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ADP-Induced Platelet Activation

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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.

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); AMP*CCl*₂P, α,β -dichloromethylene-adenosine 5'-diphosphate; AMP*CF*₂P, α,β -difluoromethylene-adenosine 5'-diphosphate; AMPNP, α,β -imido-adenosine 5'-diphosphate; AMPPCP, β,γ -methylene-adenosine; Ap_nA, α,ω -diadenosine polyphosphate; WR-K, Woodward's Reagent-K, *N*-ethyl-5-phenylisooxazolium-3'-sulfonate; NaBH₄, 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-9 α ,11 α -methanepoxy prostaglandin F2 α ; vWF, von Willebrand Factor; PAF, platelet activating factor; PMA, phorbol 12-myristate 13-acetate; [Ca²⁺]_i, intracellular calcium ion concentration; AA, arachidonic acid; PIP₂, phosphatidylinositol-4,5-bisphosphate; IP₃, inositol-1,4,5-triphosphate; IP₄, inositol-1,3,4,5-tetrakisphosphate; DAG, diacylglycerol; PKC, protein kinase C; PLC, phospholipase C; PLA₂, phospholipase A₂; 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. Helleman 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 platelets¹⁸ 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, Ca²⁺ 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 phosphokinase (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 Ticlopidine⁴⁰ 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 spasmotic 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.

logical 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 categories: 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 A₁- and A₂-receptors⁵² or R₁- and R₂-receptors.⁵³ A₁- and A₂-receptors appeared to be similar to R₁- and R₂-receptors.⁵⁴ This classification has been amended to accommodate additional pharmacological responses mediated by adenosine receptors⁵⁵ and places them in the seven transmembrane (TM) superfamily⁵⁶ of which crystalline bacteriorhodopsin is structurally archetypical.

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 pharmacolo-

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 ($\alpha,\beta\text{-Me-ATP}$) > $\beta,\gamma\text{-Me-ATP}$ > ATP = 2-methylthio-ATP (2-MeS-ATP).⁶⁰ $\beta,\gamma\text{-Me-L-ATP}$ (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 cells⁶⁴ and vas deferens cells⁶⁷ 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+}].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 > $\beta,\gamma\text{-Me-ATP}$. GTP, CTP, UTP, and ADP are as potent in inducing relaxation of *taenia coli*.⁵⁹ Suramin is a nonselective 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 P2Y₁ (human),⁷³ P2Y₂ (human),⁷⁴ P2Y₃ (chicken),⁷⁵ P2Y₄ (human),^{76,77} P2Y₅ (chicken),⁷⁸ P2Y₆ (rat),⁷⁹ and P2Y₇ (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 TM7⁵⁶ 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 ATP⁴⁻.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 ATP⁴ renders the cells nonselectively permeable to Na⁺, K⁺ and Ca²⁺ 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 [Ca²⁺]_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-³²P] 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 sug-

gests 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 Ca²⁺ from intracellular stores as well as influx of extracellular Ca²⁺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 aggregation¹⁰⁷

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 C₈ position results in lowering or complete loss of agonist potency. 8-Br-ADP¹¹¹ 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 C₂ produces ADP derivatives that are either as potent as ADP or more potent than ADP in inducing platelet activation. 2-Cl-ADP, 2-N₃-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 respons-

es.¹¹⁴ The effect of substitution at C₂ 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 N⁶ 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 (*ro*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 $[\text{Ca}^{2+}]_i$.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 *ro*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-

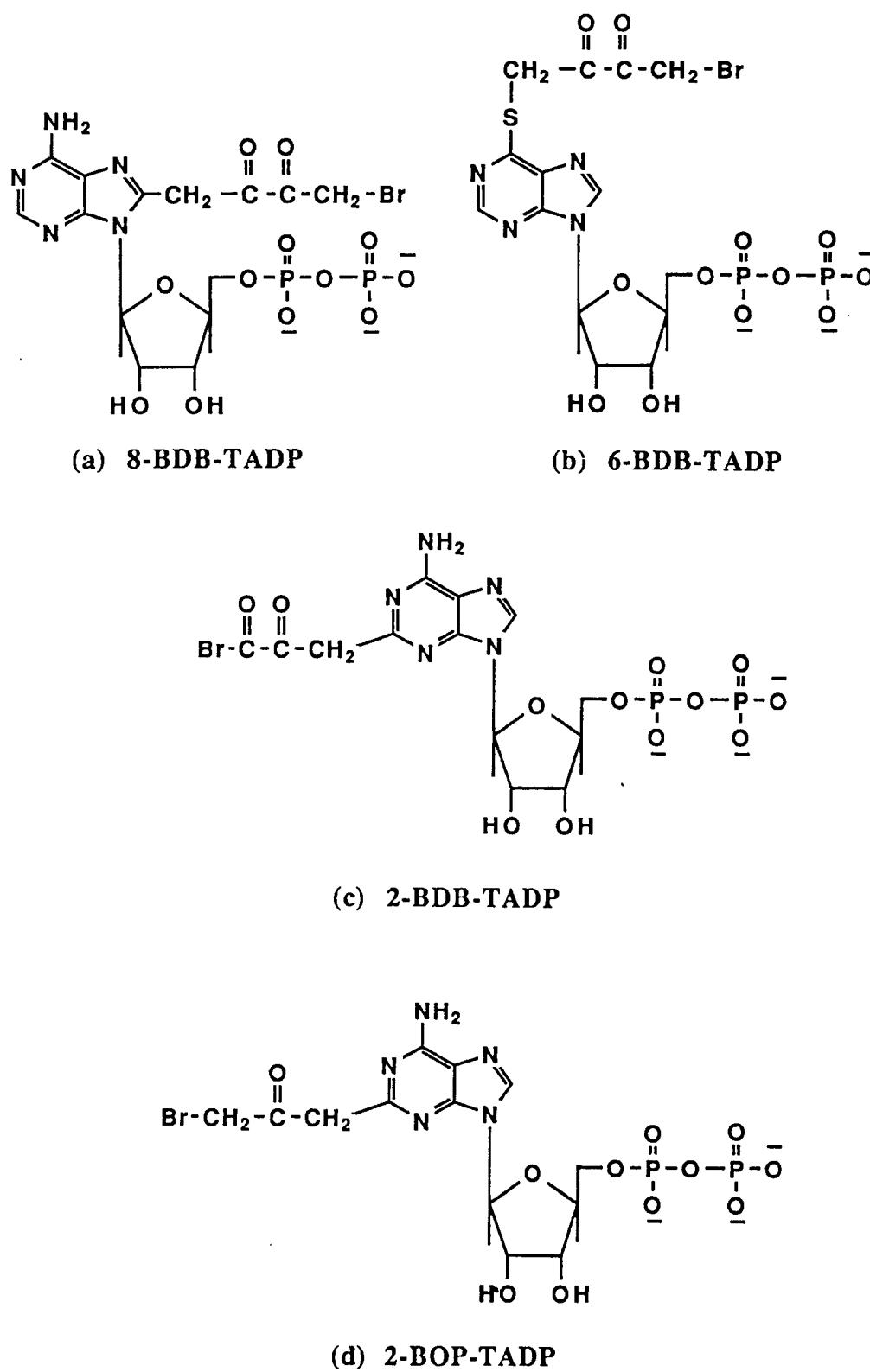
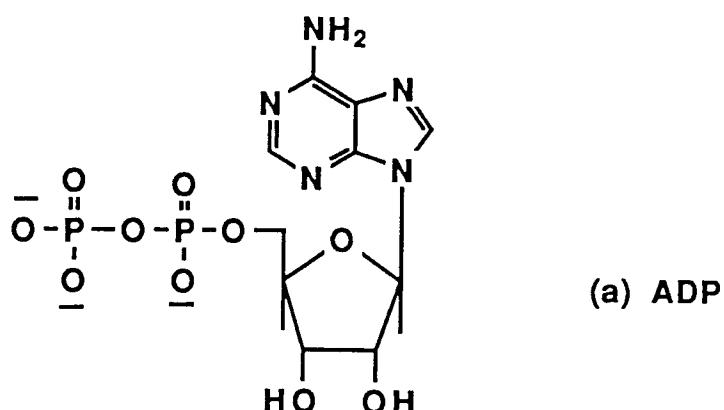
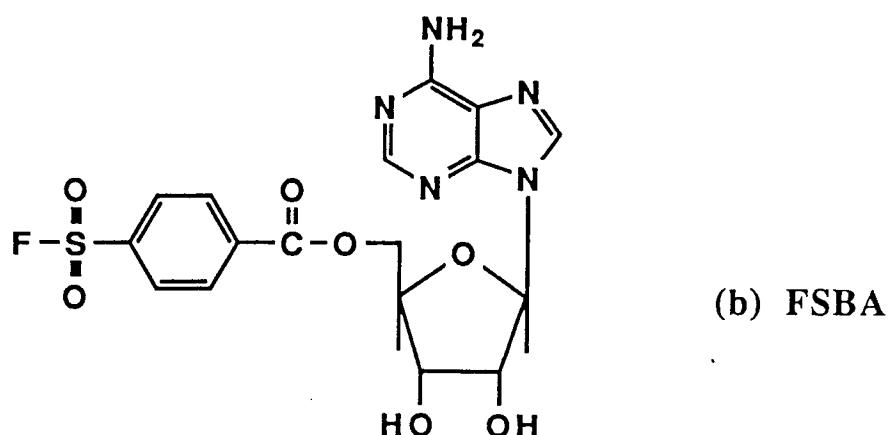


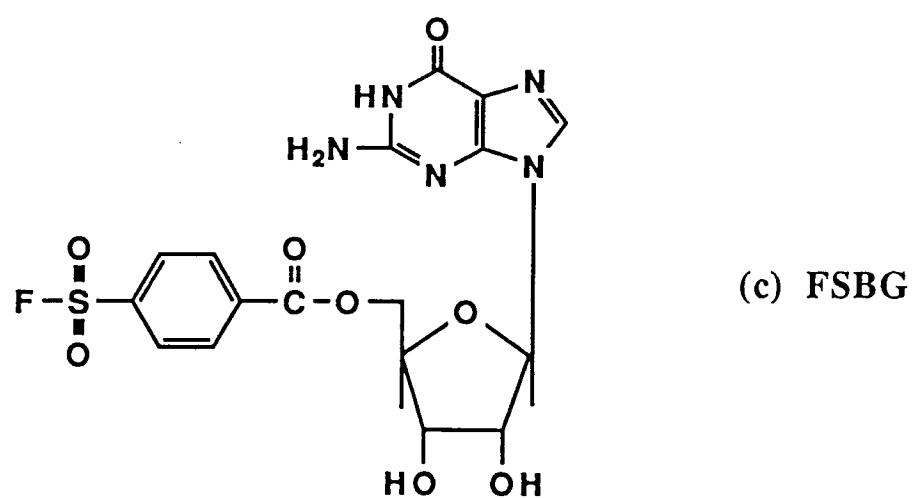
FIGURE 1. Chemical representations of chemically reactive ADP-affinity analogs. 8-BDB-TADP (a),¹¹² 6-BDB-TADP (b),¹¹⁴ 2-BDB-TADP (c),¹¹⁴ and 2-BOP-TADP (d).¹²⁸ For complete name of the nucleotide analogs, please refer to abbreviations.



(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.

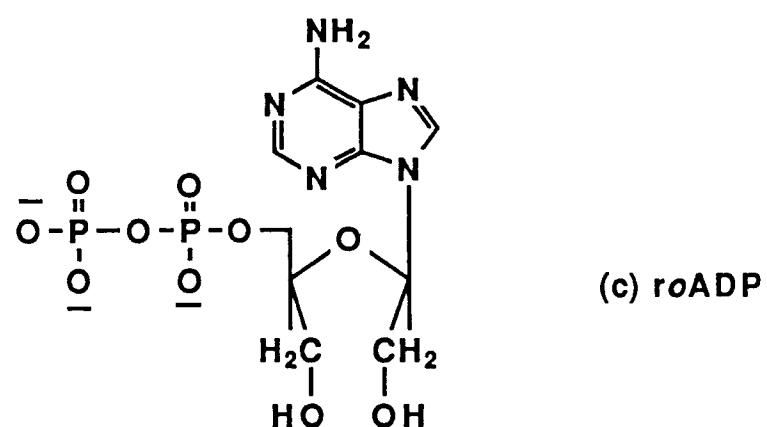
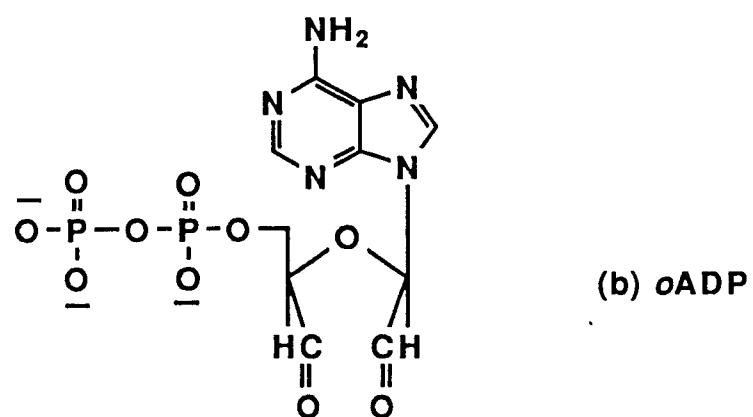
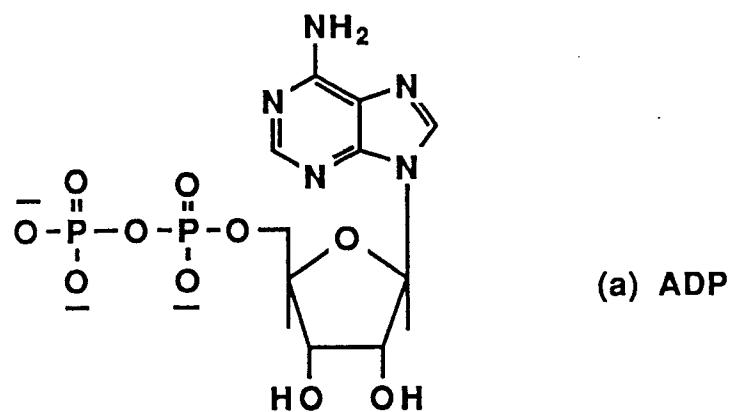


FIGURE 3. Chemical representations of ADP-affinity analogs modified in the ribose moiety. *o*ADP (b)^{116,117} and roADP (c).^{116,117} ADP (a) is included for comparison.

tive antagonist of ADP-induced inhibition of stimulated adenylate cyclase activity.^{118,119} α,β -Dichloromethylene-ADP [AMPCCl₂P] is equipotent and α,β -difluoromethylene-ADP [AMPCF₂P] 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-chloro-adenosine-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}

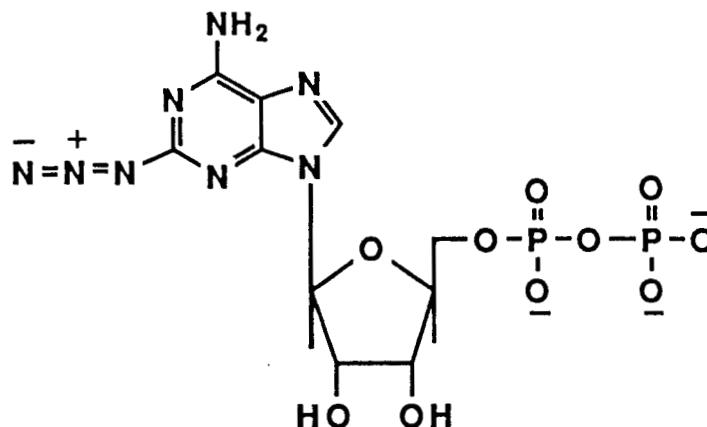
α,ω -Diadenosine polyphosphates (Ap_nA) have been shown to be antagonists of ADP-induced platelet aggregation.¹⁰⁸ Ap₄A has been shown to be the most potent of all the Ap_nA analogs (n = 2 – 6), as an inhibitor of ADP-induced platelet aggregation.¹⁰⁸ Due to the presence of an axis of symmetry in the structure of Ap₄A, 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_nA (n = 2 – 6) showed that base stacking was minimum in Ap₂A and Ap₃A.¹⁰⁹ It remains to be determined that it is also true of the platelet bound Ap₂A and Ap₃A. Therefore, the molecular basis for the strong inhibitory action of Ap₄A 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-N₃-L-ADP and 2-Cl-L-ADP are completely inactive as agonists or antagonists at P2T receptor.¹²³ In marked

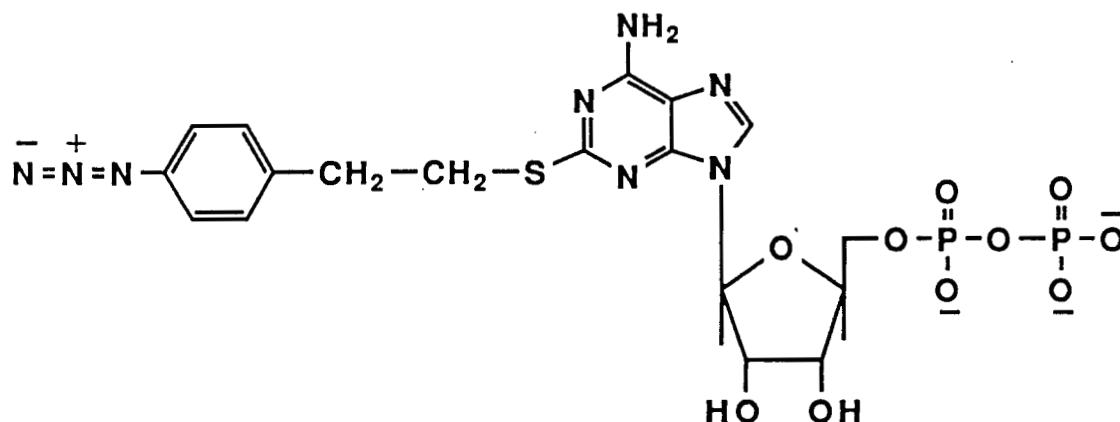
contrast, 2-N₃-L-ATP, 2-Cl-L-ATP and 2-MeS-L-ATP are 25- to 125-fold more potent than their enantiomeric counterparts as agonists at P2Y receptor.¹²⁴

V. 2-SUBSTITUTED ADP ANALOGS

Substitution, large or small, at C₂ in ADP produces compounds that have similar apparent affinities at P2T receptor.⁵⁸ 2-N₃-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-ADP¹²⁶ (Figure 4b), 2-aminopropyl-thioadenosine-5'-diphosphate,¹²⁷ and 2-BDB-TADP¹²⁸ 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-N₃-ADP, and 2-MeS-ADP not only contain substituents at C₂ that have different sizes but also are covalently bound to C₂ 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 C₂-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



(a) 2-N₃-ADP



(b) 2-AzPET-ADP

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-N₃-ADP¹²⁴ (Figure 4a) and 2-AzPET-ADP¹²⁶ (Figure 4b), although they carry the same reactive functionality, the azido group, but are bonded to C₂ 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 [Ca²⁺]_i.¹¹⁴ In order to examine the effect of the size of the substitution at C₂ 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 C₂ 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 C₂ in ADP has a bearing on the potency of an ADP analog to elicit platelet responses. Moreover, the size of a substituent at C₂ 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-N₃-ADP (Figure 4a) has been shown to aggregate platelets and inhibit stimulated adenylylate cyclase activity.¹²⁵ 2-N₃-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-N₃-[β-³²P]-ADP, however,

were unsuccessful.¹³⁰ Photoaffinity labeling of platelets by 2-AzPET-[β-³²P]-ADP {2-(*p*-azidophenyl)ethylthioadenosine [β-³²P]-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 mM ADP and ATP.¹²⁶ Other prominent bands of radioactivity in the autoradiogram of platelets labeled with 2-AzPET-[β-³²P]-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 [³H]-FSBA, an ADP-affinity reagent.^{131,132} Firm evidence that the 43-kDa protein labeled by 2-AzPET-[β-³²P]-ADP is not actin has been lacking.¹²⁶

ATP-α-S is a potent antagonist of ADP-induced platelet responses⁹⁰ and also binds tightly to the ADP-binding sites on platelets.¹³³ Photoaffinity labeling of intact platelets with [³⁵S]-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 Ca²⁺,¹³⁵ but are poorly labeled with [³⁵S]-ATP-α-S.¹³⁶ Furthermore, GPIIb has been shown to be radiolabeled with [³⁵S]-GTP-α-S and [³⁵S]-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-N₃-[γ -³²P]-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-N₃-[γ -³²P]-ATP and [³⁵S]-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 nucleotides¹³⁸⁻¹⁴⁰ 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 pro-

gressive 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 Ca²⁺ signals in Jurkat T cells via tyrosine phosphorylation of PLC γ 1 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 -³²P]-ADP¹²⁶ and [³⁵S]-ATP- α -S¹³⁴ did not consider these problems. It would be more appropriate to describe these investigations either as "photo-labeling" 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 [Ca²⁺]_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 –ε_{NH₂} (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 [³H]-FSBA was shown to label a single surface-membrane protein, aggrecin (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-³²P]-ADP to platelets.¹⁵⁹ At higher concentration (300 μM), FSBA-induced platelet shape change and caused concentration-dependent rise in [Ca²⁺].¹⁶⁰ Platelet aggregation and exposure of fibrinogen binding sites induced by collagen¹¹⁵ 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 [³H]-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 thrombin¹⁶³ 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 aggrecin 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 [³H]-FSBA produce a product of relatively low specific radioactivity, thus making the process of identifying the platelet surface radiolabeled proteins cum-

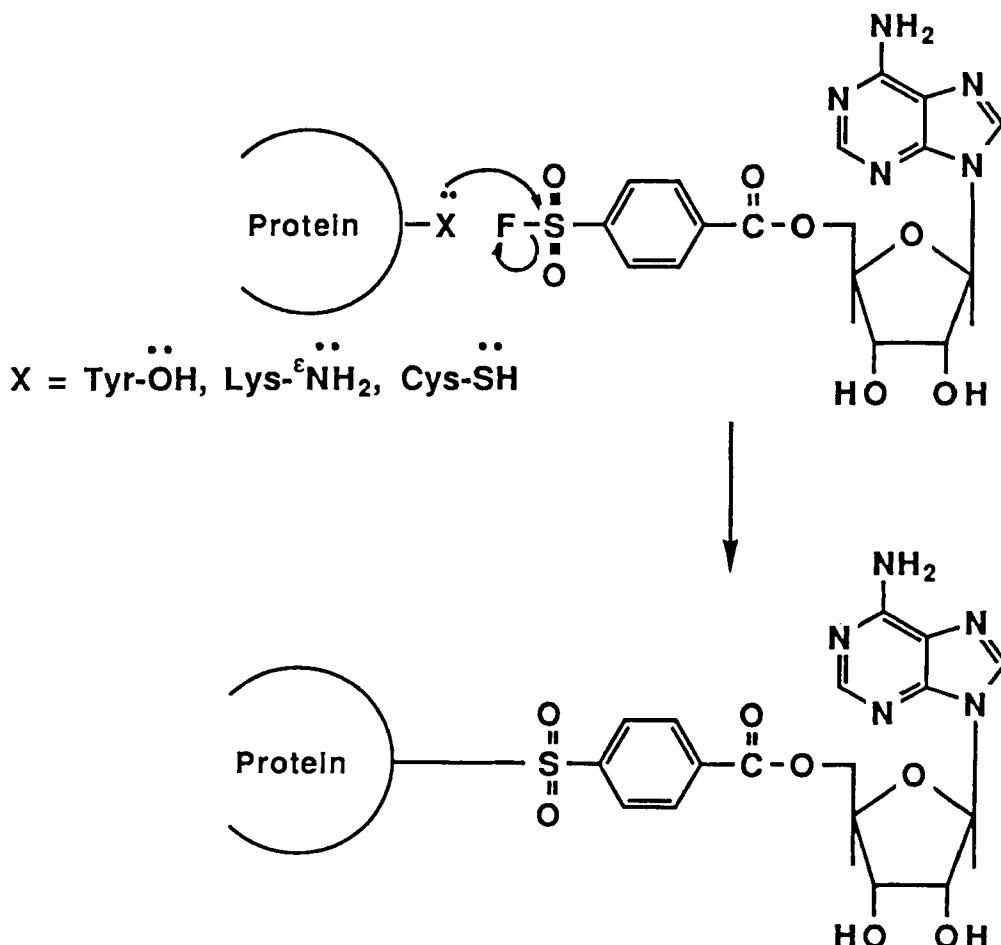


FIGURE 5. Schematic representation of covalent adduct formation between FSBA and proteins. Nucleophilic attack by the lone pair of electrons in reactive amino acids present in the functional domain(s) of proteins on the electrophilic sulfur in FSBA is accompanied by the displacement of fluoride ion leading 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 [Ca²⁺]_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 C₂, C₈, and N⁶ positions (the only positions in the ad-

enine 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

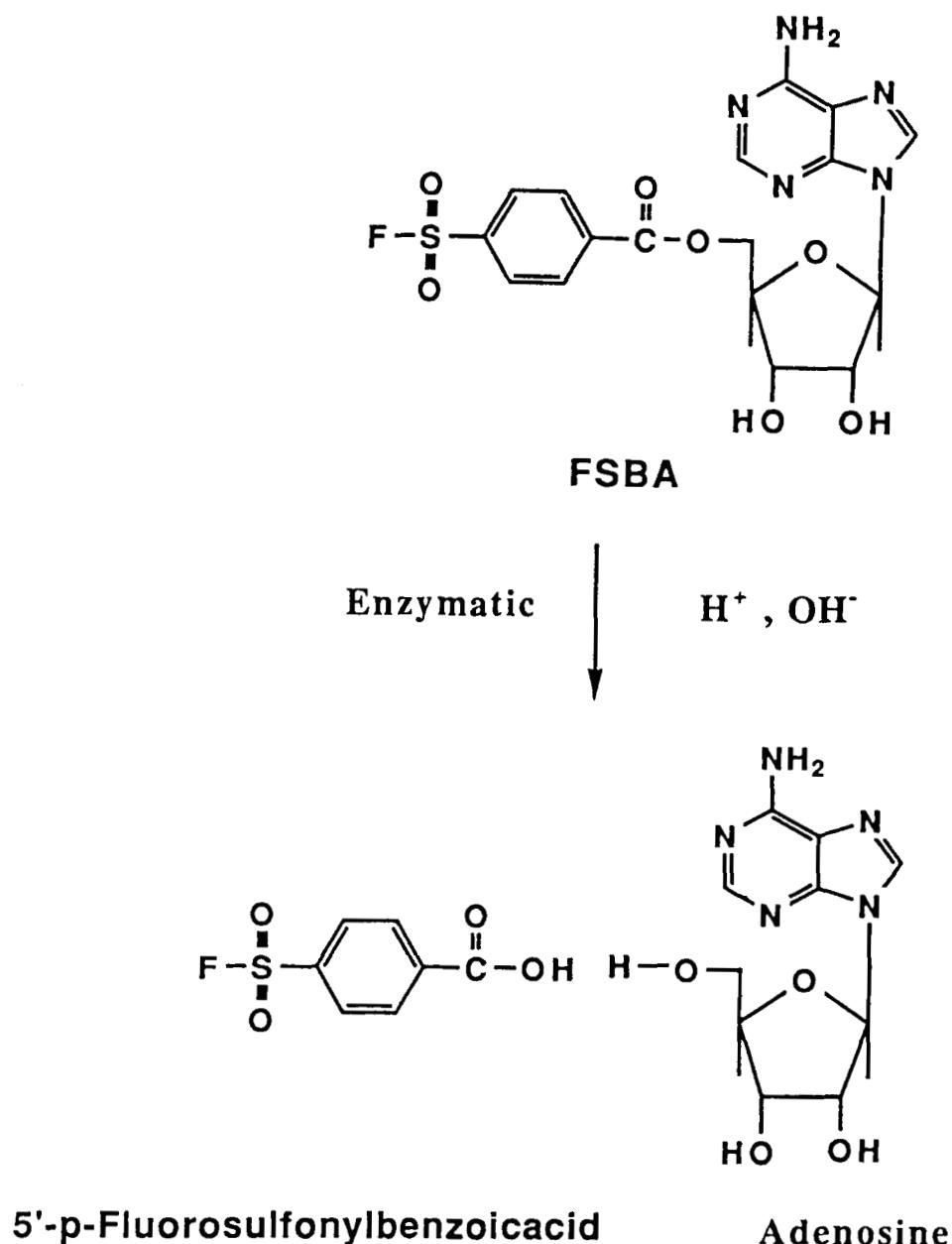


FIGURE 6. Schematic representation of the cleavage of the ester bond at the 5'-end in FSBA. Cleavage of the ester bond at 5'-end in FSBA by acids, bases, and ectonucleotidases generates adenosine and 5'-p-fluorosulfonylbenzoic acid. Adenosine is an antiplatelet agent.

compounds can be synthesized as ³²P-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.

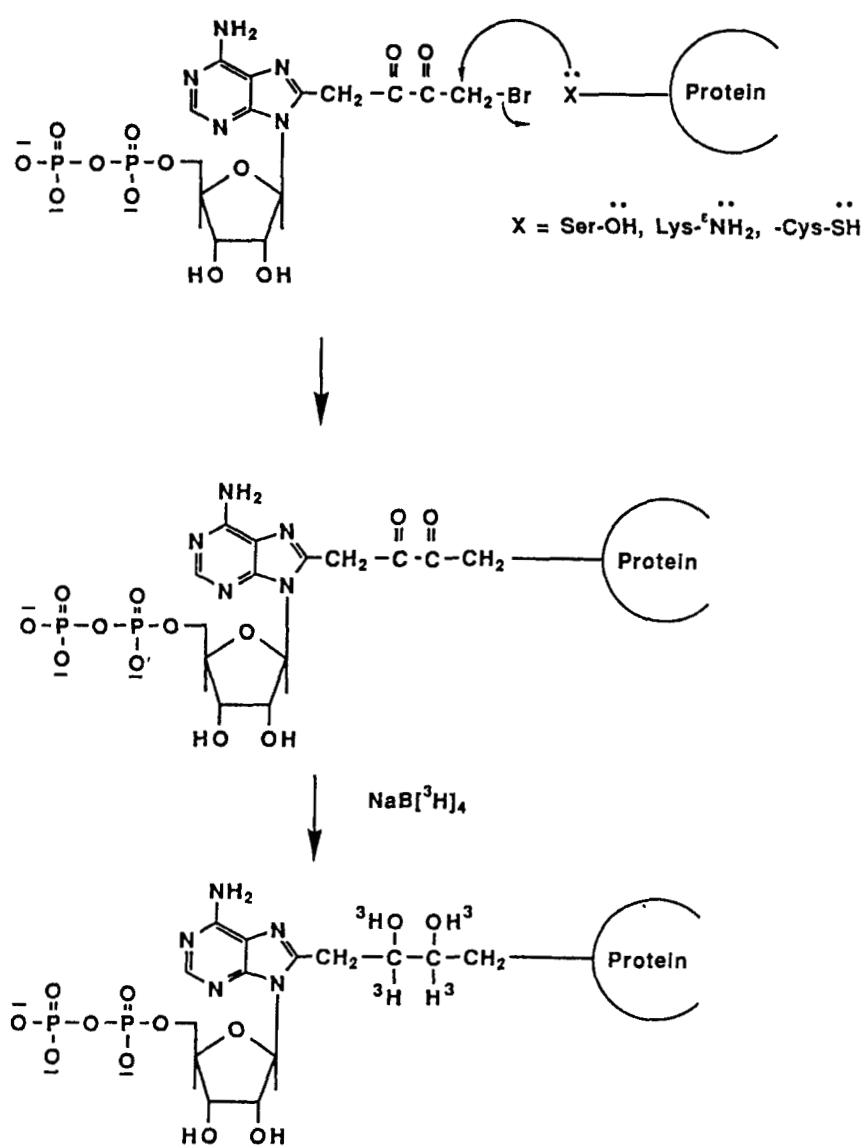
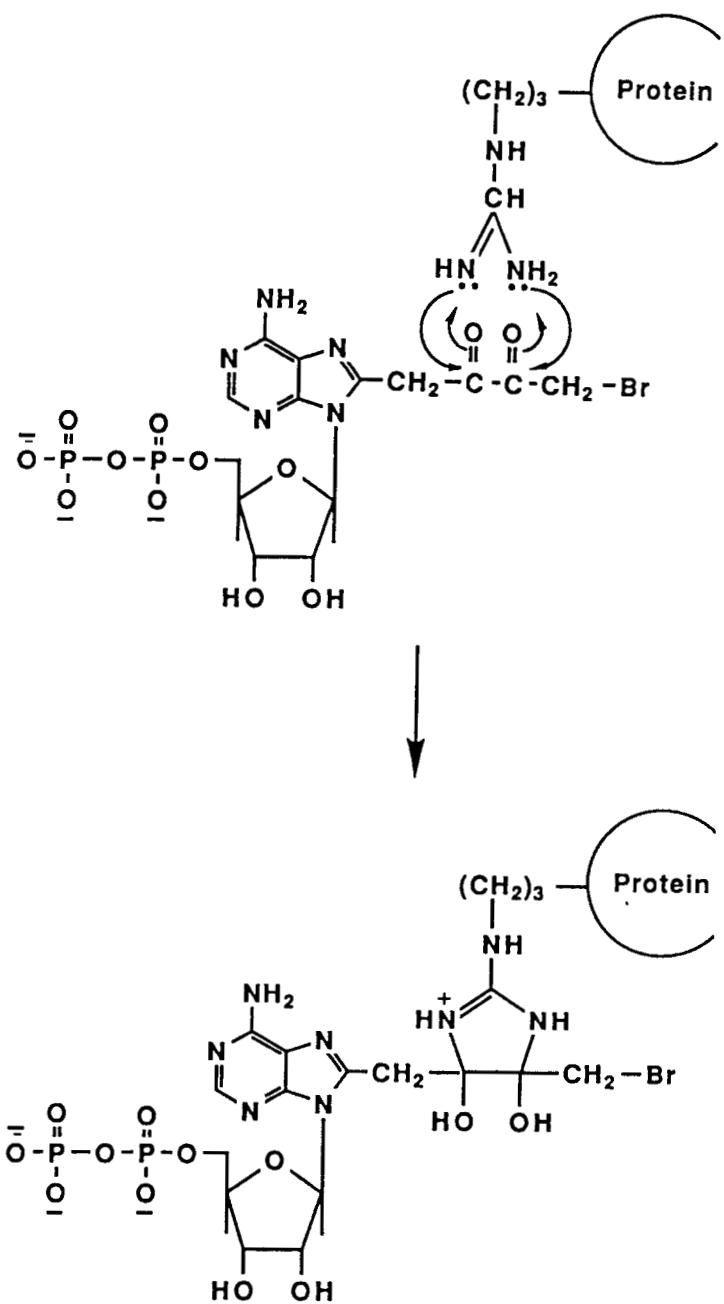


FIGURE 7. Schematic representation of the possible modes of adduct formation between 8-BDB-TADP and proteins.

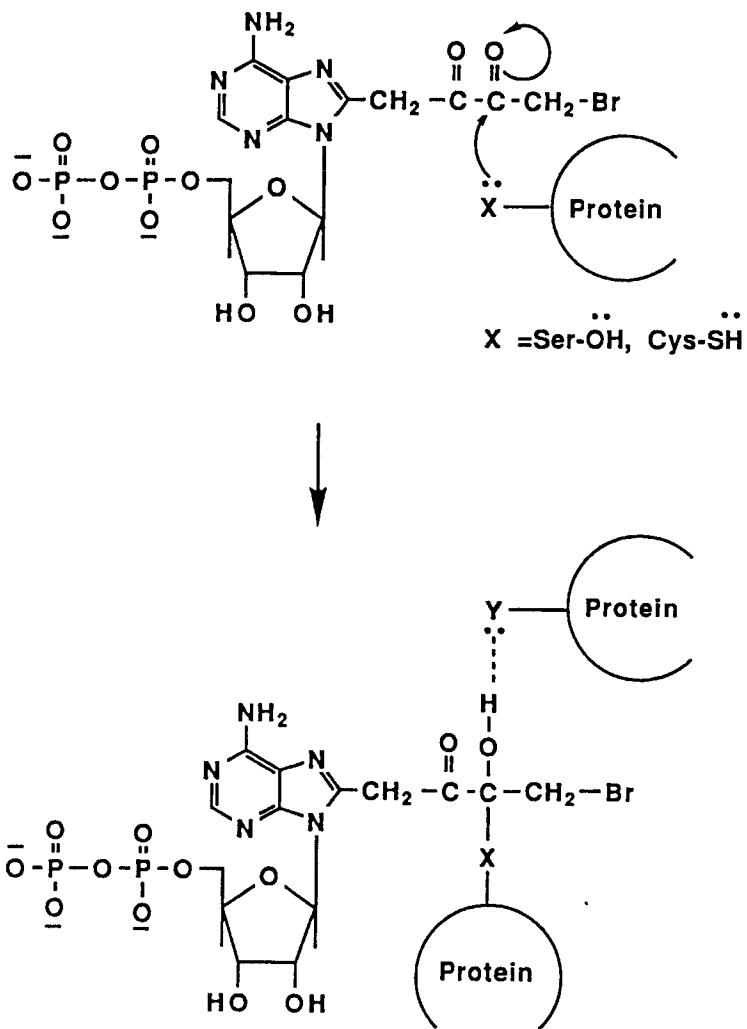
Scheme 1. Covalent adduct formation by nucleophilic displacement at alpha carbon atom in 8-BDB-TADP. Nucleophilic attack by the lone pair of electrons in reactive amino acids present in the functional domain(s) of proteins on the carbon atom alpha to the diketone function in 8-BDB-TADP, is accompanied 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 $\text{NaBH}^{[3]\text{H}}_4$ (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 $\text{NaBH}^{[3]\text{H}}_4$ 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 $[^{32}\text{P}]$ -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 $\text{NaB}^{[3]\text{H}}_4$. Thus, the ability to detect formation of a radiolabeled protein adduct following treatment with $\text{NaB}^{[3]\text{H}}_4$ provides a way to distinguish between arginine and other residues being modified by the affinity analogs containing the BDB-T group.



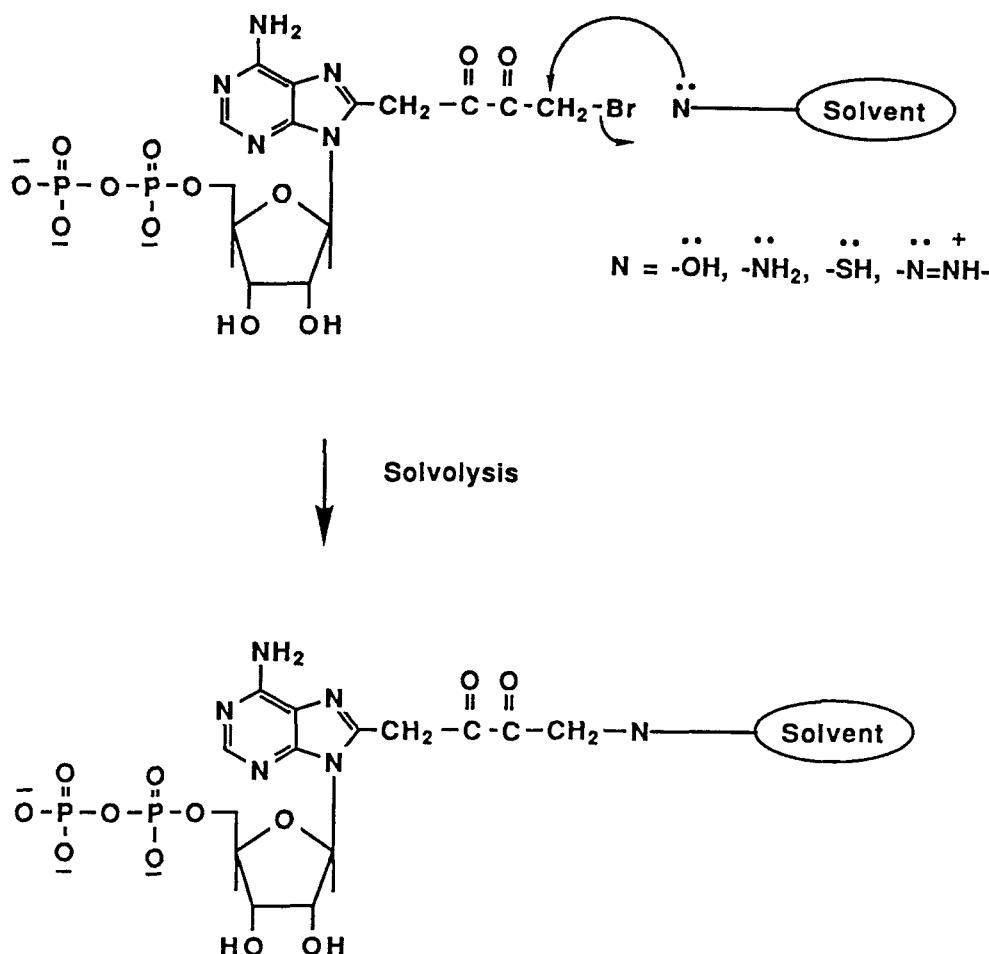
Scheme 3

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 NaBH_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 electrophilic 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 ($-\text{OH}$, $-\text{SH}$, $-\epsilon_{\text{NH}_2}$) 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 NaBH_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 adduct¹⁶⁵ 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 kinase¹⁶⁶ and glutamate dehydrogenase.¹⁶⁷ 8-BDB-TADP did not induce platelet shape change and aggregation or mobilization of [Ca²⁺]_i.¹¹² 8-BDB-TADP inhibited ADP-induced platelet shape change, aggregation, exposure of fibrinogen binding sites, secretion, mobilization of [Ca²⁺]_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 -³²P]-ADP or 8-BDB-TADP and NaB[³H]₄ showed the presence of a single radiolabeled protein band at 100 kDa (Fig-

ure 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 aggrecin, 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 –NH₂ 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 C₂ 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 [Ca²⁺]_i.¹¹⁴ When platelets were incubated with 2-BDB-TADP for longer time periods, ADP-induced platelet responses, including ADP-induced inhibition of stimulated adenylyl 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 Ca²⁺ 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

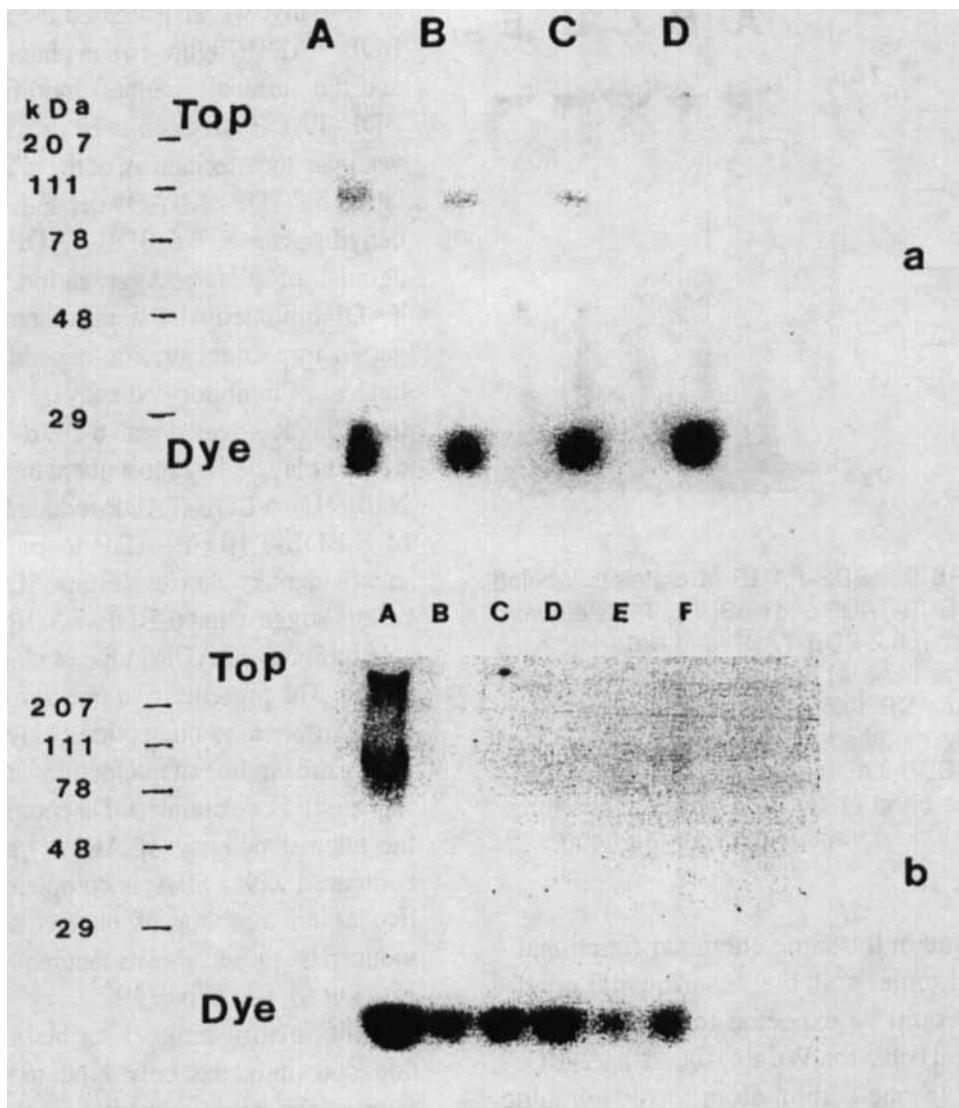


FIGURE 8. SDS-PAGE of platelets labeled with [β - ^{32}P]-8-BDB-TADP. Panel a) autoradiogram of platelets labeled with [β - ^{32}P]-8-BDB-TADP in the absence (lanes A through C) and in the presence of ADP (lane D).¹¹² Panel b) platelets were labeled with [β - ^{32}P]-8-BDB-TADP in the absence (lane A) 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

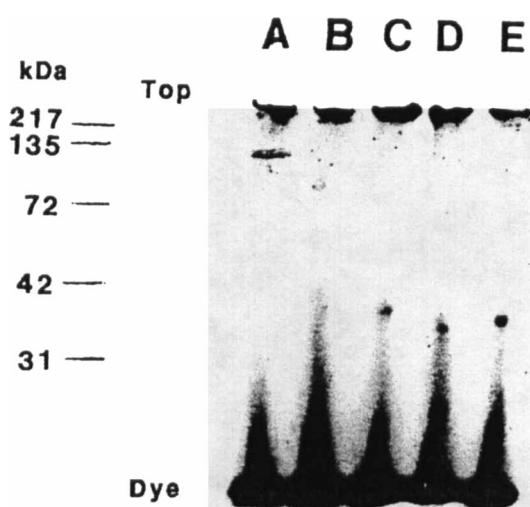


FIGURE 9. SDS-PAGE of platelets labeled with 2-BDB-TADP and $\text{NaB}^{[3]\text{H}}_4$. Platelets were labeled with 2-BDB-TADP and $\text{NaB}^{[3]\text{H}}_4$ in the absence (lane A) and presence of ATP (lane D) and ADP (lane E).¹¹⁴ Platelets were covalently modified with FSBA before labeling with 2-BDB-TADP and $\text{NaB}^{[3]\text{H}}_4$ (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-TADP¹²⁸ and equivalent to ADP. 2-BOP-TADP (Figure 1d) and $\text{NaB}^{[3]\text{H}}_4$ 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 $\text{NaB}^{[3]\text{H}}_4$. 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 N⁶ 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 C₂, C₈, and N⁶ 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 C₂ 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 GPIIb¹³⁴ and GPIIIa.¹⁷²

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

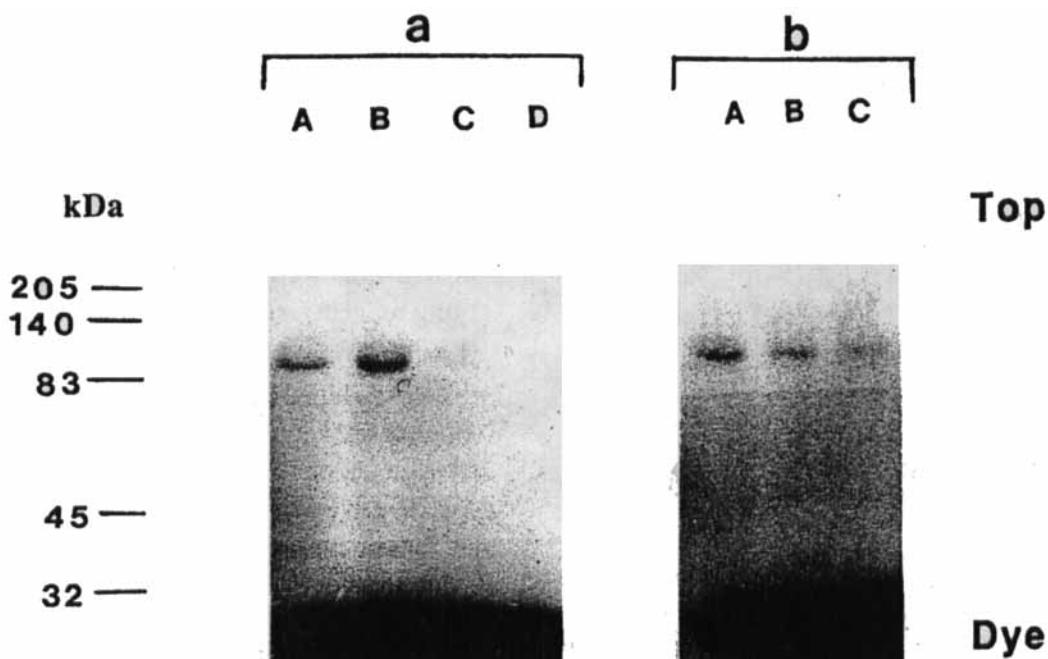


FIGURE 10. SDS-PAGE of platelets labeled with $[\beta\text{-}^{32}\text{P}]\text{-8-BDB-TADP}$ in the absence and presence of 2-BDB-TADP and 6-BDB-TADP. Panel a) platelets were labeled with $[\beta\text{-}^{32}\text{P}]\text{-8-BDB-TADP}$ in the absence (lanes A and B) and presence (covalently modified) of 2-BDB-TADP (lanes C and D).¹¹⁴ Panel b) platelets were labeled with $[\beta\text{-}^{32}\text{P}]\text{-8-BDB-TADP}$ 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, *ro*ADP (Figure 3c) and *ro*ATP were found to induce platelet shape change but not aggregation and mobilization of $[\text{Ca}^{2+}]_i$.^{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 Wrzeszczynski and Colman demonstrated that adenosine 5'-O[S-(4-bromo-2,3-dioxobutyl)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.

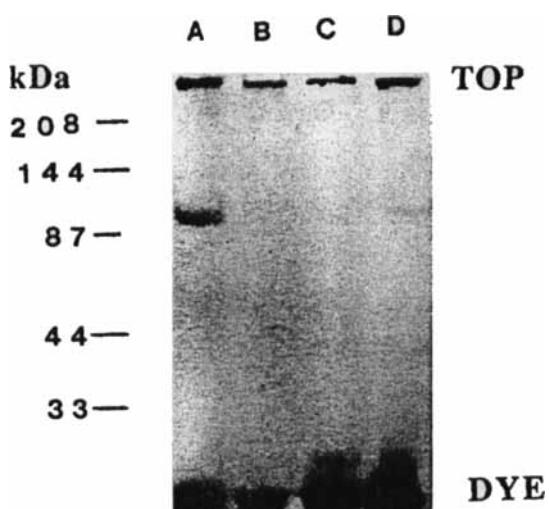


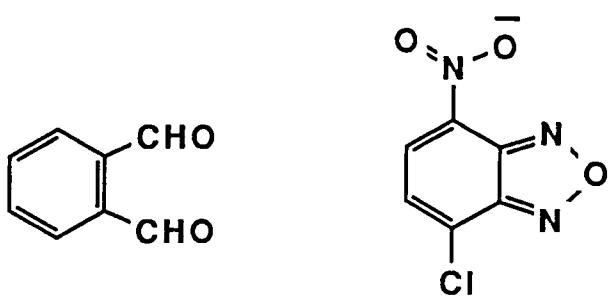
FIGURE 11. SDS-PAGE of platelets labeled with 2-BOP-TADP and $\text{NaB}^{[3]\text{H}}_4$. Platelets were labeled with 2-BDB-TADP and $\text{NaB}^{[3]\text{H}}_4$ in the absence (lane A) and presence of ADP (lane B) and ATP (lane C).¹²⁸ Platelets were covalently modified with FSBA before labeling with 2-BOP-TADP and $\text{NaB}^{[3]\text{H}}_4$ (lane D). The band at 100 kDa corresponds to radiolabeled aggrecin 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-iodoacetamido-salicylic acid.¹⁷⁵ Another reagent OPTH (Figure 12a) that covalently forms an isoindole adduct with closely situated lysine and cys-

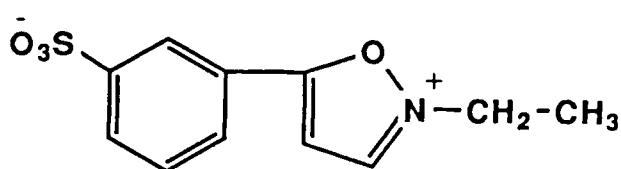
teine residues has been used to investigate the catalytic subunit of cAMP-dependent protein kinase¹⁷⁶ and catalytic and regulatory sites of cGMP-dependent protein kinase.¹⁷⁷ Because ADP-induced platelet aggregation is inhibited by sulphydryl group-modifying reagents, such as *N*-ethylmaleimide (NEM)^{178,179} 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 $[^3\text{H}]\text{-FSBA}$. Taken together the results suggest that covalent modification of essential cysteine and lysine residues by OPTH at or near the ADP-binding domain in aggrecin 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 kinase¹⁸² 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 $[^{14}\text{C}]\text{-NBD-Cl}$ 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 aggrecin in platelets exposed to 8-BDB-T-[β - ^{32}P]-ADP.¹⁸⁵ Taken together, the results show that covalent modification of aggrecin 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⁺-Mg²⁺-ATPase¹⁸⁶ 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 sulfhydryl 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 sulphydryl group-modifying reagents, such as pCMBS, NBD-Cl, and OPTH. Platelets covalently modified by WR-K were not radiolabeled with 8-BDB-T-[β-³²P]-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 [³H]-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[³H]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 affin-

ity-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

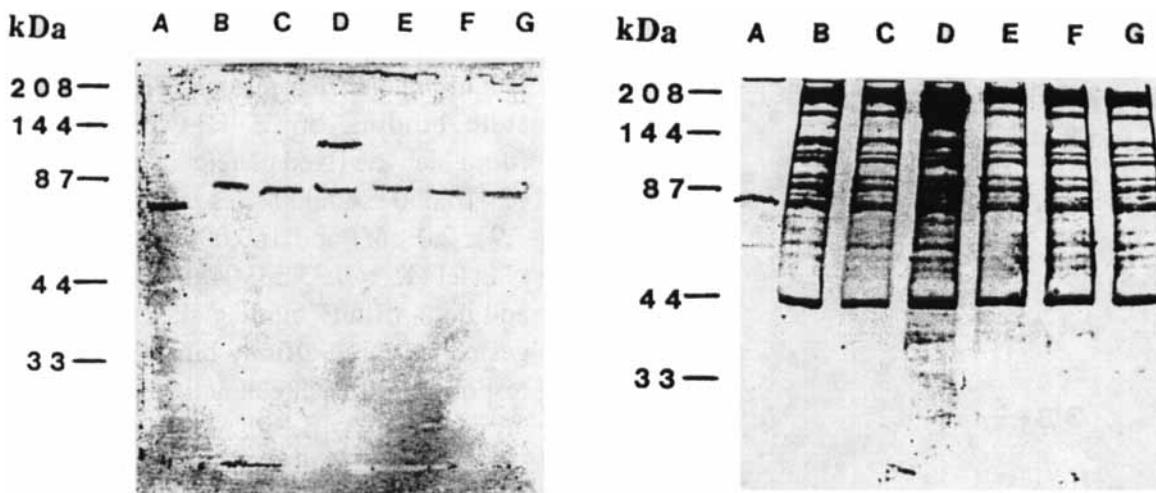


FIGURE 13. Immunoblot of platelets labeled with FSBA in the absence and presence of nucleotides. Immunoblots were prepared by SDS-PAGE of platelets, transferring the proteins from the gel onto a polyvinylidenefluoride membrane and developing the membrane with a polyclonal antibody to FSBA-labeled glutamate 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 [³H]-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 $6.5 \mu M$.¹⁹² Scatchard

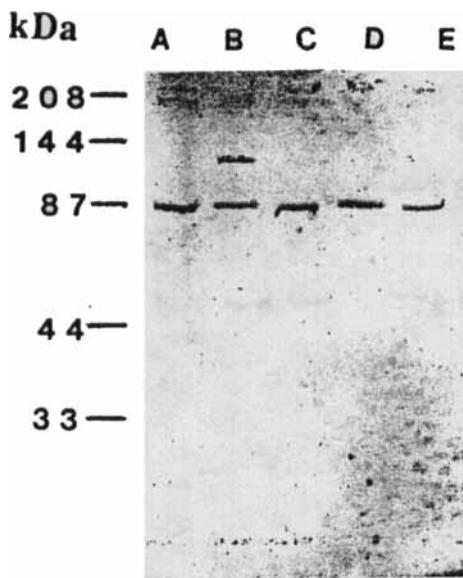


FIGURE 14. Immunoblot of platelets labeled with FSBA in the presence of chemically reactive ADP-affinity analogs. Immunoblots were prepared as described in the legend to Figure 13. The lanes correspond to the following: platelets (lane A); FSBA-modified platelets (lane B); platelets covalently modified 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 aggrecin, 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, aggrecin (100 kDa).

analysis of the binding parameters for [³H]-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 -³²P]-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 -³²P]-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 [2-³H]-ADP to paraformaldehyde fixed platelets yielded values of $410,000 \pm 40,000$ sites per platelet ($K_d = 7.9 \pm 2.0 \mu M$) and $160,000 \pm 20,000$ sites/platelet ($K_d = 0.35 \pm 0.04 \mu M$) 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 -³²P]-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 [¹⁴C]-ADP yielded values of 46,000 sites per platelet ($K_d = 3.1 \mu M$) and 50,000 sites per platelet ($K_d = 3.5 \mu M$) for the patients; a median number for normal volunteers was found to be 65,000 sites per platelet ($K_d = 3.1 \mu M$).⁴³ Binding studies using [2-³H]-ADP and paraformaldehyde-fixed platelets from one patient in the same investigation yielded values of 32,000 sites per platelet ($K_d = 0.14 \mu M$) for the high-affinity binding sites and 77,000 sites per platelet ($K_d = 2.2 \mu M$) for the low-affinity binding sites; a median value of $55,800 \pm 14,900$ sites per platelet ($K_d = 0.5 \pm 0.25$) for the high-affinity binding sites and $546,000 \pm 86,000$ sites per platelet ($K_d = 12.5 \pm 3.4$) 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-[³H]-ADP yielded values of 30 ± 17 sites per platelet ($K_d = 2.07 \pm 0.25$ nM) and 836 ± 126 sites per platelet ($K_d = 2.16 \pm 0.79$ nM) for the patient and controls, respectively.⁴⁴

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

mal healthy volunteers and normal rats yielded values of 600 sites per platelet ($K_d = 5.4 \pm 2.1 \text{ nM}$) and 1200 ± 55 sites per platelet ($K_d = 4.0 \pm 0.5 \text{ nM}$), respectively.¹⁹³ In rat platelets only, binding of 2-MeS-[³H]-ADP was found to be time-dependent and saturable; Scatchard analysis of the data yielded a value of 940 ± 30 sites per platelet ($K_d = 0.78 \pm 0.05 \text{ nM}$) 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, $K_d = 0.9 \text{ nM}$) a single class of ADP binding sites when compared with control rats (960 sites per platelet, $K_d = 0.77 \text{ nM}$). 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 $[\text{Ca}^{2+}]_i$. At higher concentration (300 μM), FSBA induced platelet shape change and concentration-dependent increase in $[\text{Ca}^{2+}]_i$.¹⁶⁰ It was suggested that FSBA-induced platelet shape change was mediated by aggrecin 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 [³H]-FSBA into aggrecin 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 -³²P]-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 demonstrated¹⁰¹ and binding characteristics of ADP to promegakaryoblasts (RPM), human erythroleukemia (HEL) cells, U937 and K562 cells have been investigated.¹⁰¹ Scatchard analysis of the saturation binding isotherms using [³H]-ADP gave values of $399,000 \pm 170,000$ sites per cell ($K_d = 775 \pm 230 \mu\text{M}$) for RPM, $81,900 \pm 32,500$ sites per cell ($K_d = 21.5 \pm 8.4 \mu\text{M}$) for HEL cells, $53,900 \pm 28,000$ sites per cell ($K_d = 13.7 \pm 3.9 \mu\text{M}$) and $11,500 \pm 2300$ sites per cell ($K_d = 22.0 \pm 5.3 \mu\text{M}$) 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 non-hydrolyzable 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 Ca²⁺, [γ -³²P]-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 Ca²⁺.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, aggrecan (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 nucle-

otides 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 [Ca²⁺]_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 Ca²⁺ 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 aggrecan 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-N₃-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 -³²P]-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 -³²P]-ADP showed the presence of an intense band of radiolabeled protein at 100 kDa.¹²⁶ The avail-

able 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 $[\text{Ca}^{2+}]_i$ from a 80 to 100 nM 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]-IP₃ from human blood platelets has been reported.²⁰⁷ The increase in the radiolabeled IP₃ was only twofold over a basal value of 150 nM and was unaffected by the presence or absence of external Ca^{2+} .²⁰⁷ One possibility is that ADP stimulates interconversion of inositol (IP, IP₂, IP₃) instead of PLC activation that leads to the formation of IP₃.²⁰⁸

Continuous-flow measurements using electroporated platelets loaded with [^3H]-inositol showed that shear forces acting alone caused a rapid burst in the synthesis of IP₃ before 0.5 s.²⁰⁹ Thrombin and ADP caused an initial (130 to 200 ms) burst of IP₃ and IP₄ (inositol-1,3,4,5-tetrakisphosphate), but IP₃ was liberated first.²⁰⁹ Other important findings of this investigation are as follows: (1) formation of IP₃ and IP₄ 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 $[\text{Ca}^{2+}]_i$.²¹⁰ Although shear forces in combination with thrombin or ADP cause intracellular rise in Ca^{2+} , both stimuli acting alone cause formation of IP₃ and IP₄.²⁰⁹ 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 IP₃ and IP₄ formation and rise in intracellular levels of Ca^{2+} either remain uncertain or unestablished. It is possible that

ADP-mediated initial burst of IP₃ 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 IP₃ 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. Caffeine (which operates through ryanodine 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 $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_i$ is released by IP₃ 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 $[\text{Ca}^{2+}]_i$, DAG, and IP₃ 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} Thapsigargin (Tg) and 2,5-di(*t*-butyl)-1,4-benzo-hydroquinone (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 Gardner²²⁶ and Irvine²²⁷ 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 $\text{Na}^+/\text{Ca}^{2+}$ exchange mechanism, implying the presence of IP_3 -receptors ($\text{IP}_3\text{-R}$) in plasma membrane.²²⁸ $\text{IP}_3\text{-R}$'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 $\text{IP}_3\text{-R}$'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 open-

ing 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 platelets²³³ 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 $^{22}\text{Na}^+$ but not $^{36}\text{Cl}^-$, 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 IP₃). 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 IP₃ 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 A₂ (PLA₂).²⁴³ Thus, it has been suggested that ADP-induced activation of PLC is secondary to the activation of PLA₂.^{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 PLA₂ possesses an alkaline pH (a consequence of Na⁺/H⁺ antiport) optimum for its enzymatic activity.^{244,245} ADP-induced mobilization of [Ca²⁺]_i was inhibited in indomethacin-treated platelets that had been preincubated with Na⁺/H⁺ exchanger inhibitor ethylisopropylamiloride: alkalinization by NH₄Cl overcame this inhibition.²⁴⁶ The results suggest that platelet activation induced by ADP and TXA₂ involve Na⁺/H⁺ exchange as a common step and enhance Ca²⁺ mobilization independently of PLA₂ activity.²⁴⁶ Stopped-flow fluorimetry showed that initial kinetics of ADP-induced rise in [Na⁺]_i and [Ca²⁺]_i in human platelets loaded with sodium binding benzofuran isophthalate were similar.²⁴⁷ Patch-clamp recordings in the absence and presence of Ca²⁺ showed the presence of a single channel. SK&F 96365, a blocker of receptor-mediated Ca²⁺ 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 Ca²⁺ mobilization without activating PLA₂.²⁴⁶

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 lipoxygenase 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-Cl¹⁸⁴ 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 carbamoylalpiperidinoalkane 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} Perturbation 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

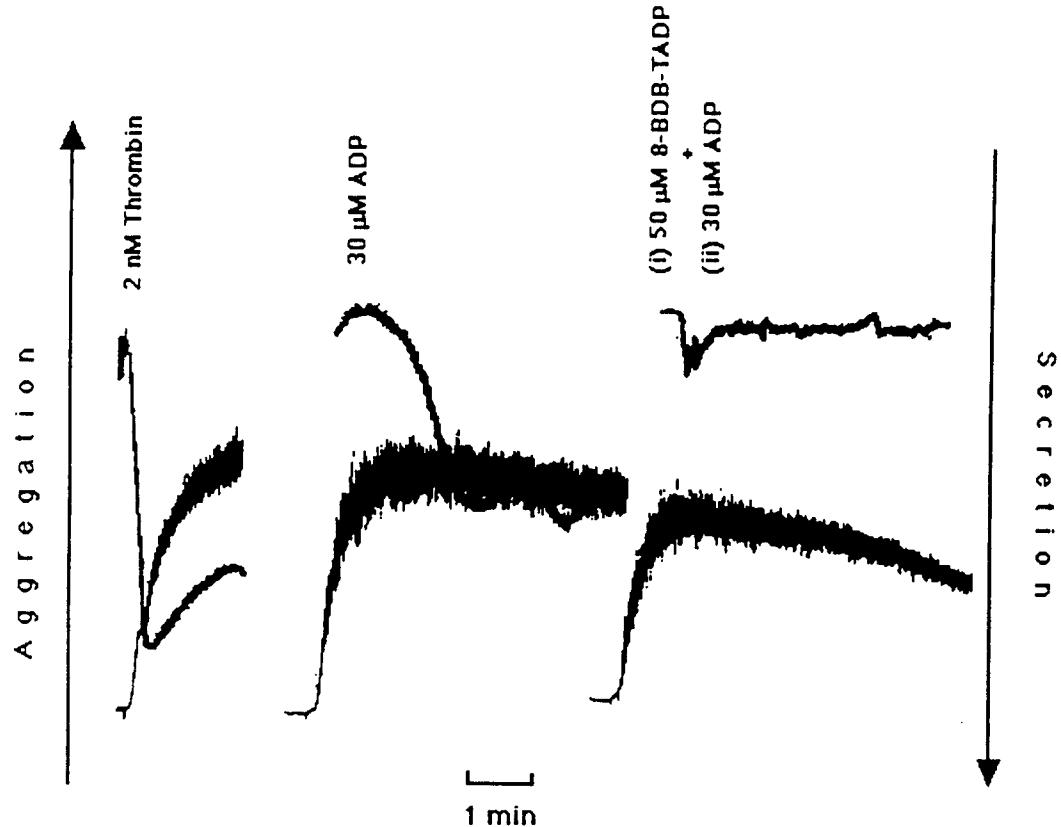


FIGURE 15. Effect of 8-BDB-TADP on ADP-induced platelet secretion. ATP release following exposure of platelets to thrombin and ADP was measured by the commercial luciferase-luciferin reagent.¹¹² Platelets were either challenged with thrombin or ADP and the agonist-induced platelet secretion of nucleotides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

lipases to liberate phosphatidic acid (PA) and AA²⁶² or can be phosphorylated to PA, which is a substrate for PLA₂ 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 PLA₂, (3) release and conversion of AA to TXA₂, (4) activation of PLC by a feedback mechanism involving TXA₂, (5) PLC-mediated formation of IP₃ 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 PLA₂ activation. It has also been demonstrated that activation of PKC by oleoylacylglycerol, a synthetic analog of DAG, facilitates labilization and fusion

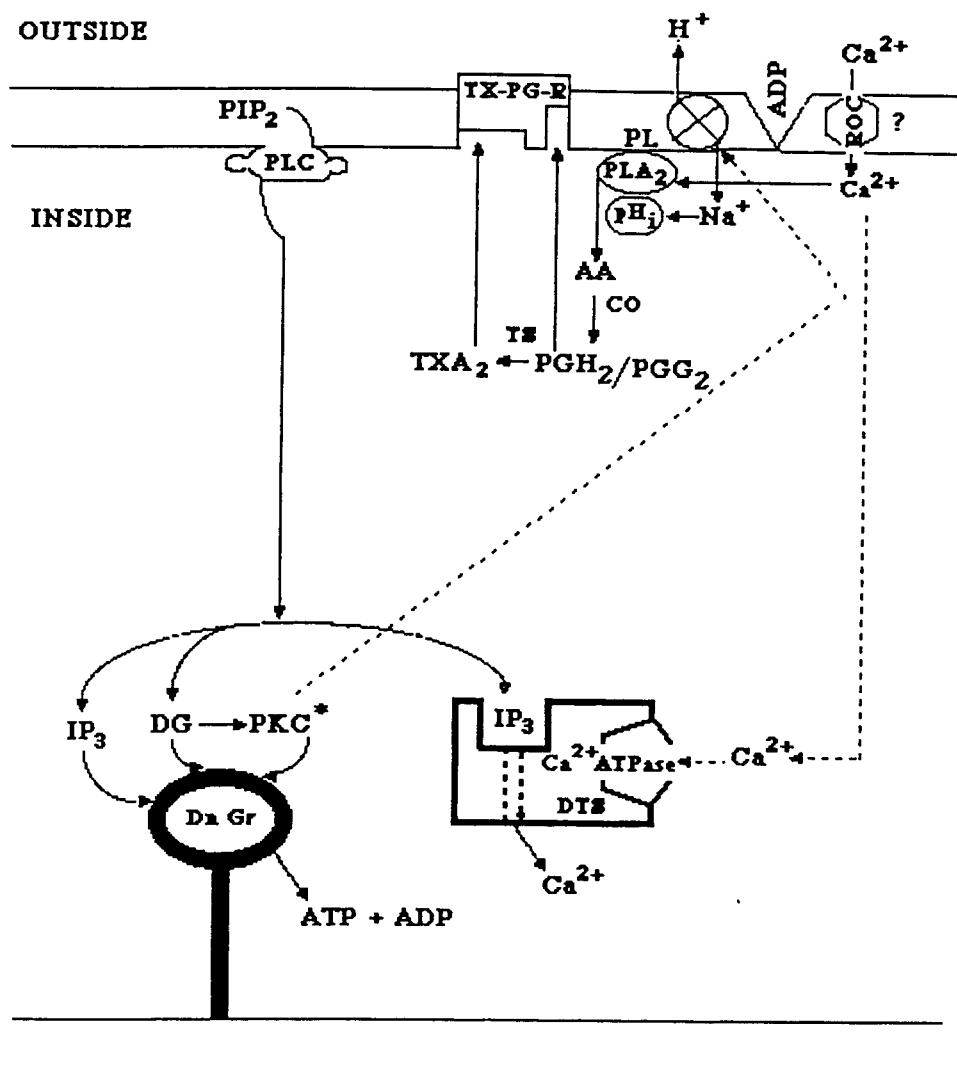


FIGURE 16. Schematic representation of ADP-induced platelet activation. Binding of ADP to its receptor on platelet surface activates Na⁺/H⁺ exchanger (mechanism?) leading to a cytosolic pH gradient (pHi) gradient favorable for the activation of phospholipase A₂ (PLA₂). The activation of PLA₂ is also aided by the ADP-induced influx of Ca²⁺ by a receptor operated channel (ROC) or by some other unknown mechanism. The activated PLA₂ converts phospholipids (PL) such as phosphatidylcholine, to arachidonic acid (AA), which is cyclized to prostaglandin endoperoxides PGG₂/PGH₂ by cyclooxygenase (CO) followed by their conversion to thromboxaneA₂ (TXA₂) catalyzed by thromboxane synthetase (TS). In a feedback mechanism receptor (TX-PG-R)-mediated activation of phospholipase C (PLC) by the prostaglandin endoperoxides PGG₂/PGH₂ and TXA₂ (which diffuse out of the membrane) leads to the formation of phospholipase C hydrolysis products inositol-1,4,5-triphosphate (IP₃) and diacylglycerol (DG) from phosphoinositol-4,5-bisphosphate (PIP₂). Protein kinase C is activated (PKC*) by DG, its physiological activator. IP₃ and DG, and PKC* then mediated release of nucleotides (ATP+ADP) from the dense granules (Dn Gr). IP₃ also releases Ca²⁺ by binding to its receptors through receptor-mediated channel in dense tubular system (DTS). Depleted stores in DTS can be replenished with Ca²⁺ by Na⁺, H⁺/Ca²⁺ATPase pump. Activation of protein kinase C (PKC*) by DG may also provide a mechanism to sustain PLA₂ 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 heterotrimeric 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 suppress cAMP formation and are regulated by the binding of pertussis toxin. There are three different kinds of $G\alpha$ 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 [³²S]-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 [³²S]-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 -³²P]-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 G_q and inhibit adenyl 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 Ticlopidine⁴⁰ 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-[³²P]-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 $\beta\gamma$ -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 $\beta\gamma$ -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 $G\alpha$ and $G\beta\gamma$ may come from two different G-proteins.²⁷³

XIV. CLONING

Recently, cloning of two human P2Y₁ Receptors has been reported.^{280,281} The second P2Y₁ receptor²⁸¹ differs from the first²⁸⁰ by only one serine residue. The second P2Y₁ 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 P2Y₁ receptor mRNA was reported in human platelets and several cell lines of megakaryocytic origin.²⁸¹ Rank order of potency for Ca²⁺ movements in Jurkat cells transfected with the P2Y₁ 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 Ca²⁺ 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 Ca²⁺ response. It should be noted that agonist-induced Ca²⁺ 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 P2Y₁ receptor.²⁸¹ No data concerning responses other than the Ca²⁺ movement in Jurkat cells transfected with the human P2Y₁ 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 P2Y₁ receptors. The human P2Y₁ 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 P2Y₁ 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 phosphoinoside hydrolysis had no effect on the agonist potencies. Fur-

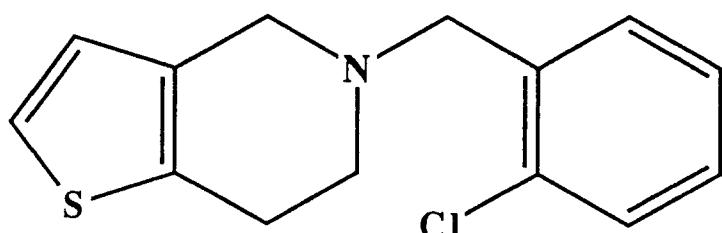
thermore, 2-MeS-ATP neither induced nor inhibited cAMP accumulation in 1321N1 cells expressing the human P2Y₁ receptor.²⁸⁴ In order to confirm that their P2Y₁ receptor was not coupled to adenylyl cyclase, it was ascertained that 2-MeS-ATP-induced inhibition of cAMP accumulation was essentially indistinguishable between the wild-type C₆ cells (that are coupled to adenylyl cyclase) and C₆ cells expressing the human P2Y₁ receptor.²⁸⁴ These results suggest that the human P2Y₁ receptor cloned by Leon et al.²⁸² is less likely to be a candidate for the platelet P2T receptor.

XV. PURINORECEPTORS AND DRUGS

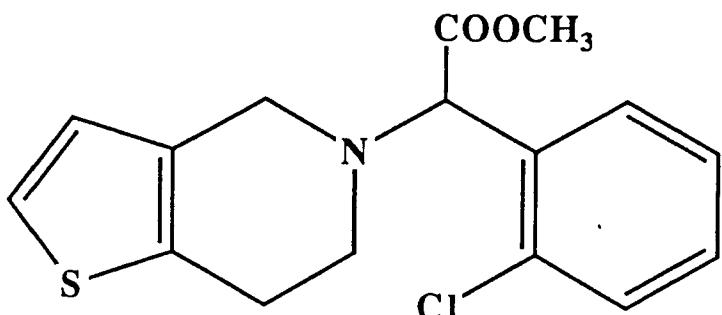
Exposure of conformationally competent fibrinogen-binding sites, the GPIIb-IIIa complex, by ADP (a weak agonist) and thrombin (a strong agonist), and all the other agonists with varying degree of potency, constitutes a central mechanism involved in platelet aggregation. Pharmaceutical industries have 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

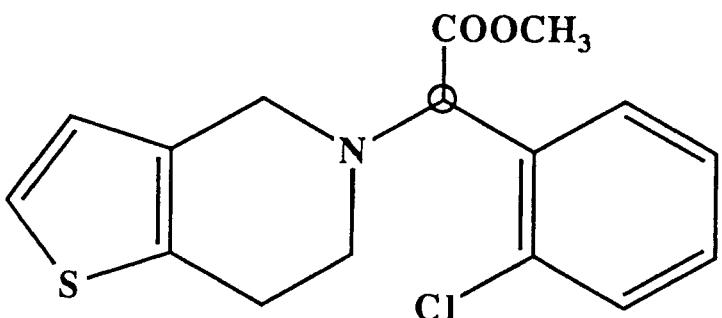
from PCR 4099 racemate. Collectively, these compounds are referred to thienopyridines in literature and have been reviewed extensively.^{38,40,41,285-290} Ticlopidine, PCR 4099, and Clopidogrel are specific inhibitors of the primary and secondary waves of ADP-induced



(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 (O). 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 hydrophobic 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

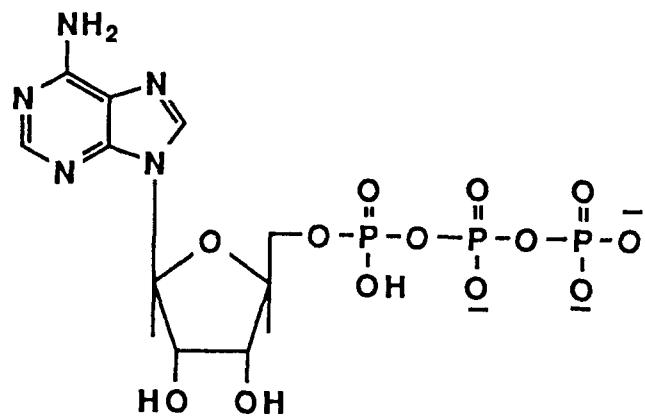
ADP, ATP, and serotonin). Platelet aggregation by low concentration of thrombin is probably ADP-dependent because it is shifted to higher thrombin concentration in the presence of CP/CPK. In platelets obtained from Sprague-Dawley rats administered PCR 4099, platelet aggregation required higher concentrations 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-

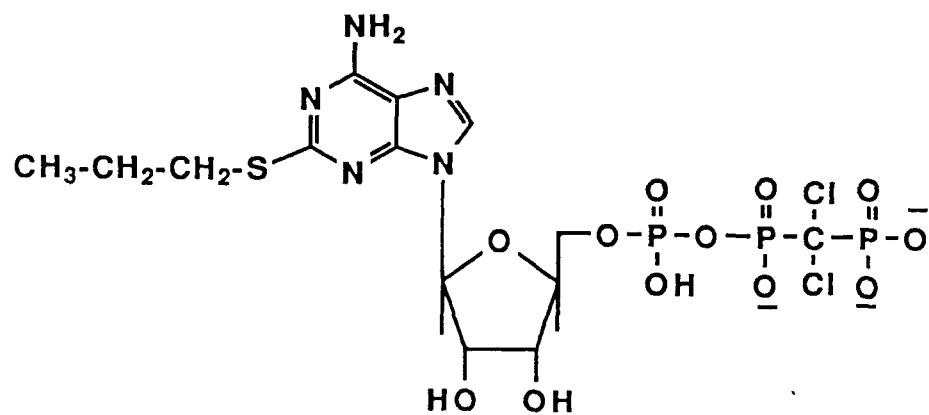
longed by ticlopidine in healthy volunteers was shortened by desmopressin.²⁹² Potentiation by desmopressin of shear-induced platelet aggregation may be one of the mechanisms by which desmopressin shortens the prolonged bleeding time. The results suggest that ticlopidine is a useful drug in minimizing 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 aggrega-

tion.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

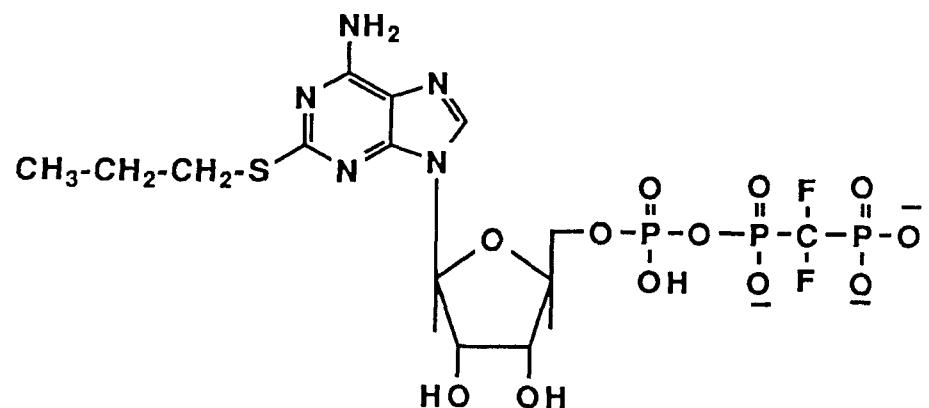
The development of nucleotide based antagonists at P2T receptor has been long overdue. ATP (Figure 18a), a natural antagonist at P2T receptor, cannot be used in clinical settings for two reasons: (1) it is active as an agonist at other P2-purinergic receptors, and (2) it is metabolized by ectonucleotidases. 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 C₂ increases their potency



(a) ATP



(b) FPL 67085



(c) FPL 66096

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, A₁-, A₂-, and A_{2b}- receptors for adenosines; α_1 -, α_2 - and β_2 -adrenergic receptors for catecholamines; 5-HT₂-receptor for serotonin; AT₁-receptor for angiotensin II and EP₂-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

Ap₄A (Figure 19a), a component of platelet dense granules,¹⁰⁴ is a competitive

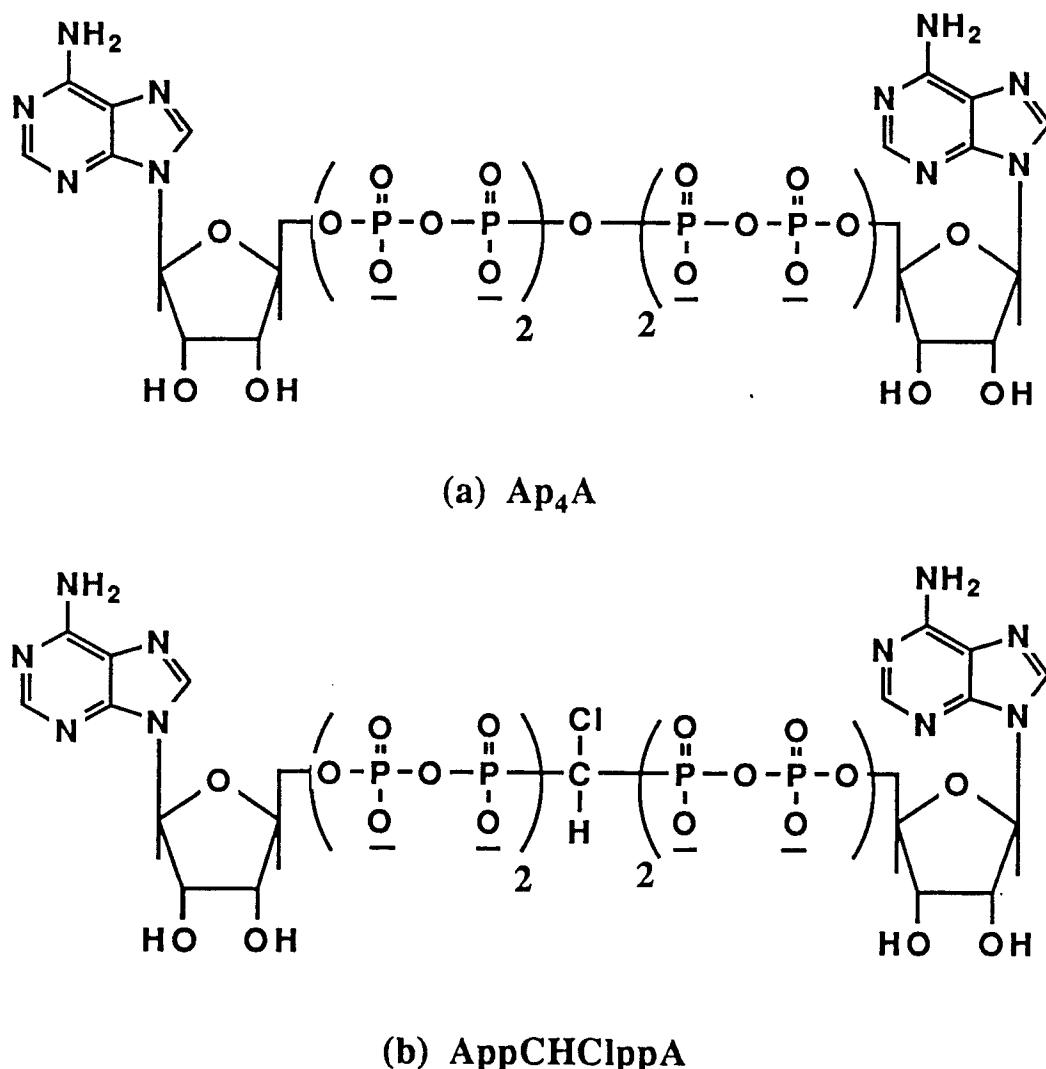


FIGURE 19. Chemical representation of diadenosine polyphosphates. (a) Ap₄A (diadenosine 5',5''-P₁,P₄-tetraphosphate or diadenosine tetraphosphate) and (b) AppCHClppA (β,β' -monochloromethylenediamenosine 5',5''-P₁,P₄-tetraphosphate).⁴⁷

inhibitor of ADP-induced platelet aggregation^{107,108,302} and has been shown to have antithrombotic effects in rabbit carotid artery model. However, Ap₄A has a short half-life time in blood because it is degraded by phosphodiesterases.³⁰³ These observations were helpful in designing β,β'-monochloromethyl-enediadenosine 5',5'''-P¹-P⁴-tetraphosphate (AppCHClppA)⁴⁷ (Figure 19b), which is far more potent than Ap₄A³⁰² 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 septicemia.

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 acti-

vation.^{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 method¹⁹⁰ to show that ADP-induced platelet responses are mediated by the binding of ADP to aggrecan,^{157,158} 100-kDa surface membrane protein, a putative ADP receptor. Other investigators have claimed that a 43-kDa surface protein¹²⁶ might represent the adenylyl cyclase binding site on human blood platelets.

There are also disagreements concerning ADP-induced formation of PLC products IP₃ 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 PLA₂; the products of PLA₂, AA, is then converted by cyclooxygenase pathway to TXA₂, which in a feedback mechanism activates PLC.^{201–203} There are also disagreements concerning mechanisms of ADP-induced rise in [Ca²⁺]_i in platelets. Whether this occurs by ligand-gated Ca²⁺ 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 Ca²⁺ precedes mobilization of Ca²⁺ from intracellular stores.^{94,95,231} Recently, the presence of P2X₁ receptors on the platelet surface that mediate rapid phase of ADP- and ATP-evoked influx of Ca²⁺ 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. Gα_{i2} protein has been identified as the G-protein coupled to the ADP-receptor on the platelet surface.²⁷⁸ Gα_{i2} protein has been shown to couple α₂-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 67085³⁰⁰ 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 ba-

sis 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.

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