognized by the polyclonal antibody to FSBA-GDH indeed corresponds to aggregin, a putative ADP-receptor and the chemically reactive ADP-affinity analogs, FSBA, 8-BDB-TADP, 2-BDB-TADP, and 2-BOP-TADP covalently modify the same platelet surface protein, aggregin (100 kDa).

analysis of the binding parameters for [^{3}H]-ADP to solubilized ADP membrane fraction yielded a value of 38 μM for the K_d of a single class of binding sites.[191] Binding of 2-N_3-[β-^{32}P]-ADP was reversible and followed saturation kinetics and a value of 400 to 600 sites per platelet was determined.[130] Equilibrium binding studies using 2-MeS-[β-^{32}P]-ADP showed that binding was saturable and reversible and yielded a value of 400 to 1200 sites per platelet for single class of binding sites; a value of 6 and 15 nM was determined for K_d in washed platelets and platelets in platelet-rich plasma (PRP).[99] Steady state binding of [2-^{3}H]-ADP to paraformaldehyde fixed platelets yielded values of 410,000 ± 40,000 sites per platelet (K_d = 7.9 ± 2.0 μM) and 160,000 ± 20,000 sites/platelet (K_d = 0.35 ± 0.04 μM) for the low- and high-affinity binding sites; it was suggested that high-affinity binding sites were responsible for platelet activation.[111] Binding of 2-AzPET-[β-^{32}P]-ADP to platelets was reversible and saturable and a value of 400 sites per platelet (apparent affinity constant of 11 nM) was determined for a single class of binding sites.[126]

Recently, two patients with defects in the ADP binding and/ ADP receptor have been reported.[43,44] In one of these investigations, binding isotherms for [^{14}C]-ADP yielded values of 46,000 sites per platelet (K_d = 3.1 μM) and 50,000 sites per platelet (K_d = 3.5 μM) for the patients; a median number for normal volunteers was found to be 65,000 sites per platelet (K_d = 3.1 μM).[43] Binding studies using [2-^{3}H]-ADP and paraformaldehyde-fixed platelets from one patient in the same investigation yielded values of 32,000 sites per platelet (K_d = 0.14 μM) for the high-affinity binding sites and 77,000 sites per platelet (K_d = 2.2 μM) for the low-affinity binding sites; a median value of 55,800 ± 14,900 sites per platelet (K_d = 0.5 ± 0.25) for the high-affinity binding sites and 546,000 ± 86,000 sites per platelet (K_d = 12.5 ± 3.4) for the low-affinity binding sites were assigned to the control.[43] In another clinical investigation, Scatchard analysis of binding isotherms generated by using 2-MeS-[^{3}H]-ADP yielded values of 30 ± 17 sites per platelet (K_d = 2.07 ± 0.25 nM) and 836 ± 126 sites per platelet (K_d = 2.16 ± 0.79 nM) for the patient and controls, respectively.[44]

A simultaneous investigation of binding of 2-MeS-[^{3}H]-ADP to platelets from nor-

mal healthy volunteers and normal rats yielded values of 600 sites per platelet (K_d = 5.4 ± 2.1 n*M*) and 1200 ± 55 sites per platelet (K_d = 4.0 ± 0.5 n*M*), respectively.[193] In rat platelets only, binding of 2-MeS-[^{3}H]-ADP was found to be time-dependent and saturable; Scatchard analysis of the data yielded a value of 940 ± 30 sites per platelet (K_d = 0.78 ± 0.05 n*M*) for a single class of binding sites.[194] The same group of investigators showed that clopidogrel, a potent and specific inhibitor of ADP-induced platelet aggregation *in vivo*, reduced by 70% (280 sites per platelet, K_d = 0.9 n*M*) a single class of ADP binding sites when compared with control rats (960 sites per platelet, K_d = 0.77 n*M*). It was concluded that rat platelets consist of two populations of a single class of binding sites, which could be discriminated on the basis of their resistance to clopidogrel.[195]

FSBA, in the presence of adenosine deaminase, was shown to inhibit ADP-induced platelet shape change, but it did not antagonize the ADP-induced inhibition of stimulated adenylate cyclase activity.[159] On the other hand, pCMBS was shown to block ADP-induced inhibition of accumulation of cAMP in platelets. FSBA did not inhibit ADP-induced mobilization of $[Ca^{2+}]_i$. At higher concentration (300 μ*M*), FSBA induced platelet shape change and concentration-dependent increase in $[Ca^{2+}]_i$.[160] It was suggested that FSBA-induced platelet shape change was mediated by aggregin and the intracellular mobilization of Ca^{2+} and antagonism of the ADP-induced inhibition of stimulated adenylate cyclase activity was mediated by a second ADP receptor or two sites on one receptor of different affinities.[160] Incorporation of [^{3}H]-FSBA into aggregin in platelets from human volunteers administered clopidogrel was unaffected compared with controls.[196] However, ADP-induced platelet aggregation was severely impaired.[196] Binding of 2-MeS-[β-^{32}P]-ADP to the platelets from volunteers administered clopidogrel was reduced (199 ± 78 sites per platelet) compared with controls (534 ± 44 sites per platelet) without a consistent change in binding affinity.[196] The results show that clopidogrel administration to human volunteers reduced the number of those ADP binding sites that are involved in the antagonism of intracellular cAMP formation. We have not made any attempt to estimate the number of ADP binding sites on platelets because the ADP affinity analogs containing the BDB-T and BOP-T moieties to identify the ADP receptor covalently modify the receptor. These reagents are biochemical probes used to identify the ADP receptor protein and relate the chemical modification of the receptor protein with biochemical platelet responses induced by ADP. They are unsuitable to evaluate the pharmacological characteristics of the receptor.

The presence of P2T receptors on hematopoietic cell lines has been demonstrated[101] and binding characteristics of ADP to promegakaryoblasts (RPM), human erythroleulekmia (HEL) cells, U937 and K562 cells have been investigated.[101] Scatchard analysis of the saturation binding isotherms using [^{3}H]-ADP gave values of 399,000 ± 170,000 sites per cell (K_d = 775 ± 230 μ*M*) for RPM, 81,900 ± 32,500 sites per cell (K_d = 21.5 ± 8.4 μ*M*) for HEL cells, 53,900 ± 28,000 sites per cell (K_d = 13.7 ± 3.9 μ*M*) and 11,500 ± 2300 sites per cell (K_d = 22.0 ± 5.3 μ*M*) for U937 cells.[101] The single class of ADP binding sites on these cells were displaced by antagonists with potency rank order of ATP > ADP > AMP > adenosine.[101]

Soslau et al. have presented experimental evidence that shows that there might be receptors for ATP on platelets that modulate platelet functions and are different from those mediated by the binding of ADP at P2T receptor.[197,198] The potency rank order β,γ-Me-ATP, α,β-Me-ATP > ATP suggests the presence of P2X purinergic receptors on human blood platelets.[198] These investigators

also suggest that inhibition of ADP-induced platelet aggregation by ATP, in part, is a consequence of increase in endogenous cAMP level mediated by an unknown G_s-coupled protein.[198] They claim that ATP and its nonhydolyzable analogs inhibit collagen- and U46619-induced platelet aggregation. Yes, but by which mechanism? It has been demonstrated that collagen-[115] and U46619-induced platelet aggregation,[161] in part, involves binding of ADP to its receptor. Therefore, binding of ATP at a P2T receptor would inhibit collagen- and U-46619-induced platelet aggregation. In the presence of Ca^{2+}, [γ-^{32}P]-ATP was found to label platelet surface proteins. The extent of phosphorylation of an individual protein and the number of phosphorylated proteins increased with increasing concentration of Ca^{2+}.[198] These results are difficult to explain. The radiolabeled ATP compounds previously have been shown to label GPIIb and GPIIIa.[134,137] It was suggested that binding of nucleotides to GPIIb and GPIIIa serves to regulate platelet functions that depend on the exposure of GPIIb-IIIa complex, the fibrinogen receptor.[137] We reemphasize that ADP and ADP analogs have not been shown to label the fibrinogen receptor. In our laboratory all of the ADP affinity analogs used were shown to bind to a single surface-membrane protein, aggregin (100 kDa).

The information presented in this section clearly shows disagreement among investigators concerning the nature and number of ADP binding sites on platelets. There is not only variation in results obtained with platelets from the same species but also with those obtained with different species. Cultured cell lines show an unusually high number of ADP binding sites with low binding affinities, but these results can in part be explained on the basis of large surface area of these cells.[101] One factor contributing to the differences in the results concerning the number of ADP binding sites on platelets may be due to the use of metabolically active nucleotides that are known to be degraded by ectonucleotidases on cell surfaces. In some of the investigations, formalin-fixed platelets have been used to attempt to circumvent this problem. The variation in results may also be due the methods employed to isolate and wash platelets that might influence secretion of nucleotides from the intracellular stores. Another factor contributing to this disparity may be due to the methods (silicon oil method, sucrose cushion method, and filter paper-retention method) employed to separate cell-bound radioactivity from unbound radioligands. In our judgment, the mysterious nature of the ADP receptor protein has contributed the most to the disagreement. Is binding of the nucleotides being determined to a 43, 61, 100 kDa protein, GPIIb, GPIIIa or yet another unknown ADP-binding protein?

The two-receptor hypothesis for the platelet ADP receptor has been in existence for quite some time. First, ADP induces platelet shape change and aggregation (which in turn depends on the exposure of fibrinogen binding sites) and mobilization of $[Ca^{2+}]_i$; second, ADP antagonizes stimulated adenylate cyclase activity. However, the degree of ADP-induced platelet aggregation by agonists at P2T does not follow the same order as the inhibition of stimulated adenylate cyclase activity. At low concentration, FSBA inhibits ADP-induced platelet shape change, aggregation, and exposure of fibrinogen binding sites. At higher concentration FSBA induces platelet shape change and mobilizes Ca^{2+} from intracellular stores. At low concentration, pCMBS blocks the ability of ADP to antagonize increase in endogenous levels of cAMP mediated by prostaglandins. At higher concentration pCMBS inhibits ADP-induced platelet shape change, aggregation, and labeling of aggregin by 2- and 8-BDB-TADP. pCMBS has been shown to inhibit binding of ADP to the high affinity-binding sites on platelets. At a given concentration, the potency of 2-Cl-ADP, 2-N_3-ADP, and 2-MeS-ADP to

aggregate platelets is different from their potency to inhibit stimulated adenylate cyclase activity. Clopidogrel blocks only by 70% the ADP binding sites on platelets, while leaving the other 30% resistant. The results presented by various investigators either favor only one class of high-affinity ADP binding sites or two classes of sites differing in their affinity for ADP. What are the chemical identities of the two proteins that bind to ADP?

Investigations carried out in our laboratory show that 2-BDB, 8-BDB-TADP, and 2-BOP-TADP under appropriate experimental conditions inhibit all of the ADP-induced platelet responses and modify a single surface-membrane protein of 100 kDa. 2-AzPET-ADP inhibits binding of 2-MeS-[β-^{32}P]-ADP to a single class of high-affinity binding sites on platelets and antagonizes cAMP formation. It labels several proteins on platelet surface, but labeling of a 43-kDa protein is prevented by ADP and ATP, suggesting that the 43-kDa protein represents the adenylate cyclase receptor for ADP on platelets. These results imply that the 43-kDa protein also mediates ADP-induced platelet shape change, aggregation, exposure of fibrinogen binding sites, and intracellular mobilization of Ca^{2+}; however, no experimental evidence has been obtained to support this. An important point that needs to be emphasized is that under photolytic conditions there may be a mixture of populations of intact 2-AzPET-ADP molecules and nitrenes derived from 2-AzPET-ADP, and the two would react differently with platelet surface proteins. We believe that intact 2-AzPET-ADP, such as 2-BDB-TADP and 8-BDB-TADP, would and should bind to aggregin, a 100-kDa protein. Whether it is also true for the binding of 2-AzPET-derived nitrenes needs careful experimental investigation. An autoradiogram of the platelets labeled with products of photolysis of 2-AzPET-[β-^{32}P]-ADP showed the presence of an intense band of radiolabeled protein at 100 kDa.[126] The available experimental evidence does not favor the presence of two different ADP binding receptor proteins in platelets. The results obtained by various investigators can be explained on the basis of one ADP receptor protein that may have two sites differing in their affinity for ADP. That epinephrine increases by 10-fold the avidity of ADP for the ADP binding sites without changing their number supports the one-receptor hypothesis. It is also possible that ADP-induced platelet responses are manifestations of two different signal transducing mechanisms that follow binding of ADP at P2T.[6,10,12] Until such times when the identity of two ADP binding proteins has been firmly established, the one-receptor hypothesis serves a reasonable working model for ADP-induced platelet activation. Extensive investigations carried out in our laboratory using ADP affinity analogs, pseudo-affinity compounds, and immunoaffinity method has shown labeling of a single ADP binding surface-membrane protein, aggregin (100 kDa) that modulates all of the platelet responses.

VIII. PHOSPHOLIPASE ACTIVATION, INOSITOL-PHOSPHATE FORMATION, AND INTRACELLULAR Ca^{2+} MOBILIZATION

Stimulation of platelets by agonists results in the rise in $[Ca^{2+}]_i$ from a 80 to 100 n*M* basal value to low micromolar levels.[92] Ionophores, such as A23187, have been shown to mobilize Ca^{2+} from intracellular stores that proceed without any receptor occupancy.[199]

Binding of agonists such as thrombin or thromboxane to platelet surface receptors leads to G-protein-mediated stimulation of PLC that brings about the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) to yield IP_3 and DAG. Binding of IP_3 to intracellular receptors is accompanied by release

of Ca^{2+} from dense tubular system.[200] In the case of ADP, this relationship has not been established. An open question has been the relationship between receptor occupancy and ADP-induced increase in the intracellular concentration of Ca^{2+}.

Experimental evidence obtained suggests that ADP does not directly stimulate membranous PLC in human blood platelets.[201–203] However, other investigators have gathered evidence that supports the claim that ADP activates PLC.[204–206] ADP-induced liberation of [^{32}P]-IP_3 from human blood platelets has been reported.[207] The increase in the radiolabeled IP_3 was only twofold over a basal value of 150 n*M* and was unaffected by the presence or absence of external Ca^{2+}.[207] One possibility is that ADP stimulates interconversion of inositols (IP, IP_2, IP_3) instead of PLC activation that leads to the formation of IP_3.[208]

Continuous-flow measurements using electroporated platelets loaded with [^{3}H]-inositol showed that shear forces acting alone caused a rapid burst in the synthesis of IP_3 before 0.5 s.[209] Thrombin and ADP caused an initial (130 to 200 ms) burst of IP_3 and IP_4 (inositol-1,3,4,5-tetrakisphosphate), but IP_3 was liberated first.[209] Other important findings of this investigations are as follows: (1) formation of IP_3 and IP_4 was detected earlier than any increase in intracellular Ca^{2+} could be detected, and (2) shear forces only in combination with thrombin or ADP induced mobilization of $[Ca^{2+}]_i$.[210] Although shear forces in combination with thrombin or ADP cause intracellular rise in Ca^{2+}, both stimuli acting alone cause formation of IP_3 and IP_4.[209] The importance of shear forces in mechanisms governing physiologic and pathologic processes in regulating cellular functions *in vivo* has been reviewed in detail elsewhere.[211] It is generally agreed that ADP causes activation of PLC, but the causal and temporal relationships between IP_3 and IP_4 formation and rise in intracellular levels of Ca^{2+} either remain uncertain or unestablished. It is possible that ADP-mediated initial burst of IP_3 may cause some release of Ca^{2+} from the dense tubular system and then release more Ca^{2+} by a different cellular mechanism similar to calcium-induced calcium release (CICR).[212–214] Single cell investigations carried out with fluorescent indicators suggest that formation of IP_3 is coupled with Ca^{2+} oscillations and CICR mechanism is required for the propagation of the oscillatory signal involving coordinated control of uptake and release cycles.[215] Investigations carried out by Tsuneda et al. [216] and Heemskerk et al. [217] have confirmed a similar phenomenon taking place in platelets. Caffine (which operates through rynodine receptor operating CICR mechanism) has been shown to elevate intracellular levels of Ca^{2+} in platelets but does not cause any functional responses. Therefore, the existence of CICR mechanism in mobilizing $[Ca^{2+}]_i$ in platelets also remains uncertain. Additional mechanism(s) for the release of Ca^{2+} may be necessary in light of the fact that only 40 to 70% fraction of the total $[Ca^{2+}]_i$ is released by IP_3 in permeabilized cells and isolated membranes.[218] This suggestion also finds support in the investigations carried out by Murphy et al., which show poor correlation between platelet-activating, factor-induced increase in $[Ca^{2+}]_i$, DAG, and IP_3 in rabbit platelets.[219] We believe that ADP by itself (without shear forces) does not make a major contribution to calcium mobilization.

IX. Ca^{2+} INFLUX

Activation of platelets by ADP is also accompanied by influx of Ca^{2+} from the extracellular medium. Mechanisms of ADP-induced Ca^{2+} influx are even more poorly understood than those responsible for mobilization of Ca^{2+} from intracellular stores.

In the capacitative model proposed by Putney, depletion of intracellular stores some-

how regulates (or signals) the opening of Ca^{2+} channels in plasma membrane.[220,221] Thapsigarin (Tg) and 2,5-di(*t*-butyl)-1,4-benzohydroquinone (tBuBHQ), inhibitors of Ca^{2+}-ATPase, have been shown to cause an increase in cytosolic Ca^{2+} in a large number of cell types, including platelets.[222] The mechanism of action of Tg and tBuBHQ involves Ca^{2+} entry by depletion of intracellular stores in the absence of second messenger generation. Cytochrome P-450 has been suggested to link store depletion with Ca^{2+} entry in platelets, and it has been shown that econazole, an inhibitor of cytochrome P450, inhibited Ca^{2+} and Mn^{2+} influx.[223,224] Sargent et al. have disputed these observations because in stopped-flow experiments econazole had no effect on the initial phase of Ca^{2+} signal.[225]

In another model, Kuno and Gardner[226] and Irvine[227] suggested that IP_3 and IP_4 may directly act at the plasma membrane receptors leading to the influx of Ca^{2+} into cytosol. Rengaswamy and Feinberg demonstrated that IP_3 could release Ca^{2+} loaded in platelet plasma membrane fractions by a Na^+/Ca^{2+} exchange mechanism, implying the presence of IP_3-receptors (IP_3-R) in plasma membrane.[228] IP_3-R's have been identified as having carbohydrate residues such as sialic acid and *N*-acetylglucosamine that are known to be covalently linked to plasma membrane proteins.[229] IP_3-R's on the surface invaginated portions of plasma membrane have been detected by a monoclonal antibody to IP_3 in a variety of cells.[230]

It should be emphasized that Ca^{2+} entry pathways mediated by second messengers such as IP_3 have been shown to exist in the case of strong agonists, such as thrombin and thromboxane. In the case of weak platelet agonists, such as ADP, it has been possible to show that Ca^{2+} influx precedes Ca^{2+} release from intracellular stores,[94,95,135] and this mechanism appears to compensate for weak second messenger mediated Ca^{2+} entry. Support for the receptor-operated channel opening mechanism in the case of ADP comes from patch-clamp investigations carried out by Mahaut-Smith et al., who demonstrated the existence of a single channel activity in platelets stimulated by ADP but not by thrombin.[96,232] The relationship of this influx of Ca^{2+} to ADP-induced aggregation of platelets is not clear at present.

The GPIIb-IIIa complex, a fibrinogen receptor, has a major Ca^{2+} binding site. GPIIb-IIIa complex has been shown to be involved in Ca^{2+} influx across plasma membrane of unstimulated platelets[233] as well as stimulated platelets.[234] While strong platelet agonists such as thrombin have been shown to engage GPIIb-IIIa in platelet calcium channel activation,[235] no data are available to show that ADP might recruit GPIIb-IIIa in Ca^{2+} translocation across the plasma membrane.

X. INFLUX OF Na^+

When Na^+/H^+ exchange is inhibited, platelet aggregation and secretion induced by ADP or low concentration of thrombin are considerably reduced.[236–239] This observation led to the proposal that exposure of platelets to physiological stimuli results in transient alkalinization of the cell interior leading to platelet activation.[240,241] Fineberg et al. made the critical observation that ADP-induced platelet aggregation is accompanied by intracellular increase in $^{22}Na^+$ but not $^{36}Cl^-$, suggesting a selective mechanism of intracellular transport of Na^+ rather than NaCl.[242] That movement of extracellular Na^+ into cytosol in platelets is coupled with extrusion of H^+ was first demonstrated by Sweatt et al., who showed that inhibitors of Na^+/H^+ blocked ADP-induced mobilization of intracellular pool of arachidonic acid.[202] Ethylisopropylamiloride (a perturbant of Na^+/H^+ exchange that functions by removing extraplatelet Na^+) has been shown to inhibit ADP-induced,

but not thrombin-induced, formation of PLC products (DAG and IP_3). ADP-induced mobilization of cyclooxygenase products was shown to be inhibited by indomethacin and SQ 29548 (an endoperoxide/thromboxane antagonist).[243] Taken together, the results suggest that inhibition of Na^+/H^+ exchange does not directly inhibit ADP-induced formation of PLC products and that ADP-induced formation of DAG and IP_3 is secondary to arachidonic acid mobilization and production of cyclooxygenase products.[202] The biochemical locus of ADP-induced Na^+/H^+ antiporter has been identified as phospholipase A_2 (PLA_2).[243] Thus, it has been suggested that ADP-induced activation of PLC is secondary to the activation of PLA_2.[202,243] These results appear to corroborate the findings of other investigators that show that ADP does not directly activate PLC.[201–203] These findings also find support from the fact that PLA_2 possesses an alkaline pH (a consequence of Na^+/H^+ antiport) optimum for its enzymatic activity.[244,245] ADP-induced mobilization of $[Ca^{2+}]_i$ was inhibited in indomethacin-treated platelets that had been preincubated with Na^+/H^+ exchanger inhibitor ethylisopropylamiloride: alkalinization by NH_4Cl overcame this inhibition.[246] The results suggest that platelet activation induced by ADP and TXA_2 involve Na^+/H^+ exchange as a common step and enhance Ca^{2+} mobilization independently of PLA_2 activity.[246] Stopped-flow fluorimetry showed that initial kinetics of ADP-induced rise in $[Na^+]_i$ and $[Ca^{2+}]_i$ in human platelets loaded with sodium binding benzofuran isophthalate were similar.[247] Patch-clamp recordings in the absence and presence of Ca^{2+} showed the presence of a single channel. SK&F 96365, a blocker of receptor-mediated Ca^{2+} entry in several nonexcitable cells, blocked ADP-induced rise in $[Na^+]_i$.[247] Taken together, the results suggest that ADP directly activates a channel in platelet plasma membrane that is permeable to Na^+ and divalent cations.[247] The results also lend credence to the suggestion made by Siffert et al. that Na^+/H^+ antiporter modulates ADP-induced Ca^{2+} mobilization without activating PLA_2.[246]

Binding of ADP promotes binding of fibrinogen to its receptor GPIIb-IIIa complex. The indirect relationship between binding of ADP to its receptor and subsequent conversion of GPIIb-IIIa complex from a low-affinity state (T-state) to a conformationally competent high-affinity state (R-state) has been termed as "pseudo-linkage" and is mediated by a third component.[248,249] The third component has been identified by Cristofaro et al. and corresponds to the Na^+/H^+ exchanger.[250] It has been demonstrated that inhibition of the Na^+/H^+ antiporter by decreasing the $[Na^+]_i$ by treatment with amiloride caused a marked decrease in fibrinogen binding sites.[250]

XI. SECRETION

Platelet α-granule secretion is associated with heterotypic interactions (platelet-leukocytes) and platelet trafficking, while dense (δ) granule secretion is associated with homotypic interactions (platelet-platelet). The mechanisms leading to the membrane fusion and secretion are thought to be similar. Platelet-induced leukocyte secretion is mediated by P-selectin released from α-granules. There is considerable interest in the role of transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF), both contained in α-granules of platelets, in the modulation of vascular injury and repair mechanism.[251–253] Failure of cyclooxygenase inhibitors and aspirin to prevent restenosis of atherosclerotic lesions after angioplasty[254,255] and aspirin-independent thromboregulation has revived interest in α-granule secretion.[256,257] When the second wave of platelet aggregation induced by weak agonists such as ADP, epinephrine, and platelet activating factor

(PAF) is impaired or lacking, it is usually accompanied by defective dense granule secretion. These platelet defects usually result in mild to moderate bleeding and prolonged bleeding times.[258,259]

Using P-selectin positive platelets as an index of α-granule release and the binding of leukocytes to P-selectin-positive platelets as a marker for P-selectin function, ADP-induced secretion of α-granules was shown to be regulated by the Na^+/H^+ antiporter.[260] Whereas the lipooxygenase inhibitor itself or in combination with aspirin had no effect, acidification of the extracellular medium or treatment with amiloride (an inhibitor of Na^+/H^+ antiporter) blocked ADP-induced secretion from α-granules.[260]

More information is available on the ADP-induced secretion from dense granules compared with α-granules. During the course of investigations of the inhibitory effect of 8-BDB-TADP on ADP-induced platelet responses, we demonstrated that the concentration of 8-BDB-TADP, which minimally affected the rate and extent of ADP-induced platelet aggregation, completely blocked ADP-induced secretion of nucleotides from the dense granules.[112] Whereas the onset and peak of thrombin-induced platelet aggregation and release from dense granules coincided, ADP-induced platelet secretion from dense granules started only after primary wave of aggregation had peaked and coincided with the peak of secondary wave of aggregation (Figure 15).[112] The dependence on aggregation could be a shear effect necessary for ADP-induced secretion but not for thrombin-induced secretion. These results show that (1) mechanisms of thrombin- and ADP-induced secretion from the dense granules of platelets are different, and (2) mechanisms of ADP-induced platelet aggregation and secretion from the dense granules are also different. These results have been confirmed during our investigations of the inhibitory effects of NBD-Cl[184] and WR-K[185] on ADP-induced platelet activation. These results are different from those obtained by Dillingham et al., who showed that aggregation-inhibitory and dense granule secretion-inhibitory potency of carbamoyalpiperidinoalkane and *N*-alkylnipectoylpiperazine derivatives on ADP-stimulated human blood platelets were nearly the same.[261] The differences in results from two investigations may be due to the chemical nature of inhibitors used to investigate ADP-induced platelet responses. Our investigation utilized ADP affinity analogs and pseudo-affinity compounds,[112,184,185] while the other investigation did not.[261]

It has been earlier discussed that Na^+/H^+ exchanger is also involved in the biochemical mechanism underlying ADP-induced secretion from dense granules in platelets. Thrombin-induced formation of IP_3 and DAG is due to primary activation of PLC, while ADP-induced formation of PLC products is also due to PLC activation but is secondary to PLA_2 activation.[202,241,242,246] Perturbance of Na^+/H^+ exchanger blocked formation of PLC products and dense granule secretion induced by ADP but not by thrombin. ADP-induced secretion from dense granules of platelets was shown to be inhibited by cyclooxygenase inhibitors and thromboxane antagonists (that block formation of thromboxane from AA produced by the action of PLA_2 on lipids). The results show that ADP induces formation of a small pool of AA by a pathway involving Na^+/H^+ antiporter, and thromboxane derived from this pool in turn activates PLC. Because the same treatment that blocks ADP-induced formation of IP_3 and DAG also blocks ADP-induced secretion from dense granules, it has been suggested that indirect activation of PLC via cyclooxygenase products may also be responsible for ADP-induced secretion from dense granules. One of the products of PLC activation is DAG, which is hydrolyzed sequentially by di- and monoglycerol

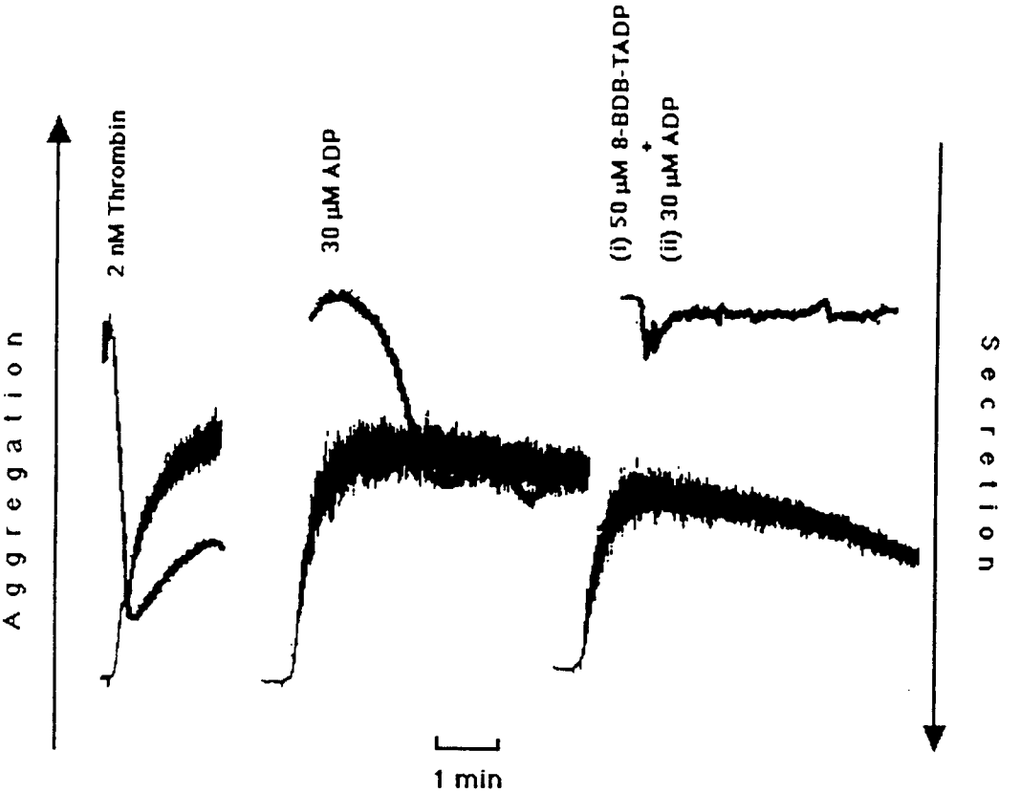


FIGURE 15. Effect of 8-BDB-TADP on ADP-induced platelet secretion. ATP release following exposure of platelets to thrombin and ADP was measured by the commercial luciferase-luciferin reagent.[112] Platelets were either challenged with thrombin or ADP and the agonist-induced platelet secretion of nucleotides was measured simultaneously with aggregation.

lipases to librate phosphatidic acid (PA) and AA [262] or can be phosphorylated to PA, which is a substrate for PLA_2 that liberates AA.[263] The sequence of events leading up to ADP-induced secretion from dense granules of platelets (Figure 16) can be summarized as, (1) Activation of Na^+/H^+ exchanger, (2) activation of PLA_2, (3) release and conversion of AA to TXA_2, (4) activation of PLC by a feedback mechanism involving TXA_2, (5) PLC-mediated formation of IP_3 and DAG, (6) DAG-mediated activation of protein kinase C (PKC) and formation of AA from DAG, and (7) dense granule secretion-mediated by products of PLC activation (Figure 16). PKC is not associated with ADP-induced primary aggregation of platelets[264,265] but is involved in granular secretion.[264-268] DAG, one of the products of PLC activation, is a physiological stimulator of PKC.[269,270] It is possible that ADP-induced activation of Na^+/H^+ exchanger initiates dense granule secretion but PKC helps sustain it by regulating the Na^+/H^+ exchanger by some unknown mechanism. ADP-induced dense granule secretion is a delayed event because activation of PLC (the products of which participate in mechanisms leading up to secretion) itself is a delayed event and is secondary to PLA_2 activation. It has also been demonstrated that activation of PKC by oleoylacetylglycerol, a synthetic analog of DAG, facilitates labilization and fusion

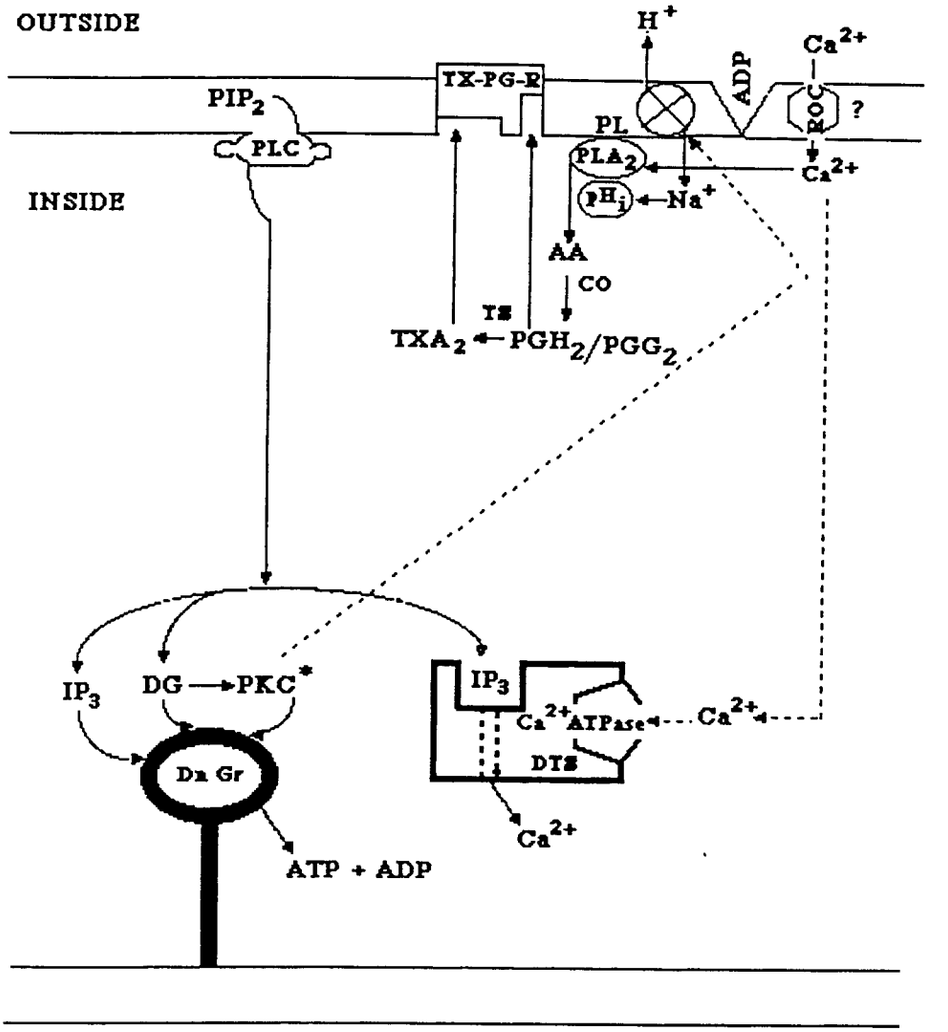


FIGURE 16. Schematic representation of ADP-induced platelet activation. Binding of ADP to its receptor on platelet surface activates Na^+/H^+ exchanger (mechanism?) leading to a cytosolic pH gradient (pH_i) gradient favorable for the activation of phospholipase A_2 (PLA_2). The activation of PLA_2 is also aided by the ADP-induced influx of Ca^{2+} by a receptor operated channel (ROC) or by some other unknown mechanism. The activated PLA_2 converts phospholipids (PL) such as phosphatidylcholine, to arachidonic acid (AA), which is cyclized to prostaglandin endoperoxides PGG_2/PGH_2 by cyclooxygenase (CO) followed by their conversion to thromboxaneA_2 (TXA_2) catalyzed by thromboxane synthetase (TS). In a feedback mechanism receptor (TX-PG-R)-mediated activation of phospholipase C (PLC) by the prostaglandin endoperoxides PGG_2/PGH_2 and TXA_2 (which diffuse out of the membrane) leads to the formation of phospholipase C hydrolysis products inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DG) from phosphoinositol-4,5-bisphosphate (PIP_2). Protein kinase C is activated (PKC*) by DG, its physiological activator. IP_3 and DG, and PKC* then mediated release of nucleotides (ATP+ADP) from the dense granules (Dn Gr). IP_3 also releases Ca^{2+} by binding to its receptors through receptor-mediated channel in dense tubular system (DTS). Depleted stores in DTS can be replenished with Ca^{2+} by Na^+, H^+/Ca^{2+}ATPase pump. Activation of protein kinase C (PKC*) by DG may also provide a mechanism to sustain PLA_2 activation by regulating the Na^+/H^+ exchanger initially activated by ADP.

of granule membranes important for secretion.[271] Although it is established that PKC is involved in ADP-induced secretion from platelet dense granules, the detailed mechanism(s) of its action remain uncertain.

XIII. SIGNAL TRANSDUCTION

G-proteins are hetrotrimeric proteins (α-, β-, and γ-subunits) that mediate transmembrane signal between the signal-generating junction (ligand bound to its receptor) and signal-terminating machinery (the effector, an enzyme, that activates second messenger). The G-proteins have been reviewed in detail elsewhere.[272,273] The G-proteins primarily belong to two categories: G_s, the G-proteins that stimulate cAMP formation by activating adenylate cyclase and are regulated by cholera toxin and G_i, the G-proteins that supress cAMP formation and are regulated by the binding of pertussis toxin. There are three different kinds of Gαi proteins in platelets, $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$; they have 85 to 90% amino acid sequence homology.[274]

2-MeS-ADP, ADP, and GDP have been shown to increase ADP-induced binding of [^{32}S]-GTP-γ-S to platelets and S_p-ATP-α-S antagonizes such binding.[275] S_p-ATP-α-S did not antagonize binding of [^{32}S]-GTP-γ-S by other agonists, indicating that platelet ADP receptor can interact with G-proteins.[275] By using techniques of photoaffinity labeling with 4-azidoanilido-[α-^{32}P]-GTP and immunoprecipitation with subtype-specific antibody,[276] the GTP binding protein in the platelets linked to ADP-induced activation was identified as $G\alpha_{i2}$.[277] In contrast, thrombin transmembrane agonist peptide (TRAP-SF7) has been shown to activate PLC through Gq and inhibit adenylyl cyclase through G_i.[274] It has been shown (discussed in previous sections) that ADP-induced formation of PLC products, IP_3 and DAG, is secondary to PLA_2 activation.[202,243] Therefore, it implies that ADP does not activate PLC through the G_q pathway.[277] Ticlopidine[40] has been shown to inhibit ADP-induced platelet aggregation and stimulated adenylyl cyclase activity.[278] Clopidogrel,[38] a thienopyridine similar to ticlopidine, was shown to block binding of 2-MeS-[^{32}P]-ADP to platelets.[194] Previously, it has been demonstrated that 2-MeS-ADP induces platelet aggregation as well as inhibits adenylyl cyclase activity.[99] Together, these results have taken to hypothesize that $G\alpha_{i2}$ represents the G-protein that mediates ADP-induced inhibition of adenylyl cyclase activity. Although no direct evidence has been obtained to support of this claim,[277] but, in an analogy to $G\alpha_{i2}$-mediated inhibition of adenylyl cyclase in platelets stimulated by the binding of epinephrine to adrenergic receptors, it might very well be the case (Reference 273; other references cited therein). It is also possible that the βγ-subunit of G_{i2} may participate in the activation of PLA_2, a step necessary in ADP-induced platelet activation (discussed in previous sections).[279] βγ-subunit of G_{i2} has been shown to modulate PLA_2 activity in other cellular systems (Reference 279; other references cited therein). It is also possible that ADP-induced platelet activation proceeds through two different signaling mechanisms and Gα and Gβγ may come from two different G-proteins.[273]

XIV. CLONING

Recently, cloning of two human $P2Y_1$ Receptors has been reported.[280,281] The second $P2Y_1$ receptor[281] differs from the first[280] by only one serine residue. The second $P2Y_1$ receptor, when expressed in Jurkat cells, exhibited pharmacological properties similar to those of a nucleoside diphosphate recep-

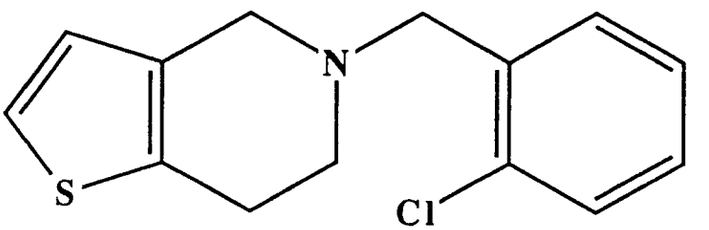
tor in platelets that modulates adenylyl cyclase activity.[281] The presence of the $2PY_1$ receptor mRNA was reported in human platelets and several cell lines of megakaryocytic origin.[281] Rank order of potency for Ca^{2+} movements in Jurkat cells transfected with the $P2Y_1$ receptor was 2-MeS-ADP > ADP > ADP-α-S and α,β-Me-ATP. UTP and adenosine were reported to be without any effect. ATP, S_p-ATP-α-S and β,γ-Me-ATP were shown to be competitive antagonists. 2-MeS-ATP and 2-Cl-ATP, even after HPLC (high-pressure liquid chromatography), were found to evoke Ca^{2+} movement in Jurkat cells transfected with the receptor. Incubation of mock-transfected Jurkat cells with 2-MeS-ATP for 5 min was shown to generate material that acted as agonist for the Ca^{2+} response. It should be noted that agonist-induced Ca^{2+} response in human platelets occurs in less than 1 s.[209] S_p-ATP-α-S, one of the most potent antagonists at P2T receptor,[90] was shown to be a partial agonist at the cloned $2PY_1$ receptor.[281] No data concerning responses other than the Ca^{2+} movement in Jurkat cells transfected with the human $P2Y_1$ receptor were reported. In this context it is worth noting that other investigators have also hypothesized that the platelet P2T receptor may be coupled to a G-protein that modulates the activity of adenylyl cyclase.[282]

Janssen et al.[283] and Schacter et al.[284] also reported cloning of human $P2Y_1$ receptors. The human $P2Y_1$ receptor transcript obtained by Janssen et al.[283] and Schacter et al.[284] appears to be very similar to that obtained by Leon et al.[282] for a 43-kDa protein. The human $P2Y_1$ receptor expressed in 1321N1 astrocytoma cells (unresponsive to nucleotides) showed an agonist potency rank order 2-MeS-ADP > ADP. 2-MeS-ATP for phosphoinositide hydrolysis.[284] The time of incubation (1 to 15 min) used for assaying incubation mixtures for phophoinoside hydrolysis had no effect on the agonist potencies. Furthermore, 2-MeS-ATP neither induced nor inhibited cAMP accumulation in 1321N1 cells expressing the human $P2Y_1$ receptor.[284] In order to confirm that their $P2Y_1$ receptor was not coupled to adenylyl cyclase, it was ascertained that 2-MeS-ATP-induced inhibition of cAMP accumulation was essentially indistinguishable between the wild-type C_6 cells (that are coupled to adenylyl cyclase) and C_6 cells expressing the human $P2Y_1$ receptor.[284] These results suggest that the human $P2Y_1$ receptor cloned by Leon et al.[282] is less likely to be a candidate for the platelet P2T receptor.

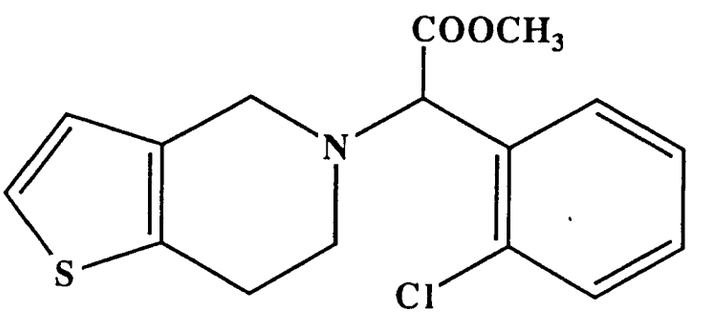
XV. PURINORECEPTORS AND DRUGS

Exposure of conformationally competent fibrinogen-binding sites, the GPIIb-IIIa complex, by ADP (a weak agonist) and thrombin (a strong agonist), and all the other agonists with varying degree of potency, constitutes a central mechanism involved in platelet aggregation. Pharmaceutical industries have been engaged with development of drugs to prevent diseases such as stroke, myocardial infarction, unstable angina, and atherosclerosis that culminate, in part, from platelet aggregation. Therefore, efforts have concentrated heavily on the development of chemically superior and pharmacologically more potent antagonists and antibodies for GPIIb-IIIa. Because of the importance of pathophysiological consequences of the biological effects resulting from binding of nucleotides to purinoreceptors, which includes ADP-induced platelet aggregation, a new sense of awareness has energized investigators to develop specific drugs that are selective at purinoreceptor. Ticlopidine {5-(2-chlorobenzyl)-4,5,6,7-tetrahydrothieno[3,2:c]-pyridine} (Figure 17a) and PCR 4099 {methyl 2-(2-chlorophenyl)-

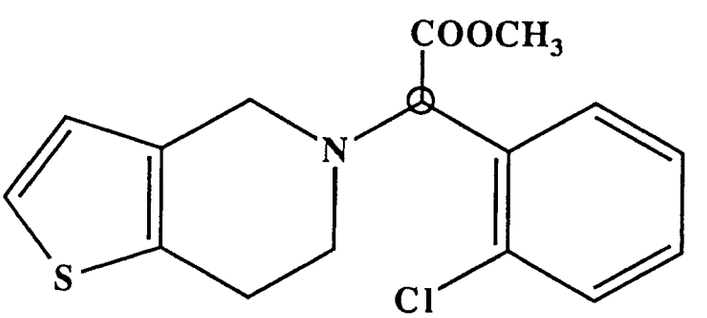
2-(4,5,6,7-tetrahydrothieno[3,2:c]-pyridine-5-yl)acetate} (Figure 17b) are structurally related compounds that specifically block ADP-induced platelet responses. Clopidogrel (*S*-SR25990c) (Figure 17c) is the biologically active dextrorotatory stereoisomer separated from PCR 4099 racemate. Collectively, these compounds are referred to thienopyridines in literature and have been reviewed extensively.[38,40,41,285-290] Ticlopidine, PCR 4099, and Clopidogrel are specific inhibitors of the primary and secondary waves of ADP-induced



(a) Ticlopidine



(b) PCR 4099



(c) *S*-SR25990c-clopidogrel

FIGURE 17. Chemical representations of thienopyridines specific for inhibition of ADP-induced platelet activation. Ticlopidine (a), PCR 4099 (b), and clopidogrel (c).[38] PCR 4099 is a C-acetyl derivative of ticlopidine, which is a racemate because it contains a chiral center (○). Clopidogrel (*S*-SR25990c-clopidogrel) is the bioactive dextrorotatory (*S*) component of racemic PCR 4099.[38]

platelet aggregation as well as ADP-induced secretion. These actions of the thienopyridines are irreversible and the drugs have to be administered orally for maximum effect that requires 3 to 5 d. Ticlopidine is more potent than PCR 4099, but clopidogrel, when administered to rats, was found to be 40 times more potent than ticlopidine.[285,291] Within the hemostatic system, the thienopyridines function mainly *in vivo* due to inhibition of platelet responses as they are inactive in conventional coagulation and fibrinolysis assays. Thienopyridines are inactive *in vitro*, suggesting that they undergo metabolic conversion to active agents. The action of clopidogrel *in vivo* involves its bioactivation in liver by cytochrome P450-1A subfamily (CYP1A1 and CYP1A2 genes).[291] The thienopyridines do not contain either the ribose or the phosphate moiety present in ADP, yet they have been demonstrated to be fairly specific for inhibiting ADP-induced platelet aggregation. These findings coupled with the fact that the thienopyridines are slow-acting drugs suggest that the biotransformed drugs probably covalently modify the ADP-receptor. It is likely that they undergo hydroxylation by the liver microsomal monooxygenases and the hydroxylated compounds are directed to the ADP receptor by hydophobic interactions between the modified thienopyridines and the ADP-binding domain of the receptor. This appears to be a reasonable explanation of their mode of action at P2T receptor in light of the investigations carried out in our laboratory concerning chemical modification of the ADP-receptor by pseudo-affinity compounds (Section XI.D).

Available evidence suggests that the thienopyridines act at the P2T receptor to selectively inhibit ADP-induced platelet aggregation. PCR 4099 completely inhibited *ex vivo* aggregation at all tested concentrations of ADP in Sprague-Dawley rats as well as Fawn-Hooded rats (that have congenital deficiency of dense granules containing ADP, ATP, and serotonin). Platelet aggregation by low concentration of thrombin is probably ADP-dependent because it is shifted to higher thrombin concentration in the presence of CP/CPK. In platelets obtained from Sprague-Dawley rats administered PCR 4099, platelet aggregation required higher concentrations of thrombin. Data gathered from human volunteers show that inhibition of ADP-induced aggregation by ticlopidine is not dependent on the inhibition of ADP-induced rise in $[Ca^{2+}]_i$ or influx of Ca^{2+}. These findings further support the proposal that P2T receptor is covalently modified by the bioactivated thienopyridines. It has been shown that there is reduced fibrinogen binding following thienopyridine treatment, but ticlopidine in no way directly modifies the fibrinogen receptor complex. Thienopyridines have been shown not to affect AA metabolism in platelets and vessel wall. A significant finding of the investigation of the action of thienopyridines is modulation of adenylyl cyclase activity plays an important role in blocking ADP-induced platelet aggregation.

Platelets can aggregate in the absence of external stimulus. Under controlled conditions of high shear stress, it is vWF rather than GPIIb-IIIa complex that supports platelet aggregation. High shear rate is known to cause severe arterial stenosis and has clinical relevance to rheological conditions existing during normal microcirculation. ADP scavengers partially inhibit formation of shear-induced platelet aggregates, suggesting that under conditions of high shear-stress, platelets release ADP from secretory granules. Desmopressin, a synthetic analog of vasopressin, increases plasma levels of vWF and factor VIII, and shortens the bleeding time of normal individuals and patients with congenital acquired defects of primary hemostasis. Ticlopidine was shown to inhibit shear-induced platelet aggregation before and after desmopressin infusion.[292] Bleeding time pro-

longed by ticlopidine in healthy volunteers was shortened by desmopressin.[292] Potentiation by desmopressin of shear-induced platelet aggregation may be one of the mechanisms by which desmopressin shortens the prolonged bleeding time. The results suggest that ticlopidine is a useful drug in minimizing arterial thrombosis due to thrombotic occlusions resulting from platelet aggregation under conditions of high shear-stress that release ADP from platelets. At sites of arterial stenosis and endothelial injury, recurrent platelet aggregation and dislodgement leads to cyclic flow variations (CFV's), which may represent a pathophysiological phenomenon akin to the one occurring in some patients with coronary artery syndromes. ADP plays an important role *in vivo* in mediating platelet aggregation, and CFVs in stenosed and endothelium-injured coronary arteries in an experimental canine model and infusion of clopidogrel to dogs following establishment of CFVs led to complete abolition of CFVs.[39] These clinical investigations underscore the role of ADP-induced platelet aggregation in coronary artery disease and emphasize the beneficial effects of thienopyridines in alleviating such pathophysiological conditions in clinical settings. Aprotonin has been shown to constitute a useful physiological antagonist of the hemorrhagic risk associated with interventional therapy under treatment with ticlopidine or clopidogrel,[293] probably due to inhibition of fibrinolysis. In another clinical investigation employing ticlopidine, lower dosage of ticlopidine with lower frequencies of side effects were demonstrated when given in combination with trapidil, a PDGF-receptor antagonist and an inhibitor of platelet aggregation.[294] Rationale of this drug combination lies in optimizing therapeutic efficacy of clopidogrel, which irreversibly inhibits platelet functions and remains inhibitory during the rest of the life span of platelets, with trapidil, which exerts reversible inhibitory effect on platelet aggregation.[294] In another interesting clinical investigation, clopidogrel administered orally was found to inhibit platelet-induced expression of tissue factor on endothelial cells, suggesting that platelet aggregation, which plays a significant role in arterial thrombosis, might also influence venous thrombosis.[295] Venous thrombosis has been shown to be associated with tissue factor expression.[295] Long treatment with ticlopidine seems to retard propagation of peripheral atheromatosis.[296] Progression of atherosclerotic disease is retarded by reduction of low-density lipoproteins (LDL).[297] Plasma lipoproteins, along with blood platelets, play an important role in the development of coronary artery disease. However, ticlopidine administration can develop risk factors because it continuously increased LDL levels in middle-aged men incapacitated with stable angina pectoris, while there was no change in high-density lipoprotein fraction.[298] It was therefore suggested that before starting with long-term treatment with platelet inhibitors like thienopyridines, their effects on cardiovascular risk factors (such as elevation of LDL levels) should be monitored.[298]

The development of nucleotide based antagonists at P2T receptor has been long overdue. ATP (Figure 18a), a natural antagonist at P2T receptor, cannot be used in clinical settings for two reasons: (1) it is active as an agonist at other P2-purinergic receptors, and (2) it is metabolized by ectonucleotidases. S_p-ATP-α-S is a very potent antagonist at P2T receptor,[90] but its clinical profiles are unknown. It has been known that that 2-MeS- and 2-EtS-ATP possess increased affinity for P2T receptor.[122] Advantage was taken of the above facts in designing 2-propylthio-β,γ-dichloromethylene-ATP (FPL 67085) (Figure 18b) and 2-propylthio-β,γ-difluoromethylene-ATP (FPL 66096) (Figure 18c), which have been found to be extremely potent antagonists at P2T receptor.[299] Introduction of a propyl group at C_2 increases their potency

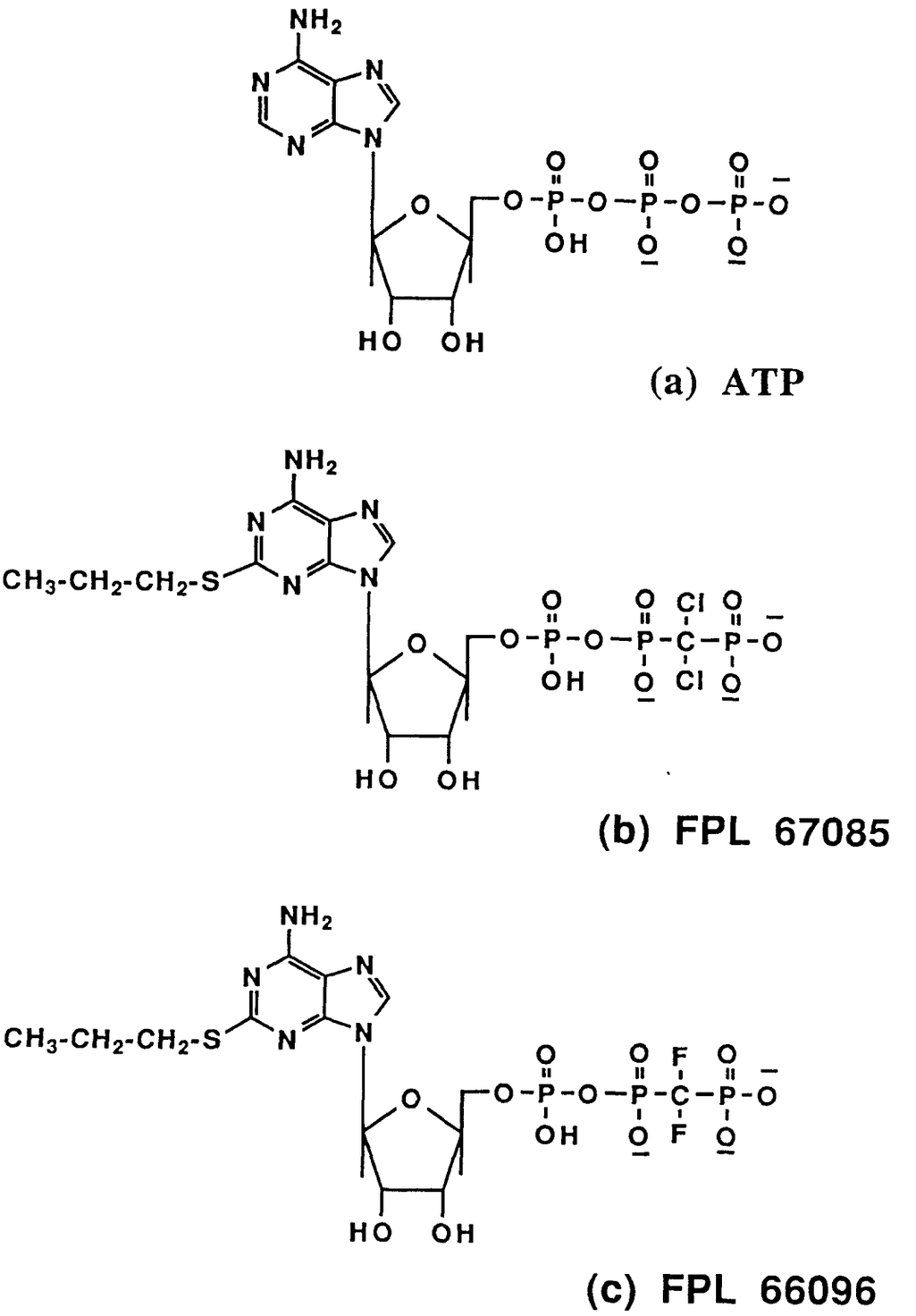
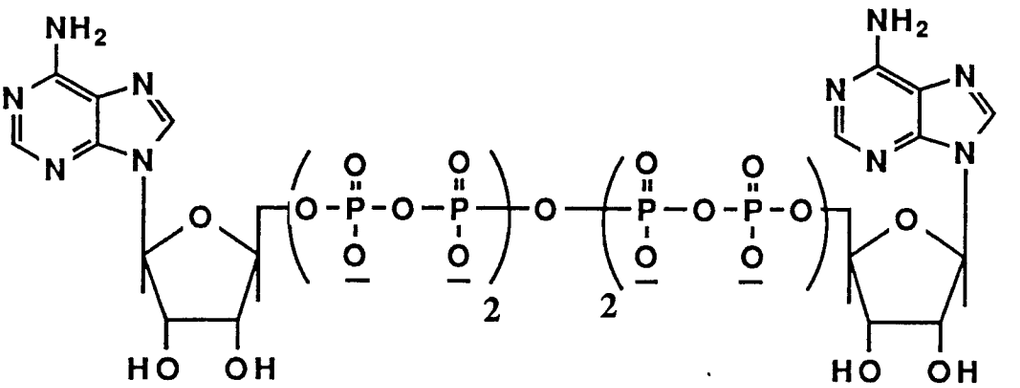


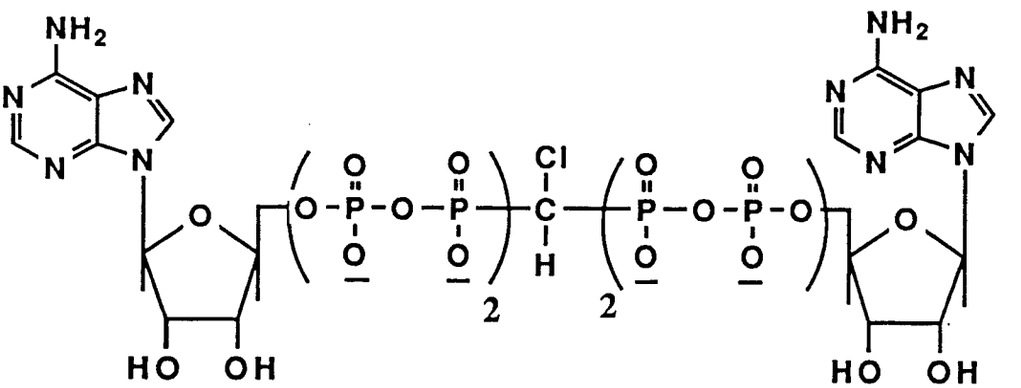
FIGURE 18. Chemical representation of nucleotide-based drugs active at P2T Purinergic receptor. FPL 67085 (b)[300] and FPL 66096 (c).[300] ATP (a) is included for comparison. It has been pointed out that designation of FPL 67085 and FPL 66096 has been changed to ARL 60785 and ARL 66096, respectively.[281]

and introduction of β,γ-dihalogroup prevents hydrolysis of FPL 66096 and FPL 67085. FPL 67085,[300] and FPL 66096,[301] have been shown to be 30,000-fold and 9000-fold more selective for P2T over P2X- and P2Y-purinergic receptors. These two compounds have no detectable activity at P2U-purinergic receptors, A_1-, A_2-, and A_{2b}- receptors for adenosines; α_1-, α_2- and β_2-adrenergic receptors for catecholamines; 5-HT_2-receptor for serotonin; AT_1-receptor for angiotensin II and EP_2-receptors for prostaglandins.[299] FPL 67085 has a pharmacokinetic profile that is desirable for use as an infusible drug in the high-risk coronary care environment.[300] In phase I clinical trials involving male volunteers, FPL 67085 was well tolerated and inhibited ADP-induced platelet aggregation *ex vivo* over a similar dose range seen in animals with maximum effect reaching in 15 min.[300] Pharmacokinetic studies showed that FPL 67085 has a half-life time of 2 min in humans.[300]

Ap_4A (Figure 19a), a component of platelet dense granules,[104] is a competitive



(a) Ap_4A



(b) AppCHClppA

FIGURE 19. Chemical representation of diadenosine polyphosphates. (a) Ap_4A (diadenosine 5′,5‴-P^1,P^4-tetraphosphate or diadenosine tetraphosphate) and (b) AppCHClppA (β,β′-monochloromethylenediadenosine 5′,5‴-P^1,P^4-tetraphosphate).[47]

inhibitor of ADP-induced platelet aggregation[107,108,302] and has been shown to have antithrombotic effects in rabbit carotid artery model. However, Ap_4A has a short half-life time in blood because it is degraded by phosphodiesterases.[303] These observations were helpful in designing β,β′-monochloromethylenediadenosine 5′,5‴-P^1-P^4-tetraphosphate (AppCHClppA)[47] (Figure 19b), which is far more potent than Ap_4A[302] in inhibiting platelet aggregation induced by ADP. Preliminary investigations showed that AppCHClppA may be a useful antithrombotic drug in hemodialysis, arteriovenous shunts, and introduction of artificial heart valves.[47] The toxicity and pharmacokinetics AppCHClppA have yet to be evaluated.

Gram-negative septic shock is lethal in human beings and its action *in vitro* can be duplicated by the action of lipopolysaccharide (LPS), endotoxin. Macrophages are inflammatory cells and release tumor necrosis factor-α, interleukin-1, and interleukin-6 when exposed to LPSs. Macrophages are likely to be exposed to extracellular ATP and ADP *in vivo*, as these compounds are released from platelets and other cells during injury and inflammation. 2-MeS-ATP, an antagonist of ADP-induced platelet activation, was found to reduce serum cytokines and protect mice from lethal endotoxemia.[304] Thus, adenine nucleotides provide a potentially new approach in controlling pathophysiology of Gram-negative septicimia.

XVI. CONCLUSIONS

As the early reports that ADP[17–20] and 2-substituted ADP-analogs[90,98,113] are platelet agonists and the ADP antagonizes stimulated adenylyl cyclase activity,[97] considerable progress has been made toward understanding the biochemical, pharmacological, and clinical aspects of ADP-induced platelet activation.[158,282,305-307] ADP-induced platelet aggregation remains at the heart of ADP-induced platelet activation. Considerable efforts have been directed to identify the ADP-receptor protein. We have used five different ADP-affinity analogs,[112,111,128,131,155] three pseudo-affinity compounds,[168,184,185] and an immunoaffinity method[190] to show that ADP-induced platelet responses are mediated by the binding of ADP to aggregin,[157,158] 100-kDa surface membrane protein, a putative ADP receptor. Other investigators have claimed that a 43-kDa surface protein[126] might represent the adenylyl cyclase binding site on human blood platelets.

There are also disagreements concerning ADP-induced formation of PLC products IP_3 and DAG. Considerable amount of experimental evidence gathered suggest that binding of ADP to its receptor activates Na^+/H^+ antiporter leading to the activation of PLA_2; the products of PLA_2, AA, is then converted by cyclooxygenase pathway to TXA_2, which in a feedback mechanism activates PLC.[201–203] There are also disagreements concerning mechanisms of ADP-induced rise in $[Ca^{2+}]_i$ in platelets. Whether this occurs by ligand-gated Ca^{2+} influx or by mobilization from intracellular stores, or both, remains a contested issue.[213,214,233,234] There is compelling evidence that ADP-induced influx of Ca^{2+} precedes mobilization of Ca^{2+} from intracellular stores.[94,95,231] Recently, the presence of $P2X_1$ receptors on the platelet surface that mediate rapid phase of ADP- and ATP-evoked influx of Ca^{2+} via a non-specific cation channel has been identified, but the identity of the receptor protein remains unknown.[308]

Our knowledge of signal transduction mechanisms of ADP-induced platelet activation remains less well understood. $G\alpha_{i2}$ protein has been identified as the G-protein coupled to the ADP-receptor on the platelet surface.[278] $G\alpha_{i2}$ protein has been shown to couple α_2-adrenergic receptors that inhibit

adenylyl cyclase without causing any change in $[Ca^{2+}]_i$. However, the increase in $[Ca^{2+}]_i$ is essential for ADP-induced platelet activation regardless of the mechanism. The two-receptor hypotheses for the ADP-induced platelet activation has been much debated. However, the existence of two different ADP-receptor proteins has never been demonstrated. Thus, it is reasonable to propose a single-receptor hypothesis that would accommodate different ADP-induced platelet responses by postulating that there are binding sites on this receptor that differ in their affinity for ADP or there are different signal transducing mechanisms associated with binding of ADP at P2T.

One of the important aspects of ADP-induced platelet activation is its ability to block cAMP formation by stimulated adenylyl cyclase,[97] but this is not the cause of ADP-induced platelet aggregation.[90,309] Therefore, the nagging question remains, What is the significance of the ability of ADP to antagonize adenylyl cyclase activity *in vivo*?

Among the most important aspects of the investigations of the P2T-purinergic receptor are the advances made in finding new, better, and potent drugs that selectively block ADP-induced platelet aggregation and thus may be used in clinical settings. The thienopyridines (ticlopidine, PCR 4099, and clopidogrel) have shown the promise of blocking ADP-induced platelet aggregation fairly selectively.[285–291] Among nucleotide-based drugs, FPL 67085[300] has been found to be very selective at P2T receptor, and its success in phase I clinical trials shows the promise of its being a useful antithrombotic drug for use in human beings.

However, much remains to be done in unequivocally identifying the ADP-receptor protein in human blood platelets. The isolation of the ADP-receptor protein is necessary to make monoclonal antibodies and clone the receptor. The cloned receptor would allow studies of the biochemical basis of the specificity for ADP and mapping of the nucleotide- binding site. Together such efforts could lead to the design of drugs that are selective, more potent, and safe, while inhibiting pathophysiological states such as arterial thrombosis resulting from the activation of the ADP-receptor protein and/or interactions of the receptor with ADP.

REFERENCES

1. Devary, A. N. and Szent-Gyorgi, A. 1929. The physiological activity of adenine compounds, with special reference to their action on heart. *J. Physiol.* **68:** 213–217
2. Su, C. 1988. Purinergic neurotransmission and neuromodulation. *Ann. Rev. Pharmacol. Toxicol.* **23:** 397–411.
3. Green, H. and Stoner, H. B. 1950. *Biological Actions of Nucleotides*, H. K. Lewis & Co., Ltd., London.
4. Burnstock, G. and Kennedy, C. 1986. A dual function of adenosine 5′-triphosphate in the regulation of vascular tone. Excitatory cotransmitter with noradrenaline from perivascular nerves and locally realesed intravascular agents. *Circ. Res.* **58:** 319–330.
5. Olson, R. A. and Pearson, J. D. 1990. Cardiovascular purinoreceptors. *Physiol. Rev.* **70:** 761–849.
6. Dubyak, G. R. 1991. Signal transduction by P_2-purinergic receptors for extracellular ATP. *Am. J. Respir. Cell Mol. Biol.* **4:** 295–300.
7. Von Kugelgen, I. and Strake, K. 1991. Noradrenaline-ATP cotransmission in symphathetic nervous system. *Trends Pharmacol. Sci.* **12:** 319–324.
8. Bean, B. P. 1990. Pharmacology and electrophysiology of ATP-activated ion channels. *Trends Pharmacol. Sci.* **13:** 87–91.

9. Burnstock, G. 1990. Purinergic mechanisms. *Ann. N.Y. Acad. Sci.* **603:** 1–17.

10. El-Moatassim, C., Dornand, J., and Mani, J. C. 1992. Extracellular ATP and cell signalling. *Biochim. Biophy. Acta* **1134:** 31–45.

11. Vassort, G., Scamps, F., Puceat, M., and Clement, O. 1992. Multiple effects of extracellular ATP in cardiac tissues. *News Physiol. Sci.* **7:** 212–215

12. Dubyak, G. R. and El-Moatassim, C. 1993. Signal transduction via P_2-purinergic receptors for extracellular ATP and other nucleotides. *Am. J. Physiol.* **265:** C577–C606.

13. Apasov, S., Koshiba, M., Redgeld, F., and Sitrovsky, M. V. 1995. Role of extracellular ATP and P1 and P2 classes of purinergic receptors in T-cell development and cytoxic T-lymphocyte effector functions. *Immunol. Rev.* **146:** 5–19.

14. Baricordi, R., Ferrair, D., Mechiorri, L., Chiozzi, P., Hanan, S., Chiari, E., Rubin, M., and Di Virgilio, F. 1996. An ATP-channel is involved in mitogenic stimulation of human T-lymphocyte. *Blood* **86:** 682–690.

15. Zheng, L., Zychlinski, A., Liu, C., Ojcius, D., and Young, J. 1991. Extracellular ATP as a trigger for apoptosis or programmed cell death. *J. Cell Biol.*. **112:** 279–285.

16. Di Virgillo, F. 1995. The purinergic P_{2Z} receptor: An intriguing role in immunity, inflamation and cell death. *Immunol. Today* **16:** 524–528.

17. Hellem, A. J. 1960. The adhesiveness of human blood platelets in vitro. *Scand. J. Clin. Lab. Invest.* **12:** 1–117.

18. Olligard, E. 1961. Macroscopic studies of platelet aggregation: nature of an aggregating factor in red blood cells and platelets. *Thromb. Diath. Haemorrh.* **6:** 86–97.

19. Gaarder, A. Jonsen, A., Laland, S., Hellem, A. J., and Owern, P. 1961. Adenosine diphosphate in red cells as a factor of human blood platelets. *Nature* **192:** 531–532.

20. Born, G. V. R. 1962. Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature* **194:** 927–929.

21. Clayton, S. and Cross, M. J. 1963. The aggregation of platelets by catecholamine and by thrombin. *J. Physiol.* **169:** 82P–83P.

22. Clayton, S., Born, G. V. R., and Cross, M. J. 1963. Inhibition of the aggregation of blood platelets by nucleosides. *Nature* **200:** 138–139.

23. Haslam, R. J. 1964. Role of adenosine diphosphate in the aggregation of human blood platelets by thrombin and fatty acids. *Nature* **202:** 765–68.

24. Haslam, R. J. 1967. Mechanisms of blood platelet aggregation. *In* Physiology of Hemostasis and Thrombosis (Johnson, S. A., and Seegers, W. H., Eds.) pp. 88–112 Charles C. Thomas, Springfield, IL.

25. Willerson, J. T., Campbell, W. B., Winniford, H. D., Schmitz, J., Apprill, P., Firth, B. G., Ashton, J., Smitherman, T., Bush, L., and Buja, L. M. 1984. Conversion from chronic to acute coronary artery disease: speculations regarding mechanisms. *Am. J. Cardiol.* **54:** 1349–1354.

26. Willerson, J. T., Hills, L. D., Winniford, M., and Buja, L. M. 1986. Speculations regarding mechanisms responsible for acute ischemic heart disease syndromes. *J. Am. Coll. Cardiol.* **8:** 245–250.

27. Willerson, J. T., Golino, P., Eidt, J., Campbell, W. B., and Buja, L. M. 1989. Specific platelet mediators and unstable coronary artry lesions: Experimental evidence and potential clinical implications. *Circulation* **80:** 198–205.

28. Davis, M. J. and Thomas, A. C. 1985. Plaque fssuring: the cause of acute myocardial infarction, sudden death, and crescendo angina. *Br. Heart J.* **53:** 363–373.

29. Fuster, V., Bradmon, L., Cohen, M., Ambrose, J. A., Bradmon, J. J., and Chesboro, J. H. 1988. Insights into pathogenisis of acute ischemic syndromes. *Circulation* **77:** 1213–1220.

30. Davis, M. J. 1994. Mechanisms of thrombosis and atherosclerosis, in *Haemostasis and Thrombosis: Basic Principles and Clinical Practice* (Colman, R. W., Hirsh, J., Marder, V. J., and Salzman, E. W., Eds.) pp. 1224–1237, J. B. Lippincott Company, Philadelphia.

31. Baumgartner, H. R. 1973. The role of blood flow in platelet adhesion and, fibrin formation of murual thrombi. *Microvasc. Res.* **5:** 167–179.

32. Baumgartner, H. R., Muggli, R., Tschopp, T. B., and Turitto, V. T. 1976. Platelet adhesion, release and aggregation in flowing blood: effect of surface properties and platelet function. *Thromb. Haemost.* **35:** 124–138.

33. Turittio, V. T., Weiss, H. J., Zimmerman, T. S., and Susman, I. L. 1985. Factor VIII/von Willebrand factor in subendothelium mediates platelet adhesion. *Blood* **65:** 823–831.

34. Stel, H. V., Sakariassan, K. S., De Groot, P. G., van Maurik, J. A., and Sixma, J. J. 1985. von Willebrand factor in the vessal wall mediates platelet adhesion. *Blood* **6:** 329–357.

35. Kieffer, N. and Phillips, D. R. 1990. Platelet membrane glycoproteins: functions in cellular interactions. *Ann. Rev. Cell Biol.* **6:** 329–357.

36. Nurden, A. T. and Nurden, P. 1993. A review of the role of platelet membrane glycoproteins in the platelet vessel wall interaction. *Balliere's Clinical Hematology* **6:** 653–690.

37. Zawilska, K. M., Born, G. V. R., and Bengent, N. A. 1982. Effect of ADP-utilizing enzymes on arterial bleeding time in rats and rabbits. *Br. J. Haematol.* **50:** 317–325.

38. Schror, K. 1993. The basic pharmacology of ticlopidine and clopidogrel. *Platelets* **4:** 252–261.

39. Yao, S.-K., Ober, J. C., McNatt, J., Benedict, C. R., Rosolowski, M., Anderson, H. V., Cui, K., Maffrand, J.-P., Campbell, W. B., Buja, L. M., and Willerson, J. T. 1991. ADP plays an important role in mediating platelet aggregation and cyclic flow variations *in vivo* in stenosed and endothelium-injured canine coronary arteries. *Circulation Res.* **70:** 39–48.

40. Panak, E., Maffrand, J. P., Picard-Fraire, C., Valles, E., Blanchard, J., and Rencucci, R. 1983. Ticlopidine: a promise for prevention and treatment of thrombus and its complications. *Haemostasis* **13 Suppl 1:** 1–54.

41. Thenault, J. McEwen, J., McCrew, A. Bouloux, C., Jacob, C., Chigot, C., Irvine, A., Kindermans, M., Maffrand, J. P., and Rencucci, R. 1986. PCR 4099. A new antithrombotic drug, evaluation of tolerence and pharmacological activity. *Thromb. Res.* **Suppl 6:** 286.

42. Maffrand, J. P., Bernat, A., Delebassee, D., Defreyn, G., Cazenave, J. P., and Gorden, J. L. 1988. ADP plays a key role in thrombogenesis in rats. *Thromb. Haemost.* **59:** 225–230.

43. Cattaneo, M., Leochi, A., Randi, A. M. N., McGregor, J. L., and Mannucci, P. M. 1992. Identification of new congenital defect of platelet function characterized by severe impairment of platelet responses to adenosine diphosphate. *Blood* **80:** 2787–2796.

44. Nurden, P., Savi, P., Heilman, E., Bihour, C., Herbert, J-M., Maffrand, J.-P., and Nurden, A. T. 1995. An inherited bleeding disorder linked to defective interaction between ADP and its receptor on platelets. *J. Clin. Invest.* **95:** 1612–1622.

45. George, J. N., Nurden, A. T., and Phillips, D. R. 1984. Molecular defects in intertactions of platelets with the vessel wall. *N. Engl. J. Med.* **311:** 1084–1098.

46. Weiss, H. J. 1994. Inherited abnormalities of platelet granules and signal transduction, in *Hemostasis and Thrombosis: Basic Principles and Clinical Practice* (Colman, R. W., Hirsh, J., Marder, V. J., and Salzman, E. W., Eds.) pp. 1224–1237, J. B. Lippincott Company, Philadelphia.

47. Kim, B. K., Zamecenik, P., Taylor, G., Guo, M. J., and Blackburn, M. 1992. Antithrombotic effect of β,β′-monochloromethylenediadenosine 5′,5‴-P^1,P^4-tetraphosphate. *Proc. Natl. Acad. Sci. U.S.A.* **89:** 11056–11058.

48. Vilen, L., Jacobsson, S., and Kutti, J. 1989. ADP-induced platelet aggregation as a function of age in healthy humans. *Thromb. Hemost.* **61:** 490–492.

49. Burnstock, G. 1972. Purinergic nerves. *Pharmacol. Rev.* **24:** 509–581.

50. Kenakin, T. P., Bond, R. A., and Bonner, T. I. 1992. Definition of pharmacological Receptors. *Pharmacol. Rev.* **44:** 351–361.

51. Burnstock, G. 1978. A basis for distinguishing two types of purinergic receptors, in *Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach* (Sraub, R. W. and Bolis, L., Eds.) pp. 107–118, Raven Press, New York.

52. Van Calker, P., Muller, M., and Hamprecht, B. 1979. Adenosine regulates via two different types of receptors; the accumulation of cyclic AMP in cultured brain cells. *J. Neurosci.* **23:** 999–1005.

53. Londos, C., Cooper, D. M. F., and Wolff, J. 1980. Subclasses of external adenosine receptors. *Proc. Natl. Acad. Sci. U.S.A.* **77:** 2551–2554.

54. Kennedy, C. and Burnstock, G. 1984. Evidence for an inhibtory prejunctional P_1-purino receptor in rat portal vein with characteristics of A_2- rather than A_1-subtype. *Eur. J. Pharmac.* **100:** 363–368.

55. Linden, J. 1991. Structure and function of A1 adenosine receptors. *FASEB J.* **5**: 2668–2776.

56. Barnard, E. A., Burnstock, G., and Webb, T. E. 1994. G-protein coupled receptors for ATP and other nucleotides: a new receptor family. *Trends Pharmacol. Sci.* **15:** 67–70.

57. Burnstock, G. and Kennedy, C. 1985. Is there a basis for distinguishing purinergic receptors? *Gen. Pharmac.* **16:** 433–440.

58. Cusack, N. J. and Hourani, S. M. O. 1990. Subtypes of P2-Purinoreceptors: Studies using analogs of ATP. *Ann. N.Y. Acad. Sci.* **603:** 172–181.

59. Gordon, J. L. 1986. Extracellular ATP: effects, sources and fate. *Biochem. J.* **233:** 309–319.

60. Fredholm, B. B., Abbracchio, M. P., Burnstock, G., Daly, J. W., Harden, T. K., Jacobson, K. A., Leff, P., and Williams, M. 1994. VI. Nomenclature and classification of purinoreceptors. *Pharmacol. Rev.* **46:** 143–156.

61. Pintor, J. and Miras-Portugal, M. T. 1995. P_2 purinergic receptors for diadenosinepolyphosphates in the nervous system. *Gen. Pharmacol.* **26:** 229–235.

62. Benham, C. D. and Tsien, R. W. 1987. A novel receptor operates Ca^{2+} permeable channel activated by ATP in the smooth muscle. *Nature* **328:** 275–278.

63. Surprenant, A., Buell, G., and North, R. A. 1995. P2X receptors bring new structure to ligand-gated ion channels. *Trends Neur. Sci.* **18:** 224–228.

64. Brake, A. G., Wagenbach, M. J., and Julius, D. 1994. New structural motif for ligand-gated ion channels defined by ionotropic ATP receptor. *Nature* **371:** 519–523.

65. Lambrecht, G., Friebbe, T., Grimm, U., Windsceif, U., Bungart, E., Hildebrandt, C., Beaumert, H. G., Spatz-kümbel, G., and Mutschler, E. 1992. PPADS, a novel functionally selective antagonist of P2 purinoreceptor mediated responses. *Eur. J. Pharmacol.* **217:** 217–219.

66. Buell, G., Michel, A. D., Lewis, C., Collo, G., Humphrey, P. P. A., and Surprenant, A. 1996. $P2X_1$ receptor activation in HL-60 cells. *Blood* **87:** 2659–2664.

67. Valera, S., Hussey, N., Evans, R. J., Adami, N., Norte, R. A., Surprenant, A., and Buell, G. 1994. A new class of ligand-gated ion channel defined by P2X receptor for extracellular ATP. *Nature* **371:** 516–519.

68. O'Connor, S. E., Dainty, I. A., and Leff, P. 1991. Further subclassification of ATP Receptors based on agonist studies. *Trends Pharmacol. Sci.* **12:** 137–141.

69. Yammada, M., Hamamori, Y., Akita, H., and Yokoyama, M. 1992. P_2-purinoreceptor activation stimulates phosphoinositide hydrolysis and inhibits accumulation of cAMP in cultured ventricular monocytes. *Circulation Res.* **70:** 477–485.

70. Boyer, J. L., Lazarowski, I. R. Cheu, X.-H., and Harden, T. K. 1993. Identification of a P2Y-purinergic receptor that inhibits adenylyl cyclase. *J. Pharmacol. Exp. Ther.* **267:** 1140–1146.

71. Bruner, G. and Murphy, S. 1990. ATP-evoked arachidonic acid mobilization in astrocytes is via a P2Y-Purinergic receptor. *J. Neurochem.* **55:** 1569–1575.

72. Hoyl, C. H., Knight, G. C., and Burnstock, G. 1990. Suramin antagonizes responses to P2-purinoreceptor agonists and purinergic nerve stimulation in the guinea-pig urinary bladder and *E. coli. Br. J. Pharmacol.* **99:** 617–621.

73. Ayanathan, K., Webb, T. E., Sandhu, A. K., Athwal, R. S., Barnard, G. A., and Kunapuli, S. P. 1996. Cloning and chromosomal localization of the human $P2Y_1$ receptor. *Biochem. Biophys. Res. Commun.* **218:** 783–788.

74. Parr, C. E., Sullivan, D. M., Paradiso, A. M., Lazarowski, G. R., Burch, L. H., Olsen, J. C., Erb, L., Weimann, G. A., Boucher, R. C., and Turner, J. T. 1994. Cloning and expression of a human P2U nucleotide receptor, a target for cystic fibrosis pharmacotherapy. *Proc. Natl. Acad. Sci. U.S.A.* **91:** 3275–3279.

75. Akabar, G. K. M., Dessari, V. P., Sheth, S., Mills, D. C. B., and Kunapuli, S. P. 1995. Identification of two calcium signalling P2T and P2U purinergic receptors in human erythroleukemia cells. *FASEB J.* **9:** 684 (Abstract).

76. Niguyen, T., Erb, L., Weisman, G. A., Murschese, A., Heng, H. H. Q., Gurrard, R. C., George, S. P., Turner, J. T., and O'Dowd, B. F. 1995. Cloning and expression, and chromosomal localiztion of the human uridine receptor gene. *J. Biol. Chem.* **270:** 30846–30848.

77. Communi, D., Priotton, S., Parmentier, M., and Boeynaems, J. M. 1995. Cloning and functional expression of a human uridine nucleotide receptor. *J. Biol. Chem.* **270:** 30849–30852.

78. Webb, T. E., Kaplan, M. H., and Barnard, E. A. 1996. Identification of 6H1 as a P2Y purinoreceptor: $P2Y_5$. *Biochem. Biophys. Res. Commun.* **219:** 105–110.

79. Chang, K., Hanaoka, K., Kumada, M., and Takuwa, Y. 1995. Molecular cloning and functional analysis of a novel P2 nucleotide receptor. *J. Biol. Chem.* **270:** 26152–26158.

80. Akbar, G. K. M., Dasari, V. R., Webb, T. E., Ayanathan, K. P., Pillarisetti, K., Sandhu, A. K., Athwal, R. S., Daniel, J. L., Ashby, B., Barnard, E. A., and Kunapuli, S. P. 1996. Molecular cloning of a novel P2 purinoreceptor from human erythroleukemia cells. *J. Biol. Chem.* **271:** 18363–18367.

81. Hilbert, M. F., Trumpp-Kallmeyer, S., Hoflock, J., and Bruinvels, A. 1993. This is not a G-protein-coupled receptor. *Trends Pharmacol. Sci.* **14:** 7–12.

82. Cockroft, S. and Gomperts, B. D. 1979. Activation and inhibition of calcium-dependent histamine secretion by ATP ions applied to mast cells. *J. Physiol.* **296:** 229–243.

83. Tatham, P. E. E., Cusack, N. J., and Gomperts, B. D. 1988. Chracterization of the ATP^4-receptor that mediates permeabilization of rat mast cells. *Eur. J. Pharmacol.* **147:** 13–21.

84. Erb, L., Lustig, K. D., Sullivan, D. M., Turner, J. T., and Weisman, G. A. 1993. Functional expression and photoaffinity labeling of a cloned P2U Purinergic receptor. *Proc. Natl. Acad. Sci. U.S.A.* **90:** 5113–5117.

85. Lustig, K. D., Shian, A. K., Brake, A. J., and Julius, D. 1993. Expression cloning of an ATP receptor from mouse neuroblastoma cells. *Proc. Natl. Acad. Sci. U.S.A.* **90:** 5113–5117.

86. Clarke, L. L., Grubb, B. R., Yankaskas, J. R., Cotton, C. U., Mekenzie, A., and Boucher, R. C. 1994. Relationship of a non-cystic fibrosis transmembrane conductance regulator-mediated chloride conductance to organ-level decreases in *cftr*(-/-) mice. *Proc. Natl. Acad. Sci. U.S.A.* **91:** 479–483.

87. Bear, C. E., Li, C., Kartner, N., Bridges, R. J., Jensen, J. R., Ramjeesingh, M., and Riorden, J. R. 1992. Purification and functional reconstitution of the cystic fibrosis transmission conductance regulator. *Cell* **68:** 809–818.

88. Mason. S. J., Paradiso, A. M., and Boucher, R. C. 1991. Regulation of trans epithelial ion transport and intracellular calcium by extracellular ATP in human normal and cystic fibrosis airway epithelium. *Br. J. Pharmacol.* **103:** 1649–1656.

89. Knowles, M. R., Lane, L., Clarke, D. V. M., and Boucher, R. C. 1991. Activation of extracellular nucleotides of chloride secretion in the airway epithelia of patients with cystic fibrosis. *N. Engl. J. Med.* **325:** 533–538.

90. Cusack, N. J. and Hourani, S. M. O. 1982. Adenosine-5′-diphosphate antagonists and human platelets: No evidence that aggregation and inhibition of adenylate cyclase are mediated by different Receptors. *Br. J. Pharmacol.* **76:** 221–227.

91. Macfarlane, D. E. and Mills, D. C. B. 1975. The effects of ATP on platelets: evidence against centeral role of released ADP in primary aggregation. *Blood* **46:** 309–320.

92. Rink, T. J. and Hallam, T. J. 1984. What turns platelets on? *Trends Biochem. Sci.* **9:** 215–219.

93. Hallam, T. J. and Rink, T. J. 1985. Response to adenosine diphosphate in human blood platelets loaded with the flourescent calcium indicator Quin-2. *J. Physiol.* **368:** 131–146.

94. Sage, S. O., Reast, R., and Rink, T. J. 1990. ADP evokes biphasic Ca^{2+} influx in Fura-2 loaded human platelets. Evidence for Ca^{2+} entry regulated by the intracellular store. *Biochem. J.* **265:** 675–680.

95. Sage, S. O. and Rink, T. 1986. Kinetic differences between thrombin- and ADP-induced calcium influx and release from internal stores in fura-2 loaded human platelets. *Biochem. Biophys, Res. Commun.* **136:** 1124–1129.

96. Mahaut-Smith, M. P., Sage, S. O., and Rink, T. J. 1990. Rapid ADP-evoked currents in human platelets recorded with nystatin permeabilized patch technique. *J. Biol. Chem.* **267:** 3060–3065.

97. Macfarlane, D. E. and Mills, D. C. B. 1981. Inhibition by ADP of prostaglandin-induced accumulation of cyclic AMP in human platelets. *J. Cyclic Nucleotide Res.* **7:** 1–11.

98. Cusack, N. J. and Hourani, S. M. O. 1982. Competitive inhibition by adenosine 5′-triphosphate of the actions of human platelets by 2-chloroadenosine 5′-diphosphate, 2-azidoadenosine 5′-diphosphate and 2-methylthioadenosine 5′-diphosphate. *Br. J. Pharmacol.* **77:** 329–333.

99. Macfarlane, D. E., Srivastva, P. C., and Mills, D. C. B. 1983. 2-methylthioadeno-

sine [β-^{32}P]diphosphate: an agonist and radioligand for the receptor that inhibits the accumulation of cyclic AMP in platelets. *J.Clin. Invest.* **71:** 420–428.

100. Fugman, D. A., Write, D. P., Jones, L. A. C., Aronow, B. J., and Liberman, M. A. 1990. In vitro establishment and characterization of a human megakaryoblastic cell line. *Blood* **75:** 1252–1261.

101. Kalambakas, S. A., Robertson, F. M., O'Connell, S. M., Sinha, S., Vishnupad, K., and Karp, G. I. 1993. Adenosine diphosphate stimulation of cultured hematopoietic cell lines. *Blood* **81:** 2652–2657.

102. Shi, X.-P., Yin, K.-C., and Gardell, S. J. 1995. Human erythroleukemic (HEL) cells express a platelet P2T-like ADP receptor. *Thromb. Res.* **77:** 235–247.

103. Murgo, A. J., Contrera, J. G., and Sistare, F. D. 1994. Evidence for seperate calcium-signalling P_{2T} and P_{2U} purinoreceptors in human megakaryoblasic Dami Cells. *Blood* **83:** 1258–1267.

104. Floddgard, H. and Klenow, H. 1982. Abundent amount of diadenosine diadenosine 5′,5‴-P^1,P^4-tetraphosphate are present and releasable but metabollically inactive in human platelets. *Biochem. J.* **208:** 737–742.

105. Luthje, J. and Ogilvie, A. 1983. The presence of diadenosine 5′,5″-P^1,P^3-triphosphate (Ap_3A) in human platelets. *Biochem. Biophys. Res. Commun.* **115:** 253–260.

106. Rodriguez Del Castillo, A., Torres, M., Dellicido, E. G., and Miras-Portugal, M. T. 1988. Subcellular distribution studies of diadenosine polyphophates- Ap_4A and Ap_5A-in bovine adrenal medulla: presence in chrimaffin granules. *J. Neurochem.* **51:** 1696–1703.

107. Luthje, J., Barringer, K., and Ogilvie, A. 1985. Effects of diadenosine triphosphate (Ap_3A) and diadenosine tetraphosphate (Ap_4A) on platelet aggregation in unfractionated human blood. *Blut* **51:** 405–413.

108. Harrison, M. J., Brossmer, R., and Goody, R. S. 1975. Inhibition of platelet aggregation and platelet release reaction by α,ω-diadenosine polyphosphates. *FEBS Lett.* **54:** 57–60.

109. Mayo, K. H., Mvele, O. M., and Puri, R. N. 1990. Proton magnetic resonance spectroscopic analysis of diadenosine 5,5′-polyphosphates. *FEBS Lett.* **265:** 97–100.

110. Castro, E., Torres, M., Miras-Portugal, M. T., and Gonzalez, M. P. 1990. Effect of diadenosine polyphosphates on catecholamine secretion from isolated chromaffin cells. *Br. J. Phatrmacol.* **100:** 360–364.

111. Jefferson, J. R., Harmon, J. T., and Jamieson, G. A. 1988. Identification of high affinity (K_d 0.35 μmol/L) and low affinity (K_d 7.9 μmol/L) platelet binding sites for ADP and competition by ADP analogs. *Blood* **71:** 100–116.

112. Puri, R. N., Kumar, A., Chen, H., Colman, R. F., and Colman, R. W. 1995. Inhibition of ADP-induced platelet responses by covalent modification of aggregin, a putative ADP-receptor, by 8-(4-bromo-2,3-dioxobutylthio)-ADP. *J. Biol. Chem.* **270:** 24482–24488.

113. Gough, G., Maguire, M. H., and Penglis, F. 1972. Analogs of adenosine 5′-diphosphate: new platelet aggregators. Influence of purine ring and phosphate chain substituents on aggregatory of adenosine 5′-diphosphate. *Mol. Pharmacol.* **8:** 170–177.

114. Puri, R. N., Colman, R. F., and Colman, R. W. 1996. Platelet activation by 2-(4-bromo-2,3-dioxobutylthio)adenosine 5′-diphosphate is mediated by its binding to a putative ADP-receptor, aggregin. *Eur. J. Biochem.* **236:** 862–870.

115. Colman, R. W., Figures, W. R., Scearce, M. L., Strimpler, A. M., Zhou, F., and Rao, K. 1986. Inhibition of collagen induced platelet activation by 5′-fluorosulfonylbenzoyladenosine. Evidence for an ADP requirement and synergistic influence of pros-

taglandin endoperoxides. *Blood* **68:** 565–570.

116. Pierce, P. H., Wright, J. M., Egan, C., and Scrutton, M. C. 1978. Interaction of human platelets with 2,3-dialdehyde and 2,3-dialcohol derivatives of adenosine diphosphate and adenosine triphosphate. *Eur. J. Biochem.* **88:** 543–554.

117. Puri, R. N. and Colman, R. W. 1997. Structural features in the ribose moiety of ADP necessary for interaction with aggregin, an ADP-receptor, during ADP-induced platelet responses: investigations using apolyclonal antibody. *FASEB J.* **9:** 329 (Abstract).

118. Cusack, N. J. and Hourani, S. M. O. 1981. Partial agonist behaviour of adenosine-5′-*O*-(2-thiodiphosphate). *Br. J. Pharmacol.* **73:** 405–408.

119. Cusack, N. J. and Hourani, S. M. O. 1981. Effects of R_p and S_p diastereoisomers of adenosine-5′-*O*-(1-thiodiphosphate) on human platelets. *Br. J. Pharmacol.* **73:** 409–412.

120. Cusack, N. J. and Petty, C. J. 1988. Effects of isopolar isosteric phosphonate analogues of adenosine 5′-diphosphate (ADP) on human platelets, in *Adenosine and Adenosine Nucleotides: Physiology and Pharmacology* (Paton, D. M., Ed.) p. 287, Taylor and Francis, London.

121. Hourani, S. M. O., Welford, L. A., and Cusack, N. J. 1986. 2-MeS-AMP-PCP and human platelets: implications for the role of adenylate cyclase in ADP-induced aggregation? *Br. J. Pharmacol.* **87:** 84P.

122. Cusack, N. J. and Hourani, S. M. O. 1982. Specific and non-compatative inhibition by 2-alkylthio analogues of adenosine 5′-monophosphate and adenosine 5′-triphosphate of human platelet aggregation by adenosine 5′-diphosphate. *Br. J. Pharmacol.* **75:** 397–400.

123. Cusak, N. J., Hickmann, M. E., and Born, G. V. R. 1979. Effect of D- and L-enantiomers of adenine, AMP and ADP and their 2-chloro and 2-azido analogues on human platelets. *Proc. R. Soc. London Ser B* **206:** 139–144.

124. Burnstock, G., Cusack, N. J., Hills, J. M., Mackenzie, A. I., and Meghiji, P. 1983. Studies on the stereoselectivity of P2-purinoreceptor. *Br. J. Pharmacol.* **79:** 907–913.

125. Cusack, N. J. and Born, G. V. R. 1977. Effects of photolysable 2-azido analogues of AMP and ADP on human platelets. *Proc. R. Soc. London Ser B* **197:** 515–520.

126. Cristalli, G. and Mills, D. C. B. 1993. Identification of a receptor for ADP on blood platelets by photoaffinity labeling. *Biochem. J.* **291:** 875–881.

127. Jefferson, J. R., Hunt, J. B., and Jamieson, G. A. 1987. Facile synthesis of 2-[(3-aminopropyl)thio]adenosine 5′-diphosphate: a key intermediate for the synthesis of molecular probes of adenosine 5′-diphosphate function. *J. Med. Chem.* **30:** 2013–2016.

128. Puri, R. N. and Colman, R. W. 1997. Modulation of platelet responses by 2-(3-bromo-2-oxopropylthio)adenosine 5′-diphosphate involves its binding to as well as covalent modification of an ADP-receptor, aggregin. *Arch. Biochem. Biophys.* **343:** 140–145.

129. Cusack, N. J. and Born, G. V. R. 1976. Inhibition of adenosine deaminase and of platelet aggregation by 2-azidoadenosine, a photolysable analogue of adenosine. *Proc. R. Soc. London Ser. B* **197:** 307–311.

130. Macfarlane, D. E., Mills, D. C. B., and Srivastva, P. C. 1982. Binding of 2-azidoadenosine[β-^{32}P]diphosphate to the receptor on intact human blood platelets which inhibits adenylate cyclase. *Biochemistry* **21:** 544–549.

131. Bennett, J. E., Colman, R. F., and Colman, R. W. 1978. Identification of adenine nucleotide proteins in human platelet membranes

by affinity labeling by 5-*p'*-fluorosulfonylbenzoyladenosine. *J. Biol. Chem.* **253:** 7346–7354.

132. Colman, R. F. 1990. Site specific modification of enzyme sites, in *Enzymes*, Vol. 19, pp. 283–321, Academic Press, New York.

133. Agarwal, A. K., Tandon, N. N., Greco, N. J., and Jamieson, G. A. 1989. Evaluation of the binding to fixed platelets of agonists and antagonists of ADP-induced platelet aggregation. *Thromb. Haemost.* **62:** 1103–1106.

134. Greco, N. J., Yamamoto, N., Jackson, B. W., Tandon, N. N., Moos, M., Jr., and Jamieson, G. A. 1991. Identification of a nucleotide-binding site on glycoprotein IIb. *J. Biol. Chem.* **266:** 13627–13636.

135. Powling, M. J. and Hardisty, R. M. 1985. Glycoprotein IIb–IIIa complex and Ca^{2+} influx into stimulated platelets. *Blood* **66:** 731–734.

136. Greco, N. J., Tandon, N. N. Jackson, B. W., and Jamieson, G. A. 1992. Low structural specificity for nucleoside triphosphates as antagonists of ADP-induced platelet activation. *J. Biol. Chem.* **267:** 2966–2970.

137. Mayinger, P. and Gawaz, M. 1992. Photoaffinity labeling of integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb-IIIa) on intact platelets with 8-azido-[γ-32P]ATP. *Biochim. Biophys. Acta* **1137:** 77–81.

138. Guthrow, C. E., Allen, J. E., and Rasmussen, H. 1972. Phosphorylation of an endogenous membrane associated cyclic adenosine 3',5'-diphosphate dependent protein kinase in human erythrocyte ghosts. *J. Biol. Chem.* **247:** 8145–8153.

139. Antonoff, R. S. and Ferguson, J. J., Jr. 1974. Photoaffinity labeling with cyclic nucleotides. *J. Biol. Chem.* **249:** 3319–3321.

140. Obrig, T. G., Antonoff, R. S., Kirwin, K. S., and Ferguson, J. J., Jr.1975. The binding of adenosine-3',5'-monophosphate by messenger ribonucleotide-like particles. *Biochem. Biophys. Res. Commun.* **66:** 437–443.

141. Schimmel, P. R. and Budzik, G. P. 1977. Photo-cross-linking of protein-nucleic acid complexes. *Methods Enzymol.* **46:** 168–180.

142. Wang, S. Y., Ed. 1974. *Photochemistry and Photobiology of Nucleotides*, Gordon & Beach, New York.

143. Oswald, R. and Changeux, J.-P. 1991. Ultraviolet light-induced labeling by non-competitive blockers of acetylcholine receptor from torpedo marmorata. *Proc. Natl. Acad. Sci U.S.A.* **78:** 3925–3929.

144. Van Prooijen, H. C., Van Marwizk-Kooy, M., Van Weelden, H., Aarts-Riemens, M. I., Borghuis, L., and Akkerman, J. W. N. 1990. Evidence of a new UVB source for irradiation of platelet concentrates. *Br. J. Haemotol.* **75:** 573–577.

145. Van Marwizk-Kooy, Van Prooijen, H. C., Van Asbeck, B. S., Borghuis, L., and Akkerman, J. W. N. 1990. Irradation of platelets with UV-B light exposes fibrinogen binding sites by an intracellualr mechanism. *Br. J. Haematol.* **75:** 573–577.

146. Van Marwizk-Kooy, M., Akkerman, J. W. N., Van Asbeck, B. S., Borghuis, L., and Van Prooijen, H. C. 1990. UVB radiation exposes fibrinogen binding sites on platelets by activating protein kinase C via reactive oxygen species. *Br. J. Haematol.* **83:** 253–258.

147. Grijzenhout, M. A., Aarts-Riemens, M. I., Akkerman, J. W. N., Nienwenhuis, H. K., Van Weelden, H., and Van Prooijen, H. C. 1993. Ultraviolet-B irradiation of platelets induces a dose-dependent increase in the expression of platelet activation markers with storage. *Br. J. Haematol.* **83:** 627–632.

148. Schieven, G. L., Kirihara, J. M., Gilliland, L. K., Uckum, F. M., and Ledbetter, J. A. 1993. Ultraviolet radiation rapidly induces tyrosine phosphorylation and calcium signalling in lymphocytes. *Mol. Biol. Cell* **4:** 523–530.

149. Devary, Y., Gottlieb, R. A., Smeal, T., and Karin, M. 1992. The ultraviolet re-

sponse is triggered by activation of *src* tyrosine kinases. *Cell* **71:** 1081–1091.

150. Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E. A., Ahmad, M. F., Avruch, J., and Woodgett, J. R. 1994. The stress activated protein kinase subfamily of c-Jun kinases. *Nature* 369: 156–160.

151. Pombo, C. M., Boventre, J. V., Avruch, J., Woodgett, J. R., Kyriakis, J. M., and Force, T. 1994. The stress activated protein kinases are major c-Jun amino-terminal kinases activated by ischemia and reperfusion. *J. Biol. Chem.* **269:** 26546–26551.

152. Herrlich, P. 1984. The mammalian genetic stress response. *Adv. Enzyme Regul.* **25:** 485–504.

153. Roni, Z. A., Lambert, E., and Weinstein, J. B. 1990. Inducible cellular responses to ultraviolet light irradiation and other mediators of DNA damage in mammalian cells. *Cell Biol. Toxicol.* **6:** 105–126.

154. Colman, R. F. 1989. *Protein Structure and Function: A Practical Approach* (Creighton, T. A., Ed.) pp. 77–99, IRL Press, New York.

155. Figures, W. R., Niewiarowski, S., Morinelli, T. A., Colman, R. F., and Colman, R. W. 1981. Affinity labeling of human platelet membrane protein with 5′-*p*-fluorosulfonylbenzoyladenosine. *J. Biol. Chem.* **256:** 7789–7795.

156. Figures, W. R., Scearce, M. L., DeFeo, P., Stewart, G., Zhou, F., Chen, J., Daniel, J. L., Colman, R. F., and Colman, R. W. 1987. Direct evidence for the interaction of the nucleotide affinity analog 5′-*p*-fluorosulfonylbenzoyladenosine with a platelet ADP receptor. *Blood* **70:** 796–803.

157. Colman, R. W. 1990. Aggregin: a platelet ADP-receptor that mediates aggregation. *FASEB J.* **3:** 1425–1435.

158. Colman, R. W. 1992. Platelet ADP Receptors stimulating shape change and inhibiting adenylate cyclase. *News Physiol. Sci.* **7:** 274–278.

159. Mills, D. C. B., Figures, W. R., Scearce, M. L., Stewart, G. J., Colman, R. F., and Colman, R. W. 1985. Two mechanisms for inhibition of ADP-induced platelet shape change by 5′-*p*-fluorosulfonylbenzoyladenosine and covalent modification at an ADP binding site distinct from that inhibits adenylate cyclase. *J. Biol. Chem.* **260:** 8078–8083.

160. Rao, A. K. and Kowalska, M. A. 1987. ADP-induced platelet shape change and mobilization of cytoplasmic ionized calcium are mediated by distinct binding sites on platelets: 5′-*p*-fluorosulfonylbenzoyladenosine is a weak platelet agonist. *Blood* **70:** 751–756.

161. Morinelli, T. A., Niewiarowski, S., Kornecki, E., Figures, W. R., Wachtfogel, Y., and Colman, R. W. 1983. Platelet aggregation and exposure of fibrinogen receptors by prostaglandin endoperoxide analogs. *Blood* **61:** 41–49.

162. Figures, W. F., Scearce, M. L., Wachtfogel, Y., Chen, J., Colman, R. F., and Colman, R. W. 1986. Platelet ADP receptor and α_2-adrenoreceptor interaction: evidence of ADP-requirement for epinepherine-induced platelet activation and an influence of epinepherine on ADP-binding. *J. Biol. Chem.* **261:** 5981–5986.

163. Puri, R. N., Zhou, F., Bradford, H., Hu, C.-J., Colman, R. F., and Colman, R. W. 1989. Thrombin-induced platelet aggregation involves an indirect cleavage of aggregin by calpain. *Arch. Biochem. Biophys.* **271:** 346–358.

164. Puri, R. N., Zhou, F., Colman, R. F., and Colman, R. W. 1990. Plasmin-induced platelet aggregation is accompanied by cleavage of aggregin and indirectly mediated by calpain. *Am. J. Physiol.* **259:** C862–C868.

165. Patthy, L. and Smith, E. L. 1975. Identification of functional arginine residues in ribonuclease A and lysozyme. *J. Biol. Chem.* **250:** 565–569.

166. DeCamp, D. L., Lim, S., and Colman, R. F. 1988. Reaction of pyruvate kinase with the new nucleotide affinity label 8-(4-bromo-2,3-dioxobutylthio)adenosine 5′-diphosphate and 5′-triphosphate. *J. Biol. Chem.* **27:** 7651–7658.

167. Ozturk, D. H., Safer, D., and Colman, R. F. 1990. Affinity labeling of bovine liver glutamate dehydrogenase with 8-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-diphosphate and 5′-triphosphate. *Biochemistry* **29:** 7112–7118.

168. Puri, R. N. and Colman, R. W. 1991. Inhibition of ADP-induced platelet shape change and aggregation by *o*-phthalaldehyde: evidence of covalent modification of cysteine and lysine residues. *Arch. Biochem. Biophys.* **286:** 419–427.

169. Huang, Y.-C. and Colman, R. F. 1989. Aspartyl peptide labeled by 2-(4-bromo-2,3-dioxobutylthio)adenosine 5′-diphosphate in the allosteric ADP site of pig heart NAD^+-dependent isocitrate dehydrogenase. *J. Biol. Chem.* **264:** 12208–122214.

170. Huang, Y.-C. and Colman, R. F. 1984. Affinity labeleing of the allosteric ADP activation site of NAD-dependent isocitrate dehydrogenase by 6-(4-bromo-2,3-dioxobutylthio)adenosine 5′-diphosphate. *J. Biol. Chem.* **259:** 12481–124888.

171. Bhatnagar, D., Roskoski, R., Jr., Rosendahl, M. S., and Leonard, N. Adenosine cyclic 3′,5′-diphosphate dependent protein kinase: a new fluorescence displacement technique for characterizing the nucleotide binding site on the catalytic subunit. *Biochemistry* **22:** 6310–6317.

172. Colman, R. W., Figures, W. R., Wu, Q. X., Chung, X. Y., Morenelli, T. A., Tuszynski, G. P., Colman, R. F., and Niewiarowski, S. 1988. Distinction between glycoprotein IIIa and a 100 kDa protein (aggregin) mediating ADP-induced platelet activation. *Arch. Biochem. Biophys.* **262:** 298–306.

173. Zablocki, J. A., Katzenellenbogen, J. A., Carlson, K. E., Norman, M. J., and Katzenellenbogen, B. S. 1987. Estrogenic affinity labels: synthesis, irreversible receptor binding, and bioactivity of aziridine-substituted hexestrols derivatives. *J. Med. Chem.* **10:** 829–838.

174. Wrzeszczynski, K. O. and Colman, R. F. 1994. Activation of bovine liver glutamate dehydrogenase by covalent reaction of adenosine 5′-*O*-[*S*-(4-bromo-2,3-dioxobutyl)thiophosphate] with arginine-459 in an ADP-regulatory site. *Biochemistry* **33:** 11544–11553.

175. Bacon, C. R., Bender, R. A., and Colman, R. F. 1981. The reaction of 4-iodoactamidosalicylic acid with TPN-dependent isocitrate dehydrogenase from pig heart. *J. Biol. Chem.* **236:** 6593–6599.

176. Puri, R. N., Bhatnagar, D., and Roskoski, R., Jr. 1985. Adenosine cyclic 3′,5′-monophosphate dependent protein kinase: fluorescent affinity labeling of the catalytic subunit from bovine skeltal muscle with *o*-phthalaldehyde. *Biochemistry* **24:** 6499–6508.

177. Puri, R. N., Bhatnagar, D., Glass, D. B., and Roskoski, R., Jr. 1985. Inactivation of guanosine cyclic 3′,5′-monophosphate dependent protein kinase from bovine lung by *o*-phthalaldehyde. *Biochemistry* **24:** 6508–6514.

178. MacIntyre, D. E., Grainge, C. A., Drummond, A. H., and Gordon, J. L. 1977. Effect of thiol reagents on platelet transport processes and responses to stimuli. *Biochem. Pharmacol.* **26:** 319–323.

179. Sato, T., Hashizumi, T., and Fuji, T. 1987. *N*-ethylmaleimde inhibits Ca^{2+} influx induced by collagen or arachidonate in rabbit platelets. *Biochim. Biophys. Acta* **928:** 266–271.

180. Subarao, K., Kakkar, V. V., and Ganguli, P. 1978. Binding of pyridoxal phosphate to human platelets: its effect on platelet function. *Thromb. Res.* **13**: 1017–1029.

181. Ghosh, P. B. and Whitehouse, M. W. 1968. 7-chloro-4-nitro-benzo-2-oxa-1,3-diazole:

a new fluorogenic reagent for amino acids and other amines. *Biochem. J.* **108:** 155–156.

182. Hartle, F. T. and Roskoski, R., Jr., 1982. Adenosine cyclic 3′,5′-monophosphate protein kinase from bivine brain: Inactivation of the catalytic subunit and holoenzyme by 7-chloro-4-nitro-2,1,3-benzoxadiazole. *Biochemistry* **21:** 5175–5183.

183. Marquardt, D. and Center, M. S. 1991. Involvement of vascular adenosine triphosphate activity in multidrug resistance in HL-60 cells. *J. Natl. Cancer Inst.* **83:** 1098–1102.

184. Puri, R. N. and Colman, R. W. 1996. Inhibition of ADP-induced platelet activation by 7-chloro-4-nitro-benz-2-oxa-1,3-diazole by covalent modification of aggregin, a putative ADP-receptor. *J. Cell. Biochem.* **61:** 97–108.

185. Puri, R. N. and Colman, R. W. 1996. A novel method for chemical modification of functional groups other than a carboxyl group in proteins by *N*-ethyl-5-phenylisooxazolium-3′-sulfonate (Woodward's Reagent-K): inhibition of ADP-induced platelet responses involves covalent modification of aggregin, an ADP receptor. *Anal. Blochem.* **240:** 251–260.

186. Lewis, R. N., George, R., and McElhaney, R. N. 1986. Structure-function investigation of the membranes (Na^+-Mg^{2+})-ATPase from *Acholeplasma laidlawii* B: studies of reactive amino acid residues using group specific reagents. *Arch. Biochem. Biophys.* **247:** 201–210.

187. Jennings, M. L. and Anderson, M. P. 1987. Chemical modification and labeling of glutamate residues at the stilbenedisulfonate site of human red blood cell band 3 protein. *J. Biol. Chem.* **262**: 11435–11445.

188. Johnson, A. R. and Dekker, E. E. 1996. Woodward's Reagent K inactivation of Escerichia coli L-thronine dehydrogenase: Increases absorbance at 340–350 nm is due the modification of cysteine and histidine residues, not aspartate or glutamate carboxyl groups. *Protein Science* **5:** 382–390.

189. Anestario, M., Jr., Harrison, M. L., and Gaehlen, R. L. 1990. Immunochemical detection of adenine nucleotide binding proteins to 5′-*p*-fluorosulfonylbenzoyladenosine. *Anal. Biochem.* **190:** 60–65.

190. Puri, R. N. and Colman, R. W. 1997. Immunoaffinity method to identify aggregin, an ADP receptor in human blood platelets. *Arch. Biochem. Biophys.*, **347:** 263–270.

191. Adler, J. R. and Handin, R. I. 1979. Solubilization and characterization of a platelet membrane ADP-binding protein. *J. Biol. Chem.* **254:** 1866–1872.

192. Nachman, R. L. and Ferris, B. 1974. Binding of adenosine diphosphate by isolated membranes from human platelets. *J. Biol. Chem.* **249:** 704–710.

193. Gachet, C., Cattaneo, M. Ohlman, P., Hechler, B., Lecchi, A., Chevalier, J., Cassel, D., Mannuchi, P. M., and Cazenave, J.-P. 1995. Purinoreceptors on blood platelets: further pharmacological and clinical evidence to suggest the presence of two ADP receptors. *Br. J. Haematol.* **91:** 434–444.

194. Savi, B., Laplace, M. cI., Maffrand, J. P., and Herbert, J. M. 1994. Binding of [^{3}H]-2-methylthio-ADP to rat platelets-effect of clopidogrel and ticlopidine. *J. Pharm. Exp. Therp.* **269:** 772–777.

195. Savi, P., Laplace, M. cI., and Herbert, J. M. 1994. Evidence for the existance of two different ADP-binding sites on rat platelets. *Thromb. Res.* **76:** 157–169.

196. Mills, D. C. B., Puri, R. N., Hu, C.-J., Minniti, C., Grana, G., Freedman, M. D., Colman, R. F., and Coalman, R. W. 1992. Clopidogrel inhibits binding of ADP analogues to the receptor mediating platelet adenylate cyclase. *Arterio. Thromb.* **12:** 431–436.

197. Soslau, G. and Parker, J. 1989. Modulation of platelet function by extracellular adenosine triphosphate. *Blood* **74:** 984–993.

198. Soslau, G., Brosky, I., and Parker, J. 1993. Occupancy of P2 purinoreceptors with unique properties modulates the function of human platelets. *Biochim. Biophys. Acta* **1177:** 199–207.

199. White, J. G., Rao, G. H. R., and Gerrad, J. M. 1974. Effects of ionophore A23187 on blood platelets. *Am. J. Pathol.* **77:** 135–149.

200. Berridge, M. J. and Irvine, R. F. 1989. Inositol phosphates in cell signalling. *Nature* **341:** 197–205.

201. Fisher, G. J., Bakshian, S., and Baldssare, J. J. 1985. Activation of human platelets with ADP causes a rapid rise in cytosolic free calcium without hydrolysis of phosphatidyl-4,5-bisphosphate. *Biochem. Biophys. Res. Commun.* **129:** 958–964.

202. Sweatt, J. D., Blair, I. A., Cragoe, E. J., and Limbird, L. E. 1986. Inhibition of Na^+/H^+ exchange block epinephrine- and ADP-induced stimulation of human phospholipase C by blockade of arachidonic acid release at a prior step. *J. Biol. Chem.* **261:** 8660–8666.

203. Vickers, J. D., Kinlough-Rathbone, R. L., and Mustard, J. F. 1986. The decrease in phosphatidyl-4,5-bisphosphate in ADP-stimulated washed rabbit platelets is not primarily due to phospholipase C activation. *Biochem. J.* **237:** 327–332.

204. Llyod, J. V., Nishizawa, E. E., Joist, J. H., and Mustard, J. F. 1973. Effect of ADP-induced aggregation on $^{32}PO_4$ incorporation into phophatidic acid and phosphoinositides of rabbit platelets. *Br. J. Haematol.* **24:** 589–604.

205. Vickers, J. D., Kinlough-Rathbone, R. L., and Mustard, J. F. 1982. Change in phosphatidyl-4,5-bisphosphate 10 seconds after stimulation of washed platelets with ADP. *Blood* **60:** 1247–1250.

206. Feliste, R., Simon, M. F., Chap, H., Douste-Blazy, L., Defreyn, G., and Maffrand, J. P. 1988. Effect of PCR 4099 on ADP-induced calcium movements and phosphatidic acid in rat platelets. *Biochem. Pharmacol.* **37:** 2559–2564.

207. Daniel, J. L., Dangelmeyer, C. A., Selak, M., and Smith, J. B. 1986. ADP stimulates IP_3 formation in human platelets. *FEBS Lett.* **206:** 299–303.

208. Vickers J. D., Kinlough-Rathbone, R. L., Packham, M. A., and Mustard, J. F. 1990. Inositol phospholipid metabolism in human blood platelets stimulated by ADP. *Eur. J. Biochem.* **193:** 521–528.

209. Gear, A. R. L. and Raha, S. 1993. Calcium signalling and phosphoinosidite metabolism in platelets: subsecond events revealed by quenched flow techniques, In *Mechanisms of Platelet Activation and Control* (Authi, K. S., Ed.) pp. 57–67, Plenum Press, New York.

210. Jones, G. D. and Gear, A. R. L. 1988. Subsecond calcium dynamics in ADP- and thrombin-stimulated platelets: a continuous-flow approach using Indo-1. *Blood* **71:** 1539–1543.

211. Kroll, M. K., Hellums, J. D., McIntyre, L. V., Schafer, A. I., and Moake, J. L. 1996. Platelets and shear stress. *Blood* **88:** 1625–1541.

212. Eudo, M. 1977. Calcium release from sarcoplasmic reticulum. *Physiol. Rev.* **57:** 71–108.

213. Galione, A., Lee, H. C., and Busa, W. B. 1991. Ca^{2+}-induced Ca^{2+} release from sea urchin egg homogenates: modulation by cyclic ADP-ribose. *Science* **253:** 1143–1146.

214. Galione, A. 1992. Ca^{2+}-induced Ca^{2+} release and its modulation by cyclic ADP-ribose. *Trends Pharmacol. Sci.* **13:** 304–306.

215. Berridge, M. J. 1991. Cytoplasmic calcium oscillations. A two pool model. *Cell Calcium* **12:** 63–72.

216. Tsenoda, Y., Matsuno, K., and Tashiso, Y. 1988. Spatial distribution and temporal

change of cytoplasmic calcium in human platelets. *Biochem. Biophys. Res. Commun.* **156:** 1152–1159.

217. Heemskerk, J. W. M., Hoyland, J., Mason, W. T., and Sage, S. O. 1992. Spiking in cytosolic calcium concentrations in single fibrinogen bound Fura 2 loaded platelet. *Biochem. J.* **283:** 379–383.

218. Authi, K. S. 1993. Ca^{2+} homeostasis and intracellular pools in human platelets, in *Mechanisms of Platelet Activation and Control* (Authi, K. S., Ed.) pp. 83–104, Plenum Press, New York.

219. Murphy, C. T., Elmore, M., Kellie, S. and Westwick, J. 1991. The relationship between cytosolic Ca^{2+}, *sn*-1,2-diacylglycerol and inositol-1,4,5-triphosphate in platelet-activating factor stimulated rabbit platelets. *Biochem. J.* **278:** 255–261.

220. Putney, J. W., Jr. 1986. A model for receptor regulated calcium entry. *Cell Calcium* **7:** 1–12.

221. Putney, J. W., Jr. 1990. Capacitative calcium entry revisited. *Cell Calcium* **11:** 611–624.

222. Authi, K. S., Bokkala, S., Patel, Y., Kakkar, V. V., and Munkonge, F. M. 1993. Ca^{2+} release from platelet intracellular stores by thapisgargin and 2,5-di-(*t*-butyl) 1,4-benzhydroquinone. Relationship to Ca^{2+} pools and relavence in platelet activation. *Biochem. J.* **294:** 119–226.

223. Alonso, M. T., Alvarez, J., Montero, M., Sanchez, A., and Garcia-Sancho, J. 1990. Agonist-induced Ca^{2+} influx into human platelets is secondary to emptying the intracellular Ca^{2+} stores. *Biochem. J.* **280:** 783–789.

224. Alvarez, J., Montero, M., and Garcia-Sancho, J. 1992. Cytochrome P-450 may link intracellular Ca^{2+} stores with plasma membrane Ca^{2+} influx. *Biochem. J.* **274:** 193–197.

225. Sargent, P., Clarkson, W. D., Sage, S. O., and Heemskerk, J. W. M. 1992. Calcium influx evoked by Ca^{2+} store depletion in human platelets is more susceptible to cytochrome P-450 inhibitors than receptor regulated Ca^{2+} entry. *Cell Calcium* **13:** 553–564.

226. Kuno, M. and Gardner, P. 1989. Ion channels activated by inositol-1,4,5-triphosphate in plasma membrane of human T-lymphocytes. *Nature* **326:** 301–304.

227. Irvine, R. F. 1990. "Quantal" calcium release and the control of Ca^{2+} entry by inositol phosphates—a possible mechanism. *FEBS Lett.* **263:** 5–9.

228. Rengaswamy, A. and Feinberg, H. 1988. Inositol-1,4,5-triphosphate induced Ca^{2+} release from platelet plasma membrane vesicles. *Biochem. Biophys. Res. Commun.* **150:** 1021–1026.

229. Klan, A. A., Steiner, J. P., and Snyder, S. H. 1992. Plasma membrane inositol-1,4,5-triphosphate receptor of lymphocytes; Selective enrichment of sialic acid and unique binding specificity. *Proc. Natl. Acad. Sci. U.S.A.* **89:** 2849–2853.

230. Fujimoto, T., Nakada, S., Miyawake, A., Mikoshiba, K., and Ogawa, K. 1992. Localization of inositol-1,4,5-triphosphate receptor-like protein plasmalemmal caveolae. *J. Cell Biol.* **119:** 1507–1513.

231. Sage, S. O., Merrit, J. E., Hallam, T. J., and Rink, T. J. 1989. Receptor mediated Ca^{2+} entry in Fura-2 loaded human platelets stimulated with ADP and thrombin. Duel wavelength studies with Mn^{2+}. *Biochem. J.* **258:** 923–926.

232. Mahaut-Smith, M. P., Sage, S. O., and Rink, T. J. 1990. Receptor activated single channels in intact human platelets. *J. Biol. Chem.* **265:** 10479–10483.

233. Suldan, Z. and Brass, L. F. 1991. Role of glycoprotein IIb.IIIa complex in plasma membrane Ca^{2+} transport: a comparison of results obtained with platelets and human erythroleukemia cells. *Blood* **78**: 2887–2893.

234. Yamaguchi, A., Yamamoto, N., Kitagawa, H., Tanoue, K., and Yamazaki, H. 1987. Ca^{2+} influx mediated through the GPIIb/IIIa complex during platelet activation. *FEBS Lett.* **225:** 228–232.

235. Fujimoto, T., Fujimura, K., and Kuramoto, A. 1991. Electrophysiological evidence that glycoprotein IIb-IIIa complex is involved in calcium channel activation on human platlete plasma membrane. *J. Biol. Chem.* **266:** 16370–16375.

236. Horne, W. C. and Simons, E. R. 1978. Effects of amiloride on the response of human platelets to bovine α-thrombin. *Thromb. Res.* **13:** 599–603.

237. Connolly, T. M. and Limbird, L. E. 1988. Removal of extra platelet Na^+ eliminates indomethacin-sensitive secretion from human platelets stimulated by epinephrine, ADP, and thrombion. *Proc. Natl. Acad. Sci. U.S.A.* **80:** 5320–5324.

238. Sweatt, J. D., Johnson, S. L., Cragoe, J. E., and Limbird, L. E. 1985. Inhibitors of Na^+/H^+ exchange block stimulus provoked arachidonic acid release in human platelets. *J. Biol. Chem.* **260:** 12910–12919.

239. Siffert, W., Gengenbach, S., and Sheid, P. 1986. Inhibition of platelet aggregation by amiloride. *Thromb. Res.* **44:** 235–240

240. Zavoico, G. B., Cragoe, E. J., and Feinstein, M. B. 1986. Regulation of intracellular pH in human platelets. *J. Biol. Chem.* **261:** 13160–13167.

241. Siffert, W. and Akkerman, J. W. N. 1988. Na^+/H^+ exchange as a modulator of platelet activation. *Trends Biochem. Sci.* **13:** 148–151.

242. Feinberg, H., Sandler, W. C., Scorer, M., LeBreton, G. C., Grossman, B., and Born, G. V. R. 1977. Movement of sodium into human platelets induced by ADP. *Biochim. Biophys. Acta* **470:** 317–324.

243. Sweatt, J. D., Connolly, T. M., Cragoe, E. J., and Limbird, L. E. 1986. Evidence that Na^+/H^+ exchange regulates receptor-mediated phospholipase A_2 activation in human platelets. *J. Biol. Chem.* **261:** 8667–8673.

244. Apitz-Castro, R. J., Mas, M. A., Cruz, M. R., and Jain, M. K. 1979. Isolation of homogeneous phospholipase A_2 from human platelets. *Biochem. Biophys. Res. Commun.* **91:** 63–71.

245. Jain, M. K. and Apitz-Castro, R. 1978. Lag phase during the action of phospholipase A_2 on phosphatidylcholine modified by alkanols. *J. Biol. Chem.* **253:** 7005–7010.

246. Siffert, W., Siffert, G., Scheid, P., and Akkerman, J. W. N. 1990. Na^+/H^+ exchange modulates Ca^{2+} mobilization in human platelets stimulated by ADP and the thromboxane mimetic U46619. *J. Biol. Chem.* **264:** 719–725.

247. Sage, S. O., Rink, T. J., and Mahout-Smith, M. 1991. Resting and ADP-evoked changes in cytosolic free sodium concentration in human platelets loaded with indicator SBFI. *J. Physoiol.* **44:** 559–573.

248. De Cristofaro, R., Landolfi, R., De Candia, E., Castagnola, M., Di Cera, E., and Wyman, J. 1988. Allosteric equilibria in the binding of fibrinogen to platelets. *Proc. Natl. Acad. Sci. U.S.A.* **85:** 8473–8476.

249. De Cera, E., Gill, S. J., and Wyman, J. 1988. Canonical formulation of linkage thermodynamics. *Proc. Natl. Acad. Sci. U.S.A.* **85:** 5077–5081.

250. De Cristofaro, R., Landolfi, R., De Cera, E., Di Candia, E., Castagnola, M., De Candia, E., and Bizzi, B. 1989. Inhibition of fibrinogen binding to human platelets by blockage of Na^+/H^+ exchange. *Biochem. Biophys. Res. Commun.* **161:** 1282–1232.

251. Ross, R. 1986. The pathogenesis of atherosclerosis-An update. *N. Engl. J. Med.* **314:** 488–500.

252. Border, W. A. and Russiatiti, L. R. 1992. Transforming growth factor-β in diseases. The dark side of tissue repair. *J. Clin. Invest.* **90:** 1–7.

253. Amento, E. P., Ehsami, N., Palmer, H., and Libby, P. 1991. Cytokines and growth factors positively and negatively regulate interstitial collagen gene expression in human vascular smooth muscle cells. *Arterioscler. Thromb.* **11:** 1223–1230.

254. Schwartz, L., Bourassa, M. G., Lesperance, J., Aldridge, H. E., Kazim, F., Savatore, E. A., Henderson, M., Bonem, E., and Kabid, P. R. 1988. Aspirin and dipyridamole in prevention of restonosis after percutaneous transluminal angioplasty. *N. Engl. J. Med.* **318:** 1714–1719.

255. Thornton, M. A., Gruentzig, A. R., Hollman, J., King, S. B., III, and Douglas, J. S. 1984. Coumadin and aspirin in prevention of recurrence after transluminal coronary angioplasty. 1984. *Circulation* **69:** 721–727.

256. Brockman, M. J., Eiroa, A. M., and Marcus, A. J. 1991. Inhibition of human platelet reactivity by endothelium-derived relaxing factor from human umbilical vein endothelial cells in suspension. Blockade of aggregation and secretion by aspirin-insensitive mechanism. *Blood* **78:** 1033–1040.

257. Marcus, A. J., Safier, L. B., Hajjar, K. A., Ullman, H. L., Islam, N., Brookman, M. J., and Eiroa, A. M. 1991. Inhibition of platelet activation by aspirin-insensitive endothelial cell ADPase: thromboregulation of endothelial cells. *J. Clin. Invest.* **88:** 1690–1696.

258. Rao, A. K. 1990. Congenital disorders of platelet function. *Hematol. Oncol. Clin. North Am.* **4:** 65–86.

259. Weiss, H. J. 1994. Inherited abnormalities of platelet granules and signal transduction, in *Hemostasis and Thrombosis: Basic Principles and Clinical Practice* (Colman, R. W., Hirsh, J., Marder, V. J., and Saltzman, E. W., Eds.), pp. 673–684, Lippincott, Philadelphia, PA.

260. Rinder, C. S., Student, L. A., Bonan, J. L., Rinder, H. M., and Smith, B. R. 1993. Aspirin does not inhibit adenosine diphosphate-induced platelet α-granule release. *Blood* **82:** 505–512.

261. Dillingham, E. O., Lasslo, A., Cater-Burks, G., Bond, S. E., and Gollamundi, R. 1989. Relationship between chemical structure and inhibition of ADP-stimulated human thrombocyte release of serotonin and platelet factor-4. *Biochim. Biophys. Acta* **990:** 128–132.

262. Bell, R. L., Kennerly, D. A., Stanford, N., and Majerus, P. W. 1979. Diglyceride lipase: a pathway for arachidonate release from human platelets. *Proc. Natl. Acad. Sci. U.S.A.* **76:** 3228–3241.

263. Billah, M. M., Lapetina, E. G., and Cutarocasas, P. 1981. Phospholipase A_2 activity specific for phosphatidic acid. A possible mechanism for the production of arachidonic acid in platelets. *J. Biol Chem.* **256:** 831–837.

264. Packham, M. A., Livine, A.-A., Ruben, D. H., and Rand, M. L. 1993. Activation of phospholipase C has little involvement in ADP-induced primary aggregation of human platelets: effects of diacylglycerols, diacylglycerol kinase inhibitor R59022, staurosporine and okodaic acid. *Biochem. J.* **290:** 849–856.

265. Pulcinelli, F. M., Ashby, B., Gazzauiiga, P. P., and Daniel, J. L. 1995. Protein kinase C activation is not a key step in ADP-mediated exposure of fibrinogen receptors on human platelets. *FEBS Lett.* **364:** 87–90.

266. Friesen, L. L. and Gerrard, J. M. 1985. The effects of 1-oleoyl-2-acetylglycerol on platelet phosphorylation and platelet ultrastructure. *Am. J. Pathol.* **121:** 79–87.

267. Gerrard, J. M., Israelis, S. J., and Friesen, L. L. 1983. Protein phosphorylation and platelet secretion. *Nouv. Fr. Haematol.* **27:** 267–273.

268. Kaibuchi, K., Takai, Y., Sawamura, M., Hosijima, M., Fujikara, T., and Nishizuka, Y. 1983. Synergistic functions of protein phosphorylation and calcium mobilization

in platelets. *J. Biol. Chem.* **258:** 6701–6704.

269. Takai, Y., Kishimoto, A., Kikkawa, U., and Nishizuka, Y. 1979. Unsaturated diacylglycerol as a possible messenger for the calcium-activated, phospholipid dependent protein kinase system. *Biochem. Biophys. Res. Commun.* **91:** 1218–1224.

270. Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U., and Nishizuka, Y. 1980. Activation of calcium and phospholipid dependent protein kinase by diacylglycerol, its possible relation to phosphatidylinositol turnover. *J. Biol. Chem.* **255:** 2273–2276.

271. Gerrard, J. M., Beattie, L. L., Park, J., Israels, S. J., McNicol, A., Lint, D., and Cragoe, E. J., Jr. 1989. A role of protein kinase C in membrane fusion necessary for platelet granule secretion. *Blood* **74:** 2405–2413.

272. Gilman, A. G. 1987. G-proteins: transducers of receptor generated signals. *Ann. Rev. Biochem.* **56:** 615–649.

273. Raymond, J. R. 1995. Multiple mechanisms of receptor-G protein signalling specificity. *Am. J. Physiol.* **38:** F141–F158.

274. Brass, L. F., Hoxie, J. A., and Manning, D. R. 1993. Signalling through G proteins and G protein-coupled receptors during platelet activation. *Thromb. Haemost.* **70:** 217–223.

275. Gachet, C., Cazenave, J.-P., Ohlman, P., Gerhard, H., Wieland, T., and Jacob, K. H. 1992. ADP-receptor-induced activation of guanine-nucleotide-binding proteins in human platelet membranes. *Eur. J. Biochem.* **207:** 259–263.

276. Laugwitz, K.-L., Spicher, K., Scultz, G., and Offermanns, S. 1994. Identification of receptor-activated G-proteins: selective immunoprecipitation of photolabeled G-protein α-subunit. *Methods Enzymol.* **237:** 283–294.

277. Ohlman, P., Laugwitz, K.-L., Nurnberg, B., Spicher, K., Scultz, G., Cazenave, J.-P., and Gachet, C. 1995. The human platelet ADP-receptor activates G_{i2} proteins. *Biochem. J.* **312:** 775–779.

278. Gachet, C., Cazenave, J.-P., Ohlmann, P., Bouloux, C., Defrreyn, G., Driot, F., and Maffrand, J.-P. 1990. The thienopyridine ticlopidine selectively prevents the inhibitory effects of ADP but not of adrenaline on cAMP levels raised by stimulation of the adenylyl cyclase of human platelets by PGE_1. *Biochem. Pharmacol.* **40:** 2683–2687.

279. Clapham, D. E. and Neer, E. J. 1993. New roles for G-protein βγ-dimers in transmembrane signalling. *Nature* **365:** 403–406.

280. Léon, C., Vial, C., Cazenave, J.-P., and Gachet, C. 1996. Cloning and sequencing of a human cDNA encoding endothelial $P2Y_1$ purinoreceptor. *Gene* **171:** 295–297.

281. Léon, C., Hechler. B., Vial, C., Leray, C., Cazenave, J.-P., and Gachet, C. 1997. The $P2Y_1$ receptor is an ADP receptor antagonized by ATP and expressed in platelets and megakaryoblastic cells. *FEBS Lett.* **403:** 26–30.

282. Mills, D. C. B. 1996. ADP Receptors on platelets. *Thromb. Haemost.* **76:** 835–56.

283. Janssens, R., Communi, D., Pirotton, S., Samson, M., Parmentier, M., and Boeynames, J.-M. 1996. Cloning and tissue distribution of the human $P2Y_1$ receptor. *Biochem. Biophys. Res. Commun.* **221:** 588–591.

284. Schacter, J. B., Li, Q., Boyer, J. L., Nicholas, R. A., and Harden, T. K. 1996. Second messenger cascade specificity and pharmacologiacl selectivity of the human $P2Y_1$-purinoreceptor. *Br. J. Pharmacol.* **118:** 167–173.

285. Defreyn, G., Bernat, A., Delebasse, D., and Maffrand, J.-P. 1989. Pharmacology of toiclopidine: a review. *Semin. Thromb. Hemost.* **15:** 159–166.

286. Saltiel, E. and Ward, A. 1987. Ticlopidine: a review of its pharmacodynamic and pharmacokinetic properties and therapeutic ef-

ficay in platelet-dependent disease states. *Drugs* **34:** 22–262.

287. McTavish, D., Faulds, D., and Gao, K. L. 1990. Ticlopidine. An updated review of its pharmacology and therapeutic use in platelet-dependent disorders. *Drugs* **40:** 238–259.

288. Bruno, J. J. 1983. The mechanism of action of ticlopidine. *Thromb. Res. Suppl IV:* 59–67.

289. Maffrand, J.-P., Defreyn, G., Bernat, A., Delebasee, D., and Tissmier. 1988. Reviewed pharmacology of ticlopidine. *Angiolgie* **7:** 6–13.

290. Herbert J, M., Frechel, D., Valleé, E., Kiefer, G., Gouy, D., Berger, Y., Defreyn, G., and Maffrand, J.-P. 1993. Clopidogrel, a novel antiplatelet and antithrombotic agent. *Cardiovasc. Drug Rev.* **11:** 180–198.

291. Savi, P., Combalbert, J., Gaich, C., Rouchon, M.-C., Maffrand, J.-P., and Berger, Y. 1994. The antiaggregating activity of clopidogrel is due to a metabolic activation by the hepatic cytochrome P450-1A. *Thromb. Haemost.* **72:** 313–317.

292. Cattaneo, M., Lombardi, R., Bettega, D., Leechi, A., and Mannuchi, M. 1993. Shear-induced platelet aggregation is potentiated by desmopressin and inhibited by ticlopidine. *Arterioscl. Thromb.* **13:** 393–397.

293. Herbert, J.-M., Bernat, A., and Maffrand, J.-P. 1993. Aprotonin reduces clopidogrel-induced prolongation bleeding time in rats. *Throm. Res.* **71:** 433–441.

294. Nagakawa, Y., Akedo, Y., Orimo, H., and Yano, H. 1990. Effect of combination of antiplatelet agents in man. Combination of aspirin, trapidil, ticlopidine and dipyridamole. *Thromb. Res.* **60:** 469–475.

295. Savi, P., Bernat, A., Dumas, A., Ait-Chek, L., and Herbert, J.-M. 1994. Effects of aspirin and clopidogrel on platelet dependent tissue factor expression in endothelial cells. *Thromb. Res.* **73:** 117–124.

296. Steiger, H., Hess, H., Mietaschek, A., Trampish, H.-J., and Ingrish, H. 1984. Einfluss von ticlopidine auf die periphere obliterierendie Arteriopathie. *Dtsch. Med. Wschr.* **109:** 1240–1249.

297. Levy, R. I., Brensike, J. F., Epstein, S. E., Kelsey, S. F., Passamanti, E. R., Richardsson, J. M, Loh, I. K., Stone, N. J., Aldrich, R. F., Battaglini, J. W., Moriarty, D. J., Fisher, M. L., Friedman, L., Friedwald, W., and Detere, K. M. 1984. The influence of changes in lipid values induced by cholestyramine and diet on progression of coronary artery disease—Results of NHIBI Type II coronary intervention study. *Circulation* **69:** 325–337.

298. Berglund, U. and Wallentin, L. 1986. Influence on lipoprotein metabolism of platelet inhibitory drug ticlopidine. *Atherosclerosis* **59:** 241–246.

299. Humphries, R. G., Tomlinson, W., Clegg, J. A., Ingall, A. H., Kindon, N. D., and Leff, P. 1995. Pharmacological profile of the novel P2T-purinoreceptor antagonist, FPL 67085 in vitro and in anesthesized rats in vivo. *Br. J. Pharmacol.* **115:** 1110–1116.

300. Humphries, R. G., Robertson, M. J., and Leff, P. 1995. A novel series of P2T purinoreceptor antagonists: definition of the role of ADP in arterial thrombosis. *Trends Pharmacol. Sci.* **16:** 179–181.

301. Humphries, R. G., Tomlinson, W., Ingall, A. H., Cage, P. A., and Leff, P. 1994. FPL 66096: a novel, highly potent and selective antagonist at human P2T purinoreceptor. *Br. J. Pharmacol.* **113:** 1057–63.

302. Chao, F. and Zamecnick, P. C. 1984. Inhibition of platelet aggregation by Ap_4A *Hoppe Seyler's Z. Physiol. Chem.* **365:** 610–611.

303. Louie, S., Kim, B. K., and Zamecnik, P. C. 1988. Diadenosine 5′,5‴-P1,P4-tetraphosphate, a potential antithrombotic agent. *Thromb. Res.* **49:** 557–566.

304. Proctor, R. A., Denlinger, L. C., Leventhal, P. S., Daugherty, S. K., Van De Loo, J.-W., Tanke, T., Firestein, G. S., and Bertics, P. J. 1994. Protection of mice from endotoxic death by 2-methylthio-ATP. *Proc. Natl. Acad. Sci. U.S.A.* **91:** 6017–6020.

305. Seiss, W. 1989. Molecular mechanisms of platelet activation. *Physiol. Rev.* **69:** 58–178.

306. Gachet, C. and Cazenave, J.-P. 1991. ADP-induced blood platelet activation: a review. *Nouv. Rev. Franc. Hematol.* **33:** 347–358.

307. Hourani, S. M. O. and Hall, D. A. 1994. Receptors for ADP on human blood platelets. *Trends Pharmacol. Sci.* **15:** 103–108.

308. MacKenzie, A. B., Mahaut-Smith, M. P., and Sage, S. O. 1996. Activation of receptor-operated cation channel via P2X1 not P2T purinoreceptors in human platelets. *J. Biol. Chem.* **271:** 2879–2881.

309. McFarlane, D. E. 1987. Platelet responses and metabolism (Holmsen, H., Ed.), pp. 19–36, CRC Press, Boca Raton, FL.