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Pharmacological Receptors on Blood Platelets*

SUSANNA M. O. HOURANI AND NOEL J. CUSACK

School of Biological Sciences, University of Surrey, Guildford, Surrey, United Kingdom, and Pharmacology Department, Whitby Research Inc., Richmond, Virginia

* This paper is dedicated to Professor G. V. R. Born, F.R.C.P., F.R.S., who introduced us to platelets, on the occasion of his 70th birthday.

I. Introduction

IN the 20 years since the last major review of this field in *Pharmacological Reviews* (Mustard and Packham, 1970), there have been great advances in the understanding of platelet function. In 1970 the potent, lipid-derived, mediators TXA_2,† PGI_2, and PAF had not yet been discovered, and the mechanism of action of aspirin was still unknown. Although calcium ions (Ca^{2+}) and the plasma protein fibrinogen were known to be essential for platelet aggregation, their exact role in causing activated platelets to stick together was not known. Much more is also now known about the types and nature of the receptors that control platelet function and about the intracellular second-messenger systems that mediate the effects of excitatory and inhibitory agonists. However, we are still a long way from understanding exactly how platelets work and disentangling all the complex positive and negative feedback mechanisms involved in their responses.

Platelets are popular models for the study of stimulus-response coupling mechanisms because (*a*) they can be obtained easily and rapidly from blood as a rather homogeneous, pure cell suspension; (*b*) they respond reliably and quantifiably to many agonists of pharmacological interest; (*c*) they lack the ability to divide or synthesise proteins that could complicate investigations; and (*d*) they are one of the few cell types that can be obtained readily from healthy human volunteers. In addition, their undoubted importance in thrombosis and haemostasis, as well as their possible roles in other conditions such as asthma and migraine, means that their responses, and the pharmacological control of these responses, are of more than merely academic interest. However, although platelets are relatively easy to work with practically, the interpretation of results is not always straightforward but is complicated by the release, during platelet activation, of substances that amplify the original stimulus and may change the nature of the observed response. In addition, platelets are extremely responsive cells and can be activated by simple and common procedures such as cooling, centrifugation, gel filtration, and contact with glass.

Although platelet function can be influenced by a wide variety of substances, as discussed in the earlier review (Mustard and Packham, 1970), in this article we will concentrate on the pharmacological receptors that are thought to exist on human platelets and on the substances acting as agonists or antagonists at these receptors. Agents that act on platelets, but whose effects are not receptor mediated (such as antigen-antibody complexes, lectins, and particulate matter) and platelet biochemistry that is not directly related to stimulus-response coupling, will not be considered, nor will the pathological aspects of platelets and platelet disorders. Several books concerning platelets have been published recently that include topics not covered in this review (Phillips and Shuman, 1986; MacIntyre and Gordon, 1987; Gordon, 1981; Longenecker, 1985a; Holmsen, 1987).

A. Structure and Function of Platelets

Resting platelets are disc shaped and are the smallest of the cells in the blood, being approximately 3 μm in diameter. Although they were first observed in the middle of the 19th century, platelets were not firmly accepted as cellular constituents of blood until this century, and

†Abbreviations: TXA_2, thromboxane A_2; PGI_2, prostacyclin; PAF, platelet-activating factor; 5-HT, 5-hydroxytryptamine; AMP, adenosine 5′-monophosphate; cyclic AMP, adenosine 3′,5′-cyclic monophosphate; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; GP, glycoprotein; K_1, affinity constant; K_D, dissociation constant; PG, prostaglandin; DAG, diacylglycerol; $I(1,4,5)P_3$, inositol (1,4,5)trisphosphate; G_s, stimulatory G protein; G_i, inhibitory G protein; cyclic GMP, guanosine 3′,5′-cyclic monophosphate; GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; FSBA, 5′-*p*-fluorosulphonylbenzoyladenosine; SP-ATP-α-S, SP diastereoisomer of adenosine 5′-*o*-[1-thiotriphosphate]; LSD, *d*-lysergic acid diethylamide; CNS, central nervous system; AVP, [8-arginine]-vasopressin; $d(CH_2)_5$[Tyr(Me)^{2}AVP, [1-(β-mercapto-β,β-cyclopentamethyleneproprionic acid), 2-(0-methyl)-tyrosine, 8-arginine]-vasopressin; dVDAVP, 1-deamino-[4-valine, 8-D-arginine]-vasopressin; $d(CH_2)_5$[D-Ileu^{2}Ala^4]AVP, [1-(β-mercapto-β,β-cyclopentamethyleneproprionic acid), 2-(D-isoleucine-4-alanine,8-arginine]vasopressin; 13-APA, 13-azaprostanoic acid; CTA_2, carboxylic TXA_2; PTA_2, pinane TXA_2; R-PIA, N^6-R-phenylisopropyladenosine; CPA, N^6-cyclopentyladenosine; CHA, N^6-cyclohexyladenosine; NECA, 5′-N-ethylcarboxamidoadenosine; CPCA, 5′-N-cyclopropylcarboxamidoadenosine; MECA, 5′-N-methylcarboxamidoadenosine; XAC, xanthine amine congener; GMP-PNP, guanosine 5′-β, γ-imidotriphosphate.

as recently as 1920 it was suggested that they were artifacts precipitated from plasma (Robb-Smith, 1967). Their small size means that little can be seen of their structure using a light microscope, but electron microscopy has revealed a well-defined and complex structure, consistent with their active role. For a comprehensive review of the cell biology of platelets see White and Gerrard (1980).

Platelets are formed in the bone marrow and are derived from megakaryocytes, probably by fragmentation of the cytoplasm, both by the pinching off of parts of megakaryocyte pseudopodia and by mechanical fragmentation of larger portions of megakaryocyte cytoplasm during passage through the microcirculation of the lung (Pennington, 1981).

The discoid shape of resting platelets is maintained by a ring of microtubules running around the edge of the disc immediately below the plasma membrane. Chilling platelets or treating them with drugs such as colchicine disrupts this microtubule ring and results in the platelets losing their discoid shape and becoming spherical (White and Krivit, 1967; White, 1968). This also occurs when platelets are activated by most stimulatory agonists.

The plasma membrane of platelets is rich in glycoproteins, some of which are thought to be components of the receptors present at the platelet surface, although the precise function of most of these glycoproteins is unknown. This plasma membrane is invaginated to form the surface-connected canalicular system, which greatly increases the surface area of the platelet and which is in close proximity to a network of dense tubules derived from the smooth endoplasmic reticulum of the megakaryocytes. This association of surface invagination with the dense tubular system is similar to the association in skeletal muscle between transverse tubules and the sarcoplasmic reticulum (White, 1972). Functional similarities also may exist because the dense tubular system, like the sarcoplasmic reticulum, is a site of storage of Ca^{2+} (Skaer et al., 1974), which may be released during platelet activation. Platelets also contain actin and myosin and can be regarded as contractile cells—indeed, it is possible to measure the force generated by stimulated platelets adherent to nylon mesh using classical organ bath techniques (Yamakado et al., 1983; Salganicoff et al., 1985; Salganicoff and Sevy, 1985).

Platelets contain few mitochondria with sparse cristae but contain many glycogen granules; metabolic energy is mainly derived from glycolysis rather than oxidative phosphorylation (Holmsen, 1977). There also are three types of storage granules, the contents of which can be released on platelet activation. The *dense granules* contain high concentrations of biogenic amines (mainly 5-HT in human platelets), adenine nucleotides (ADP and ATP), and Ca^{2+}, the release of which can affect vascular tone as well as the thrombus-forming ability of other platelets. The second type of releasable granule is the heterogeneous population of *α-granules*, which mainly contain proteins that can influence blood vessel function and the clotting cascade, such as platelet factor 4, β-thromboglobulin, and platelet-derived growth factor. These granules also contain fibrinogen, which is important for platelet aggregation as well as being the substrate for thrombin which converts it to fibrin, the physical basis of the mesh of a blood clot. Platelets also contain *lysosomal granules*, the contents of which also can be released but only after the platelets are stimulated with powerful aggregating agents such as thrombin or high concentrations of collagen. In addition to the contents of these three types of granules, activated platelets can release pharmacologically active substances that are newly synthesised rather than stored, such as prostaglandins, TXA_2 and PAF, which affect vascular tone and permeability and activate other platelets.

The major physiological function of platelets is in haemostasis. At sites of vascular injury, platelets adhere to exposed subendothelial collagen and release the contents of their dense granules and α-granules, thereby recruiting more circulating platelets which then clump together to form a haemostatic plug. Platelet activation also promotes the coagulation cascade resulting in the generation of thrombin that, as well as catalysing the formation of fibrin to consolidate the plug, also acts as a powerful platelet activator and, thus, amplifies the haemostatic process. Patients with an inadequate number of platelets (thrombocytopenia) or whose platelets are for some reason incapable of participating properly in haemostasis suffer from bleeding disorders, but the more important involvement of platelets in pathology is in occlusive vascular disease. Platelets are thought to play a role in atherosclerosis, both by forming mural thrombi, which become covered with endothelium and are incorporated into atherosclerotic plaques, and by secreting mitogenic factors such as platelet-derived growth factor, which stimulate the proliferation of cells in the vessel wall; both processes lead to a narrowing of the blood vessel lumen (Sussman, 1985). Although the role of platelets in atherogenesis is not clear, their importance in thrombosis is undoubted; they form part of and may initiate the occluding thrombus. The prophylactic use of antiplatelet drugs in an attempt to reduce the occurrence of thrombosis has so far had limited success, although recent results with aspirin have been more encouraging (Fuster et al., 1989). Obviously, the commercial potential of an antithrombotic drug is considerable, and so this goal is being actively pursued and may soon be reached. Such a drug also may have other therapeutic uses because platelets have been implicated in a number of nonthrombotic conditions due to the variety and potency of the substances they release when activated (Page, 1988).

B. Platelet Responses

In a stirred suspension, platelets challenged with activating agents normally change shape, aggregate, and

release their granule contents, in that order and with increasing concentrations of agonist. (For recent reviews of platelet activation, see Zucker and Nachmias, 1985; and Siess, 1989.) Quantitative studies of platelets were much facilitated by the invention by Born (1962a,b) of the aggregometer, a simple, widely used device in which light is passed through a rapidly stirred, warmed platelet suspension and detected by a photocell. Platelet responses can be observed either in anticoagulated platelet-rich plasma, prepared simply by gently centifuging whole blood to sediment red and white cells or in buffer after carefully washing the platelets free of plasma by centrifugation and/or gel filtration.

Shape change is observed as a small decrease in light transmission as the platelets lose their discoid shape, become spherical, and extrude pseudopodia. This change in shape is associated with constriction of the ring of microtubules running around the circumference of the platelet and centralization of the organelles within the microtubule ring. Shape change is not an essential prerequisite for aggregation, because adrenaline causes aggregation without shape change (see Mustard and Packham, 1970) and cytochalasin B inhibits shape change but not aggregation (White, 1971), showing that the development of platelet stickiness is not simply a result of the change in shape of platelets exposing new adhesive sites.

Aggregation of platelets is observed as a rapid increase in light transmission (as the number of particles in the suspension effectively decreases); this increase may obscure the smaller initial decrease due to shape change. Aggregation, but not shape change, is dependent on extracellular Ca^{2+} and fibrinogen, which form the bridges between platelets, and only occurs if the suspension is stirred rapidly enough (usually at approximately 1000 rpm) to cause the platelets to collide. The rate or extent of this increase in light transmission can be measured and is related to the concentration of aggregating agent, the latter observation permitting quantitative pharmacological investigations to be carried out. Weak aggregating agents or low concentrations of stronger ones cause reversible ("primary") aggregation, whereas stronger stimuli cause an irreversible ("secondary") aggregation which is associated with prostaglandin synthesis and the release of granule contents. Aspirin inhibits both of these secondary events, suggesting that the release reaction caused by many agonists is mediated by arachidonic acid metabolism (Mustard et al., 1975b). For such agonists (e.g., ADP), it seems likely that it is the act of aggregation, rather than the agonist itself, that causes prostaglandin synthesis and the release action because unstirred platelets, or those prevented from aggregating by treatment with antibodies against GPIIb/IIIa (see below), do not release their granule contents when challenged with such agonists, whereas centrifugation of activated platelets will cause release (Massini and Lüscher, 1972; Charo et al., 1977; Balduini et al., 1988). This aggregation-induced synthesis of TXA_2 is enhanced by reduced extracellular Ca^{2+} levels such as are found in citrate-containing plasma (Packham et al., 1989). The substrates released from the dense granules, in particular ADP, also play an important role in enhancing secondary aggregation induced by other agonists.

The development of platelet stickiness and the resulting aggregation are thought to be due to the appearance of a single population of fibrinogen-binding sites. (For recent reviews, see Marguerie et al., 1987; Leung and Nachman, 1986; Nurden, 1986; and Parmentier et al., 1990.) Radiolabeled fibrinogen binds only to activated platelets, and this binding is specific and saturable and correlates with platelet aggregation, suggesting that it mediates aggregation (Mustard et al., 1978; Bennett and Vilaire, 1979; Marguerie et al., 1979; Peerschke et al., 1980). Typically, there are approximately 38,000 binding sites per platelet, with a K_D of 0.1 to 1.0 μM and a time course that may depend on the speed of mixing (Marguerie et al., 1987). Some workers have observed curvilinear Scatchard plots consistent with the existence of two binding sites, although this could instead indicate cooperative effects; this would not be unlikely for a large ligand like fibrinogen, which will form multiple contacts with the cell surface (Peerschke, 1985). Fibrinogen binding does not occur in the absence of divalent cations and is supported by Ca^{2+} or Mg^{2+} (0.1 to 1.0 mM) but not Mn^{2+}, a requirement that reflects that needed for platelet aggregation (Bennett and Vilaire, 1979; Marguerie et al., 1980; Peerschke et al., 1980). The exposure of fibrinogen receptors does not require divalent cations, however (Peerschke and Zucker, 1981), suggesting that the stimulus-response coupling for aggregation, like that for shape change, does not require extracellular Ca^{2+}.

Fibrinogen binding to activated platelets and their subsequent cross-linking mediates reversible, primary aggregation, whereas strong stimuli induce an irreversible secondary aggregation that is associated with the release of granule contents. Irreversible aggregation may be due to stabilisation of the fibrinogen bridges by thrombospondin, another adhesive protein that is released from the α-granules (Leung and Nachman, 1986).

The platelet fibrinogen receptor is thought to be a complex of two membrane glycoproteins, GPIIb and GPIIIa, whose association is Ca^{2+} dependent, but, unlike fibrinogen binding, is not supported by Mg^{2+}. This complex, which is lacking in the bleeding disorder Glanzmann's thrombasthenia, exists on resting as well as on stimulated platelets; the nature of the changes that allow it to bind to fibrinogen is not known (Marguerie et al., 1987). Two other proteins, fibronectin and von Willebrand factor, also can bind to activated platelets and are involved in platelet adhesion. Although they can bind to the GPIIb/IIIa complex, they cannot compete effectively at physiological fibrinogen concentrations and probably

have their own binding sites that mediate their adhesive actions (Sixma, 1986).

The general sequence of events that occurs following exposure to an aggregating agent is shape change, appearance of fibrinogen receptors, aggregation, and release of dense granules (resulting in enhanced activation due to released ADP) and α-granules (resulting in stabilisation of the fibrinogen bridges between platelets and irreversible aggregation). The agonist itself and/or the act of aggregation can induce arachidonic acid metabolism, leading to the formation of stimulatory prostaglandins, in particular the endoperoxides PGG_2 and PGH_2 and the prostanoid TXA_2, which further enhance aggregation and cause the release reaction. There are at least two interrelated forms of positive feedback control, release of ADP and synthesis of prostanoids, and for strong stimuli such as collagen and thrombin there may be a third factor, the synthesis and release of PAF (Chignard et al., 1979; 1980).

C. Stimulus-Response Coupling

Platelet function is controlled by two types of agonists: stimulatory, causing the responses described above, and inhibitory, which nonspecifically and noncompetitively reduce these responses. It is now generally accepted that stimulatory agonists in general act via the phospholipase C pathway to generate the second messengers Ca^{2+} and DAG, whereas inhibitory agonists act via stimulation of adenylate cyclase to generate the second messenger cyclic AMP. These effects are both thought to be coupled to receptor occupation via guanine nucleotide-binding proteins (G proteins, formerly called N proteins) and are probably mediated by protein phosphorylation. Because these events are the subject of numerous excellent recent reviews (various chapters in Westwick et al., 1985 and in MacIntyre and Gordon, 1987; Rink, 1988; Siffert and Akkerman, 1988; Huang and Detwiler, 1986; Haslam, 1987; Brass, 1988; Feinstein, 1989; Siess, 1989; and Rink and Sage, 1990), they will not be discussed in great detail here. Although this general scheme is widely accepted, there are still some areas of uncertainty and controversy, partly due to the special problems of working with platelets.

The role of Ca^{2+} in platelet activation was suggested by the finding that the Ca^{2+} ionophore A23187 can mimic the effects of stimulatory agonists and cause shape change, aggregation, and the release reaction (Feinman and Detwiler, 1974; White et al., 1974; Massini and Lüscher, 1974); activation by A23187 does not require extracellular Ca^{2+} stores (Feinman and Detwiler, 1974). However, Holmsen and Dangelmaier (1981) reported that A23187 could only activate platelets in its free form and was inactive when complexed with divalent cations, suggesting that such complexes may not be able to penetrate platelet membranes and that A23187 might not be acting simply as an ionophore. In platelets permeabilised by high voltage discharges, however, Ca^{2+} can cause release of granule contents, confirming that an increase in intracellular Ca^{2+} activates platelets (Knight and Scrutton, 1980).

The measurement of intracellular Ca^{2+} and the investigation of its role in stimulus-response coupling was revolutionized by the invention by Tsien et al. (1984) of the fluorescent Ca^{2+} indicator quin-2 and its more sensitive successor fura-2 which, in esterified form, enter intact cells and following deesterification become trapped inside. Studies with these indicators have shown that stimulation of platelets by agonists is associated with an increase in intracellular Ca^{2+} from approximately 100 nM to a maximum of approximately 1 μM (Hallam and Rink, 1985a,b; Hallam et al., 1984a; Rink and Hallam, 1984). The increases in intracellular Ca^{2+} produced by a number of agonists are greatly reduced in the absence of extracellular Ca^{2+} when using quin-2, which has a significant Ca^{2+}-buffering effect, suggesting that influx may occur as well as release from intracellular stores, although the difference was much smaller when fura-2 was used (Pollock and Rink, 1986; Rink, 1986; Haslam, 1987). Fura-2 can be used at much lower intracellular concentrations than quin-2 and so does not buffer Ca^{2+} to the same extent, and the difference in results with the two indicators suggests that influx is important as a way of maintaining increased intracellular Ca^{2+} levels rather than being the major source of Ca^{2+} (Haslam, 1987; Pollock and Rink, 1986). Further evidence for agonist-induced Ca^{2+} influx was obtained using extracellular Mn^{2+} which quenches quin-2 fluorescence and can be used to demonstrate the entry of divalent cations (Hallam and Rink, 1985b). Ca^{2+} influx occurs before mobilisation from internal stores occurs, showing that increased intracellular Ca^{2+} is not responsible for Ca^{2+} channel opening (Sage and Rink, 1986a); this influx is probably not via voltage-dependent Ca^{2+} channels because it is not triggered following depolarisation with high levels of extracellular K^+ and is not blocked by Ca^{2+} channel blockers (Hallam and Rink, 1985a,b; Sage and Rink, 1986b; Rink, 1988).

The mobilisation of Ca^{2+} from internal stores of platelets is thought to be due to agonist-induced activation of phospholipase C, which cleaves phosphatidylinositol 4,5-biphosphate to give two messengers, $I(1,4,5)P_3$, which releases Ca^{2+} from the dense tubules, and DAG, which activates protein kinase C (Drummond and MacIntyre, 1987; Nishizuka, 1986). Polyphosphoinositide breakdown occurs rapidly following the addition of stimulatory agonists (Billah and Lapetina, 1982; Vickers et al., 1982; Agranoff et al., 1983), and $I(1,4,5,)P_3$ has been shown to cause the release of Ca^{2+} from its intracellular store, the dense tubular system (O'Rourke et al., 1985; Authi and Crawford, 1985). That an increased level of intracellular Ca^{2+} was not the only mediator was suggested by a comparison of the Ca^{2+} levels induced by physiological agonists with those produced by Ca^{2+} ionophores which,

at concentrations that achieve similar platelet responses, required much higher Ca^{2+} levels (Rink et al., 1982; Hallam et al., 1984a; Hallam and Rink, 1985a; Simpson et al., 1986). This suggested that there was another mediator released by receptor agonists; this is thought to be DAG (see Haslam, 1987; Drummond and MacIntyre, 1987). DAG and phorbol esters, which also activate protein kinase C, can cause aggregation and release without any increase in intracellular Ca^{2+}, although they do not cause shape changes; the latter effect may, therefore, be a Ca^{2+}-dependent response (Rink et al., 1983). Stimulation of protein kinase C also has an inhibitory effect on phosphoinositide hydrolysis and Ca^{2+} mobilisation, indicating a possible role in the feedback control of phospholipase C activation (MacIntyre et al., 1985; Drummond and MacIntyre, 1987). Synergism between Ca^{2+} and DAG may explain why some agonists cause responses with smaller increases in intracellular Ca^{2+} than would be expected from studies using ionophores; however, there are still some unexplained features of the stimulus-response coupling to some agonists, in particular to ADP and to adrenaline.

The effects of ADP on inositol lipid metabolism are not clear-cut, and several workers have failed to detect significant effects in human platelets, although in rat or rabbit platelets changes have been found (Drummond and MacIntyre, 1987; Haslam, 1987). However, ADP does cause increases in intracellular Ca^{2+}, although this seems to be due mainly to influx rather than from internal stores and, as with other agonists, the levels achieved are lower than expected from studies with ionophores, suggesting the involvement of some other synergistic pathway for platelet activation (Hallam and Rink, 1985a; Sage and Rink, 1986a). ADP-induced Ca^{2+} influx, unlike that induced by other agonists, occurs without measurable delay and therefore appears to be due to activation of receptor-operated Ca^{2+} channels rather than to any second-messenger system (Sage et al., 1989). Indeed, patch clamp studies in intact platelets have shown that ADP opens a plasma membrane channel permeable to divalent cations which is not voltage dependent (Mahaut-Smith et al., 1990). In the case of adrenaline, no consistent activation of phospholipase C has been detected under conditions in which release of other endogenous agonists has been prevented, and no consistent elevation of intracellular Ca^{2+} has been observed; therefore, the mechanism by which adrenaline activates platelets is still unclear (Haslam, 1987; Drummond and MacIntyre, 1987).

A further complication associated with ADP administration is the apparent stimulatory effect produced by low extracellular Ca^{2+} levels, such as are found in plasma anticoagulated with citrate rather than with heparin or with hirudin, which act by antagonising thrombin rather than by chelating Ca^{2+}. In a suspension medium containing a physiological Ca^{2+} concentration, ADP causes a much reduced release of granule contents (Mustard et al., 1975a); prostaglandin synthesis also may be impaired (Lüscher et al., 1980). This is not due to an inhibition of release by heparin or by hirudin because citrate added to heparinised platelets enhances release, whereas heparin or hirudin added to citrated platelets does not inhibit it (Macfarlane et al., 1975). In addition, in washed platelets or in a platelet suspension from which Ca^{2+} has been removed by a chelating resin, physiological Ca^{2+} levels still inhibit release (Massini, 1977; Heptinstall and Taylor, 1979). Similarly, aggregation induced by adrenaline is much reduced in plasma that is anticoagulated with heparin or with hirudin rather than with citrate, and this is probably due to a stimulatory effect of reduced Ca^{2+} levels (Macfarlane et al., 1975; Lages and Weiss, 1981). More recent studies have shown that TXA_2 synthesis and 5-HT release in response to PAF are also enhanced by reduced extracellular Ca^{2+} levels (Lalau-Keraly et al., 1989). Such findings are explained by the demonstration that low extracellular Ca^{2+} enhances the stimulation of TXA_2 production caused by close contact of platelets which is induced by a variety of agonists, although if TXA_2 formation is inhibited by aspirin, then Ca^{2+} enhances (primary) aggregation (Packham et al., 1989).

There is some evidence that increased Na^+/H^+ exchange, leading to an increase in intracellular pH, may play a part in Ca^{2+} mobilisation, although its exact role is controversial (Siffert and Akkerman, 1987; 1989; Siffert et al., 1990; Haslam, 1987; Rink and Sage, 1990). Removal of extracellular Na^+, or the use of amiloride analogues which inhibit Na^+ transport, inhibits Ca^{2+} mobilisation by thrombin, whereas the ionophore monensin, which enhances Na^+/H^+ transport, enhances Ca^{2+} moblisation (Siffert and Akkerman, 1987). The activation of Na^+/H^+ exchange may be a result of protein kinase C activation (Siffert et al., 1987), but for some agonists such as adrenaline, stimulation of exchange may be more directly coupled to receptor activation (Zavoico et al., 1986). Na^+/H^+ exchange may play a role in the activation of phospholipase A_2 and in the formation of TXA_2 and, therefore, enhance Ca^{2+} mobilisation by this pathway, at least for weak stimulants such as ADP, adrenaline, and low concentrations of thrombin, because inhibitors of the exchange block arachidonic acid release (Sweatt et al., 1985); alternatively, Na^+/H^+ exchange may enhance Ca^{2+} mobilisation independently of phospholipase A_2 activation (Siffert et al., 1990). However, although ADP does stimulate Na^+/H^+ exchange, overall acidification of the cytoplasm is observed and amiloride and its analogues do not inhibit ADP-induced aggregation at concentrations that block Na^+/H^+ exchange, suggesting that this exchange is not required for aggregation (Funder et al., 1988). Also, the Ca^{2+} mobilisation caused by thrombin peaked before any alkalinisation occurred (Sage et al., 1990). Some of the confusion concerning the role of Na^+/H^+ exchange may be explained by the recent

demonstration that the amiloride analogues used have nonspecific inhibitory effects as well and the suggestion that extracellular Na^+ itself rather than Na^+/H^+ exchange may be what is required for full aggregation (Krishnamurthi et al., 1990).

Most aggregating agents also inhibit adenylate cyclase and reduce levels of cyclic AMP in platelets in which cyclic AMP was elevated, and it has been suggested that a decrease in cyclic AMP might be involved in mediating aggregation (Salzman, 1972). However, some aggregating agents, such as vasopressin and PAF, do not inhibit adenylate cyclase in intact platelets (Haslam and Rosson, 1972, 1975; Haslam et al., 1985), intracellular inhibitors of adenylate cyclase do not induce aggregation (Haslam et al., 1978; Salzman et al., 1978; Harris et al., 1979), and aggregation can still occur in the presence of increased cyclic AMP levels (Haslam and Taylor, 1971). This suggests that a decrease in cyclic AMP is neither necessary nor sufficient for aggregation, although it may enhance aggregation in the presence of an inhibitory agonist that acts by stimulating adenylate cyclase (Haslam et al., 1978; Cusack and Hourani, 1982a). Intracellular inhibitors of adenylate cyclase have been shown to enhance aggregation induced by some aggregating agents, including analogues of the prostaglandin endoperoxides that do not themselves inhibit adenylate cyclase (Salzman et al., 1978), suggesting that inhibition of adenylate cyclase might play some role in inducing aggregation and that the inability of inhibitors of adenylate cyclase to potentiate most aggregating agents is because these agents have themselves inhibited adenylate cyclase adequately. However, vasopressin (which also does not inhibit adenylate cyclase) is not potentiated (Haslam et al., 1978), and the role of the inhibition of adenylate cyclase is still unclear, although it is obvious that it is not a major route of platelet activation.

Aggregating agents increase levels of cyclic GMP in platelets (Haslam, 1975), but this is probably an effect of, rather than a cause of, platelet aggregation (Haslam et al., 1980; Huang and Detwiler, 1986; Tremblay and Hamet, 1987). 8-Bromo-cyclic GMP, which penetrates cells, inhibits platelet function, suggesting that cyclic GMP, like cyclic AMP, is indeed inhibitory in platelets and may be acting as a feedback inhibitor of platelet activation (Haslam et al., 1980; Tremblay and Hamet, 1987). In unstirred platelet-rich plasma or in the presence of ethylenediaminetetraacetic acid to chelate Ca^{2+}, aggregation does not occur and no increase in cyclic GMP is detected, suggesting that platelet-platelet contact rather than receptor activation itself may be the stimulus for increases in guanylate cyclase activity (Davies et al., 1976). Release of unsaturated fatty acids, such as arachidonic acid, caused by platelet-platelet contact may mediate this effect because fatty acids are known to stimulate guanylate cyclase both directly and through the formation of the prostaglandin endoperoxides (Murad et al., 1986; Huang and Detwiler, 1986). This is also true for nitric oxide ("endothelium-dependent relaxing factor") which is released from vascular endothelial cells and is also responsible for the vasodilator activity of the nitrates and sodium nitroprusside (Palmer et al., 1987; Radomski et al., 1987). Indeed, platelet aggregation induced by ADP, arachidonic acid, thrombin, and A23187 results in nitric oxide production from arginine; inhibition of this pathway by arginine analogues enhances aggregation (Radomski et al., 1990).

Although the mechanism by which stimulatory agonists cause platelet activation has still not been completely elucidated and other pathways may still be discovered, it is clear that inhibitory agonists act via stimulation of adenylate cyclase. The platelet adenylate cyclase system is one of the best characterised, and cyclic AMP fulfills all the necessary criteria for a second messenger. The effect of elevated cyclic AMP levels is to inhibit nonspecifically and noncompetitively platelet activation induced by aggregating agents, including Ca^{2+} ionophores, and to cause disaggregation if aggregation has already occurred (for recent reviews see Feinstein et al., 1981, 1985; Tremblay and Hamet, 1987; Jakobs et al., 1986; Rink, 1986; Huang and Detwiler, 1986; Aktories and Jakobs, 1985). Increases in cyclic AMP levels inhibit and reverse the increases in cytoplasmic Ca^{2+} caused by aggregating agents (Feinstein et al., 1983), suggesting an effect on both Ca^{2+} mobilisation and on Ca^{2+} sequestration. Increases in cyclic AMP inhibit agonist-induced phosphatidylinositol 4,5-biphosphate hydrolysis (Billah et al., 1979; Feinstein et al., 1985) and the formation of $I(1,4,5)P_3$ (Watson et al., 1984), presumably by an effect on phospholipase C, and have been reported to oppose the $I(1,4,5)P_3$-induced release of Ca^{2+} (Moos and Goldberg, 1988). Increased cyclic AMP levels also increase the activity of the dense tubule Ca^{2+}-ATPase thought to be responsible for Ca^{2+} sequestration (Käser-Glanzmann et al., 1979). However, increases in total cyclic AMP content in platelets caused by various agents do not always correlate with inhibition of platelet responses, suggesting that this second-messenger system too has its subtleties (Rink, 1986).

The coupling of platelet receptors, both stimulatory and inhibitory, to their effector mechanisms is thought to be via GTP-binding coupling proteins (G proteins), as occurs in other cells (for reviews see Haslam, 1987; Huang and Detwiler, 1986; Haslam et al., 1985). The G proteins controlling adenylate cyclase have been most studied, and two types, G_s and G_i, mediating stimulation and inhibition, respectively, have been identified. They consist of three subunits called α, β, and γ, and whereas the β and γ subunits seem to be the same for G_s and G_i, the α-subunits exist in two forms α_s and α_i which stimulate and inhibit, respectively, adenylate cyclase. The α-subunits bind the guanine nucleotides GDP and GTP and possess GTPase activity, and the currently accepted

model for G protein function is that the activity of the α-subunit is controlled by the $\beta\gamma$ complex (for reviews see Brass, 1988; Birnbaumer, 1990). In the resting state the receptor is bound to the complete G protein, which carries GDP and is inactive. When the receptor is occupied by an agonist, GDP is exchanged for GTP and the α-subunit is released from the $\beta\gamma$ complex and modulates adenylate cyclase activity. The intrinsic GTPase activity of the α-subunit hydrolyses GTP to GDP which promotes dissociation from the cyclase and reassociation with the $\beta\gamma$ subunits. Stable GTP analogues such as GTP-γ-S and GMP-PNP, therefore, allow prolonged activation of G proteins. G_i may inhibit adenylate cyclase both directly via α_i and indirectly because its released $\beta\gamma$ subunits may bind to, and thus inactivate, α_s. Two bacterial toxins (which do not penetrate intact platelets) interfere with G protein function through an ADP-ribosylation of the α-subunits: cholera toxin which blocks the GTPase activity of α_s and pertussis toxin which blocks the inhibitory effect of α_i. The G protein that mediates receptor coupling to phospholipase C and the Ca^{2+}/DAG second-messenger system has not been identified but in some cells is inhibited by pertussis toxin, suggesting similarities with G_i.

In platelets adenylate cyclase has been shown to be regulated by the G proteins G_s and G_i (Stiles and Lefkowitz, 1982; Aktories and Jakobs, 1984, 1985; Katada et al., 1984a,b), and there is evidence from permeabilised platelets for a role of a G protein in the activation of phospholipase C (for review see Haslam, 1987). Stable GTP analogues reduce the intracellular Ca^{2+} concentration necessary for platelet activation (Haslam and Davidson, 1984a) and cause increases in DAG formation (Haslam and Davidson, 1984b) and the hydrolysis of polyphosphoinositides (Lapetina, 1986; Brass et al., 1986). The identity of this G protein is still unclear, and although some studies have shown that pertussis toxin can inhibit the effects of aggregating agents in causing phosphatidylinositol breakdown in permeabilised platelets (Brass et al., 1986), others have shown either no inhibitory effect on G protein function or even an enhancement (Lapetina, 1986; Houslay et al., 1986), suggesting that this G protein is not G_i. In any case, there is no correlation between the ability of aggregating agents to inhibit adenylate cyclase and to stimulate phosphatidylinositol turnover, and it has even been suggested that these two effects are negatively correlated in that agents that are potent activators of phospholipase C (thrombin, PAF, and vasopressin) are poor inhibitors of adenylate cyclase, whereas those that are potent inhibitors of adenylate cyclase (ADP and adrenaline) activate phospholipase C weakly (Haslam et al., 1985). This implies that receptors cannot be coupled simultaneously to both G_i and the G protein controlling phospholipase activity and that receptors for different aggregating agents may bind preferentially to one or the other. However, studies in which pertussis toxin was used have shown that, under conditions in which both phospholipase C activation and adenylate cyclase inhibition are blocked, only one ADP-ribosylated α-subunit of 41 kDa can be identified. Although this subunit can be resolved into two subspecies by isoelectric focussing, they are functionally, structurally, and immunologically identical, suggesting that they are only minor variants of the same protein. Aggregating agents block ADP-ribosylation of this protein to different degrees depending on whether they can activate both signal transduction pathways or only one, suggesting that only one G protein, G_i, is involved and that some factor other than G proteins may determine which receptors couple to which effector systems (Brass et al., 1988). Various other low molecular weight GTP-binding proteins (approximately 20 kDa) have also been isolated from the platelets, but the function of these is unknown (Nagata and Nozawa, 1988; Bhullar and Haslam, 1988).

The responses of platelets to stimulatory and inhibitory agonists are thought to be mediated, in platelets as in other cells, largely by phosphorylation of proteins (for reviews see Cohen, 1988; Gerrard et al., 1985, 1986; Huang and Detwiler, 1986; Haslam, 1987). Activation of protein kinase C by DAG results in the phosphorylation of one major protein (47 kDa) (Castagna et al., 1982; Sano et al., 1983) whose function is unknown (see Haslam, 1987); increases in intracellular Ca^{2+} result in the calmodulin-dependent phosphorylation of myosin light chain (20 kDa) (Daniel et al., 1981; Hathaway and Adelstein, 1979). Protein kinase C also phosphorylates myosin light chain, although at a different site (Naka et al., 1983), and a group of proteins of 39 to 41 kDa, one of which may be G_i (Williams et al., 1987).

Aggregation can still occur, although it is much reduced, in the presence of a nonspecific inhibitor of protein kinases, implying that there is also a phosphorylation-independent pathway of platelet activation mediated by Ca^{2+} (Watson and Hambleton, 1989). Inhibitory agonists, acting via cyclic AMP, stimulate a cyclic AMP-dependent protein kinase which phosphorylates several platelet proteins (Haslam et al., 1979, 1980). Although the function of these proteins has not been identified, they may include myosin light chain kinase (Hathaway et al., 1981) as well as proteins involved in Ca^{2+} mobilisation and sequestration (see Tremblay and Hamet, 1987; Feinstein et al., 1985).

II. Receptors for Stimulatory Agonists

A. Adenosine 5'-Diphosphate

ADP was the first compound shown to aggregate platelets in vitro (Born, 1962b) and for a while was thought to mediate aggregation caused by all other aggregating agents because their effects were inhibited by adenosine and its analogues which, because of their structural similarity to ADP, were believed to be ADP antagonists (Clayton and Cross, 1963; Clayton et al., 1963; Born,

1964; Born et al., 1965; Mustard and Packham, 1970). However the realisation that adenosine noncompetitively inhibited platelet aggregation by stimulating adenylate cyclase (Mills and Smith, 1971; Haslam and Lynham, 1972; Haslam and Rosson, 1975), and the demonstration that ATP, a true ADP antagonist, did not inhibit primary aggregation induced by other agents (Macfarlane and Mills, 1975), showed that aggregating agents can induce aggregation in their own right and not simply as a result of ADP release. However, the idea that ADP has a central role in aggregation induced by adrenaline has recently been revived because of the inhibitory effect of an affinity reagent, FSBA, which has been claimed to be a selective ADP antagonist (Figures et al., 1986). However, the specificity of FSBA is doubtful (see below and Macfarlane, 1987), and it is generally believed that ADP has a role only in secondary aggregation induced by other aggregating agents. Low concentrations of ADP will enhance the effect of other aggregating agents, but this action is not confined to ADP and is a general feature of any pair of agonists, although it may in part be due to the low sensitivity of the measurement of light transmittance as an index of aggregation, as only larger aggregates are detected (Thompson et al., 1986).

Although the effects of ADP have long been known, little is understood concerning the nature of the ADP receptor, its coupling mechanisms, and its physiological importance (for reviews see Haslam and Cusack, 1981; Macfarlane, 1987; Cusack and Hourani, 1991). ADP in vitro causes shape change, aggregation, and the release of granule contents, although, as discussed above, this release is only observed in a medium containing reduced Ca^{2+} levels, and is a consequence of prostaglandin synthesis caused by platelet-platelet contact (Mustard et al., 1975b). ADP also inhibits stimulated adenylate cyclase (Cole et al., 1971; Mills and Smith, 1971; Haslam, 1973), and controversy exists as to whether aggregation and the inhibition of adenylate cyclase are mediated by one or two types of ADP receptor (see Macfarlane, 1987, and below).

1. Structure-activity relationships. Many analogues of ADP have been synthesised (see fig. 1 for some examples of structures) and tested for their ability to activate platelets, and some general conclusions as to their structural requirements can be drawn (Haslam and Cusack, 1981; Hourani and Cusack, 1985; Cusack et al., 1985; Cusack and Hourani, 1991). There is a high degree of structural specificity associated with platelet activation; the other naturally occurring nucleoside diphosphates, GDP, inosine 5′-diphosphate, cytidine 5′-diphosphate, and uridine 5′-diphosphate are very much less potent than ADP (Gaarder et al., 1961; Chambers et al., 1968; Packham et al., 1974). Modifications to the purine ring at the N^1, C^8, or C^6 positions also result in loss of activity (Gaarder and Laland, 1964; Stone et al., 1976), but substitution at the C^2 position is tolerated and even some

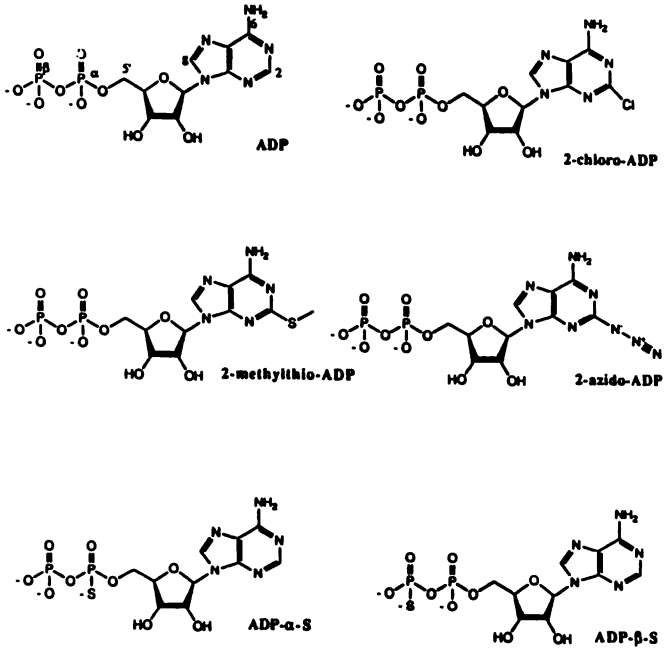


FIG. 1. Some examples of platelet ADP (P_{2T}) receptor agonists.

very large substituents do not reduce activity (Gough et al., 1972; Robey et al., 1979; Jefferson et al., 1987). Indeed, some 2-substituted analogues, such as 2-chloro-ADP, 2-azido-ADP, and 2-methylthio-ADP, are more potent than ADP as aggregating agents (Maguire and Michal, 1968; Gough et al., 1972; Cusack and Born, 1977; Cusack and Hourani, 1982d).

Modifications to the ribose sugar, such as removal or inversion of configuration of a hydroxyl group or periodate cleavage of the ring, result in weakly active compounds (Gaarder et al., 1961; Gaarder and Laland, 1964; Pearce et al., 1978). The ADP receptor is stereospecific; the unnatural L-enantiomers of ADP, 2-chloro-ADP and 2-azido-ADP (which contain L-ribose instead of the normal D-ribose), are inactive (Cusack et al., 1979).

The 5′-diphosphate chain is also important for agonist activity, and addition or subtraction of phosphates to form ATP or AMP results in the formation of competitive antagonists (Packham et al., 1969, 1972; Macfarlane and Mills, 1975). Adenosine 5′-tetraphosphate has been reported to have weak aggregating activity, although this may be due to its degradation to ADP because, when highly purified, it has only a weak antagonist action (Harrison and Brossmer, 1976). Replacement of one of the bridging oxygens by a methylene group generates analogues (α,β-methylene-ADP and homo-ADP) with only weak aggregating activity, as does replacement of one of the terminal charged oxygens by an uncharged fluoro group to give ADP-β-F (Gough et al., 1972; Horak and Barton, 1974, Cusack et al., 1985). The low potency of α,β-methylene-ADP presumably is not due to its reduced electronegativity compared to ADP, because stable analogues that are isopolar with ADP are not more potent: α,β-imido-ADP is a partial agonist, and α,β-

difluoromethylene-ADP and α,β-dichloromethylene-ADP are inhibitors of aggregation (Cusack and Pettey, 1988). Replacement of an oxygen on either the α- or β-phosphate by sulphur to give ADP-α-S and ADP-β-S generates partial agonists with an intrinsic activity of approximately 0.75 (Cusack and Hourani, 1981b,c). ADP-α-S exists as a pair of diastereoisomers which differ in potency by approximately fivefold, showing that the ADP receptor displays only weak stereoselectivity toward the diphosphate part of the molecule (Cusack and Hourani, 1981c).

Only a few of these ADP analogues have been tested for their effects on stimulated adenylate cyclase, and no other intracellular biochemical effects have been studied. ADP itself and 2-chloro-ADP are approximately equipotent as aggregating agents and as inhibitors of stimulated adenylate cyclase (Haslam and Rosson, 1975; Cusack and Hourani, 1982d). ADP-β-S is a partial agonist for the inhibition of adenylate cyclase, as it is for aggregation, but its intrinsic activity here is a little lower at approximately 0.5 (Cusack and Hourani, 1981b). However, 2-azido-ADP and 2-methylthio-ADP are more potent as inhibitors of adenylate cyclase than as aggregating agents (Macfarlane et al., 1982, 1983; Cusack and Hourani, 1982d), and ADP-α-S does not inhibit adenylate cyclase, although it is an antagonist of this action of ADP (Cusack and Hourani, 1981c). This lack of correlation between the ability of analogues to activate platelets and to inhibit stimulated adenylate cyclase does imply that these two effects are independent and not causally related. The differential potency of 2-azido-ADP and 2-methylthio-ADP has led to the suggestion that these two effects were mediated by separate receptors (Macfarlane et al., 1982, 1983). However, this differential potency could also be due to the analogues binding to only one receptor type, which can couple to two different effector mechanisms, and thereby influence this coupling in different ways. The action of ADP-α-S as a partial agonist for aggregation and an antagonist for adenylate cyclase inhibition supports the "one receptor—two coupling mechanisms" hypothesis, especially because the stereoselectivity for both these actions is similar and the EC_{50} value of the diastereoisomers as aggregating agents is approximately equal to their K_i values as antagonists of the adenylate cyclase effect (Cusack and Hourani, 1981c).

The study of the structure-activity relationships for ADP antagonists is complicated by their dephosphorylation in plasma to ADP analogues, which can enhance aggregation or make platelets refractory to ADP, and to the formation of adenosine analogues that can inhibit aggregation nonspecifically by increasing intracellular cyclic AMP levels. These problems have not always been addressed, which makes interpretation of reported results difficult, particularly if inhibitors have been preincubated with the platelets in plasma (see Haslam and Cusack, 1981). ATP is a competitive antagonist of aggregation induced by ADP, with a K_i of approximately 20 μM (Macfarlane and Mills, 1975); AMP is also a weak antagonist, but its major inhibitory effect is via its degradation product adenosine (Packham et al., 1969, 1972). ATP has also been shown to inhibit the effects of ADP-α-S, ADP-β-S, 2-chloro-ADP, 2-methylthio-ADP, and 2-azido-ADP, all with an appropriate K_i value, confirming that these analogues act at the ADP receptor (Cusack and Hourani, 1981b,c, 1982d).

Various analogues of ATP and of AMP have been reported to be antagonists (see fig. 2 for some examples of structures), but in most cases their competitivity has not been thoroughly investigated and little quantitative data are available [for review see Haslam and Cusack (1981)]. One ADP analogue, 8-bromo-ADP has also been reported to act as an ADP antagonist (Jefferson et al., 1988). A number of α,ω-diadenosine polyphosphates and their methylene analogues have been identified as ADP antagonists, and because P^1,P^4-diadenosine tetraphosphate occurs in cells it may have some physiological role (Harrison et al., 1975; Kim et al., 1989b; Andersson, 1989).

A detailed study of eight antagonists whose competitivity was established by Schild analysis showed that, as with ADP itself, 2-substitution on the purine ring is tolerated as are some modifications to the phosphate chain, toward which weak stereoselectivity is displayed

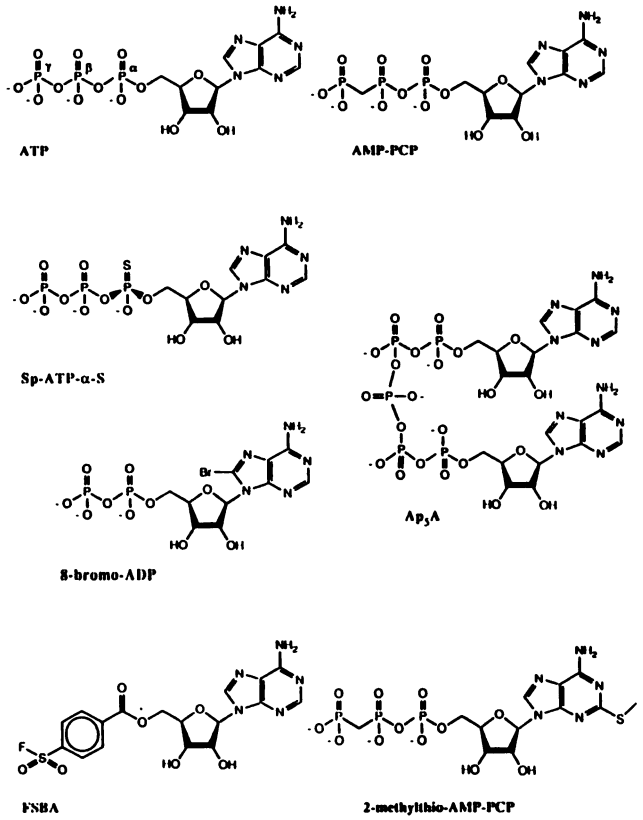


FIG. 2. Some examples of platelet ADP (P_{2T}) receptor antagonists. AMP-PCP, adenosine 5′-β,γ-methylenetriphosphonate. Ap_5A, P^1,P^5-diadenosine pentaphosphate.

(Cusack and Hourani, 1982c). As well as inhibiting ADP-induced aggregation, these antagonists also competitively inhibit the effects of ADP on adenylate cyclase, and there is a good correlation between their pA_2 values for these effects; this is strong evidence that they are both mediated by one receptor type (Cusack and Hourani, 1982c). In addition, ATP inhibits both effects of 2-chloro-ADP, 2-azido-ADP, and 2-methylthio-ADP with the same pA_2 value (Cusack and Hourani, 1982d). The most potent competitive inhibitor (K_B 4 μM) is the S_P diastereoisomer of ATP-α-S (S_P-ATP-α-S) (Cusack and Hourani, 1982c). Recently, the corresponding guanosine analogue GTP-α-S, unlike GTP itself, was found to inhibit platelet aggregation with an IC_{50} value of approximately 2.5 μM, which is similar to that of ATP (Greco et al., 1991a).

Absolute stereoselectivity is displayed toward the ribose moiety, because L-ATP and L-AMP are inactive (Cusack et al., 1979; N. J. Cusack and S. M. O. Hourani, unpublished data), and cleavage of the ribose ring results in reduction of activity (Pearce et al., 1978). An interesting group of compounds is the 2-alkylthio analogues of AMP and of ATP which, although originally described as antagonists (Gough et al., 1978; MacIntyre et al., 1977a), are noncompetitive but specific inhibitors of ADP-induced aggregation (Cusack and Hourani, 1982b). Their mode of action is unknown, but they could not achieve >50% inhibition of the aggregation induced by ADP, suggesting that perhaps they inhibit only one component of the action of ADP. Studies with a nondegradable alkylthio analogue 2-methylthioadenosine β,γ-methylenetriphosphonate suggested that inhibition of adenylate cyclase may be this component and that this inhibitory effect of ADP may, after all, play a role in stimulus-response coupling at the ADP receptor (Cusack et al., 1985; Hourani et al., 1986).

FSBA, an affinity analogue of ADP, has been claimed to act as a competitive antagonist of the effects of ADP and to inhibit only aggregation and not the effects of ADP on adenylate cyclase. This observation has been taken as evidence of the existence of two separate receptors for these two effects (Colman et al., 1980). FSBA has been used extensively by Colman et al as an ADP antagonist (for reviews, see Colman, 1986; Colman et al., 1988; Colman, 1990), but doubts as to its specificity remain (Macfarlane, 1987). It inhibits aggregation induced by prostaglandin endoperoxides, collagen, adrenaline, and thrombin, a finding taken to imply a role for ADP in aggregation caused by these agents (Colman, 1986) but which could equally be taken as evidence for the nonspecific inhibitory effect of FSBA. The compound is essentially an adenosine analogue with a reactive group attached to the 5′ position and, in an aqueous medium, rapidly breaks down to adenosine which is responsible for at least some of the drug's inhibitory effect (Mills et al. 1985). FSBA does not inhibit the increase in intracellular Ca^{2+} induced by ADP (Rao and Kowalska, 1987) and, although these authors interpreted this finding as indicating that the Ca^{2+} effect was mediated by a separate receptor from that inducing shape change (which is inhibited by FSBA), this confirms the suspicion that FSBA is not interacting solely with the ADP receptor.

Various other compounds that are not structurally related to ADP, such as nitrofurantoin, furosemide, and pyridoxal phosphate (Rossi and Levin, 1973a,b; Kornecki and Feinberg, 1980), have been reported to inhibit ADP-induced platelet aggregation, but their competitivity and specificity has not been established. Sulphydryl reagents have been reported to inhibit the effects of ADP on adenylate cyclase (Macfarlane and Mills, 1981) and on aggregation (MacIntyre et al., 1977b). In addition, ADP-removing enzyme systems, such as creatine phosphate/creatine phosphokinase, have been used to investigate the role of released ADP in platelet function (Haslam, 1964), but the mechanism by which these enzyme systems inhibit aggregation has been questioned (Huang and Detwiler, 1980; Nunn and Chamberlain, 1983).

Overall, the platelet ADP receptor does not appear to be identical with any other receptor for adenine nucleotides. Purine receptors have been divided into P_1 (which recognise adenosine) and P_2 (which recognise ADP and ATP) (Burnstock, 1978), and according to this classification the platelet receptor is clearly a type of P_2-purinoceptor (Gordon, 1986). P_2-purinoceptors have been tentatively subdivided further on the basis of agonist potencies into P_{2X} (mediating contraction of smooth muscle) and P_{2Y} (mediating relaxation) (Burnstock and Kennedy, 1985), but the platelet receptor does not fit neatly into either class and has even been called a P_{2T} receptor (Gordon, 1986). In other tissues ADP and ATP are both agonists with approximately equal potency, whereas on platelets ATP is an antagonist and indeed is a unique example of an endogenous compound active as a competitive antagonist. No true competitive antagonists at the P_{2X} and P_{2Y} receptors are known, and those receptors have different structure-activity requirements for agonists. Platelet ADP receptors have much more rigid structural requirements than do P_{2X} or P_{2Y} receptors, and they display a higher degree of stereoselectivity (for reviews, see Cusack et al., 1988; Cusack and Hourani, 1990). On P_{2X} receptors almost any structural modification is tolerated, no stereoselectivity (or even in some cases reversed stereoselectivity) is displayed, and stable phosphonate analogues are more potent than ADP. On P_{2Y} receptors 2-substitution enhances potency, whereas the methylene phosphonate analogues are less potent and some stereoselectivity is observed; therefore, the platelet receptor resembles the P_{2Y} receptor more closely than it does the P_{2X} receptor. It is possible that other tissues may be discovered that have receptors similar to the platelet ADP receptor, but at the moment it is clearly in a class of its own.

2. Binding studies and receptor isolation. Early studies of the binding of radiolabeled ADP to platelets were complicated by methodological problems including the rapid metabolism and low affinity of ADP, the variable amounts of medium trapped when intact platelets are used, and the probability that, because of the many metabolic roles of intracellular nucleotides, there are large numbers of irrelevant binding sites exposed when platelet membrane preparations are used (for review and table comparing results, see Haslam and Cusack, 1981). Estimates of the number of receptors per platelet range from 30,000 to 120,000, with affinities for ADP mainly in the micromolar range, which is similar to the EC_{50} values normally observed for the effects of ADP. A recent study of the binding of ADP to paraformaldehyde-fixed platelets showed binding to two sites, with high (0.35 μM) and low (7.9 μM) affinities (Jefferson et al., 1988). There were 160,000 high affinity sites per platelet, and binding was inhibited effectively by ADP and by ATP, weakly by AMP, and not at all by adenosine, as expected for the ADP receptor. However, although in general binding correlated with pharmacological activity, the structure-activity relationships for ADP analogues to displace ADP were not identical with their ability to act as aggregating agents and, more important, the K_i of ATP derived from these binding studies (0.4 μM) is not the same as its apparent K_B of approximately 20 μM derived from pharmacological studies (Macfarlane and Mills, 1975; Cusack and Hourani, 1982c,d). An extension of this study also showed discrepancies between binding and the pharmacological activity of both agonists and antagonists, particularly those substituted at the C^2 position whose affinities were lower than expected from their high potencies (Agarwal et al., 1989).

Recently, [^{35}S]S_P-ATP-α-S was reported to bind to fixed platelets at a high affinity site (K_D 1 nM; approximately 38,000 sites per platelet) and to a lower affinity site (K_D 314 nM; approximately 34,000 sites per platelet) (Greco et al., 1991b). Studies in which the more potent C^2-substituted ADP analogues 2-azido-ADP and 2-methylthio-ADP were used as radioligands demonstrated saturable binding to approximately 700 sites per platelet, in each case with affinities in the nanomolar range, and a pattern of displacement by nucleotides that is consistent with these sites being the ADP receptors, although only a few displacing ligands were tested (Macfarlane et al., 1982, 1983; Mills et al., 1983). These authors interpreted their results as showing binding to the "adenylate cyclase" receptor only, and not to the "aggregation" receptor, but because these compounds are potent aggregating agents they would be expected to bind to both sites. Because only one site was detected, this is more consistent with the two effects being mediated by only one type of ADP receptor.

Various attempts have also been made to label covalently the ADP receptor using affinity or photoaffinity analogues, but none of these attempts has been wholly convincing. The 2′,3′-dialdehyde analogues of ADP and of ATP labeled some platelet proteins, but there is no evidence that this labeling was specific for the ADP receptor, and because these analogues have only very weak agonist or antagonist affinity they are probably not the best choice of ligand (Pearce et al., 1978; Pearce and Scrutton, 1977). The photoaffinity analogue 2-azido-ADP (Cusack and Born, 1977) also covalently labeled various proteins on the surface of the intact platelets, but this labeling was not prevented either by ADP or by ATP and, therefore, is unlikely to represent the ADP receptor (Macfarlane et al., 1982; Mills et al., 1983).

FSBA has been used as an affinity analogue and labels a variety of proteins, including actin and myosin, when incubated with broken platelet preparations, whereas in intact platelets predominantly one polypeptide of 100 kDa (2500 sites per platelet) is labeled (Bennett et al., 1978, 1981; Figures et al., 1981). The incorporation of FSBA was prevented by ADP and by ATP, but only at a concentration of 10 mM; at this concentration adenosine and GDP also inhibited incorporation by 50% (which is not appropriate for the ADP receptor), whereas uridine 5′-diphosphate, thrombin, and adrenaline did not (Bennett et al., 1978). This 100-kDa polypeptide has been called "aggregin" and a model has been proposed in which it is closely associated with the GPIIb/IIIa fibrinogen receptor complex and sterically hinders its assembly until ADP binding or proteolytic cleavage relieves this hindrance (see Colman, 1986; Colman et al., 1988). In this model aggregation induced by thrombin is attributed to the increases in intracellular Ca^{2+} activating calpain, a Ca^{2+}-activated protease that is exposed at the platelet surface and cleaves aggregin to cause exposure of fibrinogen receptors (Colman, 1990). However, in view of doubts as to the specificity and mode of action of FSBA and, therefore, the identity of this 100-kDa polypeptide (see above and Macfarlane, 1987), proposal of this model appears premature.

Photolysis of [^{35}S]S_P-ATP-α-S with intact platelets radiolabeled a single protein of 120 kDa, identified as $GPIIb_\alpha$, and covalent binding was shown to occur in an 18-kDa domain between amino acid tyrosine-198 and glycine-358, in close proximity to the proposed binding sites for fibrinogen and for Ca^{2+} (Greco et al., 1991b). This adenine nucleotide-binding site has some of the expected properties of the ADP receptor, although the influence on radiolabeling of a variety of platelet receptor agonists has not yet been reported.

Adler and Handin (1979) isolated an ADP-binding protein from platelets by subjecting platelet membranes to freeze-thaw injury and extracting with saline. This protein has a molecular weight of 61 kDa and bound ADP with a K_D of 0.38 μM with a capacity corresponding to approximately 10,000 sites per platelet. Binding was effectively inhibited by ATP and by ADP, weakly by

AMP, and not at all by adenosine, which is consistent with this molecule being the ADP receptor, although a wider range of analogues should have been tested. However, it seems unlikely that the ADP receptor could be removed so easily from the platelet surface, and this work has not been followed up.

3. Metabolism of extracellular adenosine 5′-diphosphate. Platelets, like other cells including endothelial and smooth muscle cells, possess ectonucleotidases capable of sequentially dephosphorylating adenine nucleotides ultimately to adenosine, which is then taken up and incorporated into the platelet adenine nucleotides (Salzman et al., 1966a,b; Spaet and Lejnieks, 1966; Gordon, 1986). For discussion of the uptake of adenosine into platelets see section IIIA. The existence of an ecto-ATPase led to the suggestion (Salzman et al., 1966b) that this enzyme is responsible for maintaining platelets in the resting state and that product inhibition by ADP of this enzyme was responsible for aggregation induced by ADP, but this hypothesis is no longer considered likely (see Haslam and Cusack, 1981). The significance of these ectoenzymes on vascular cells is now thought to be their transformation of ATP released from damaged cells into ADP which triggers aggregation and then is itself degraded to the inhibitory adenosine, thereby limiting thrombus formation (Born and Kratzer, 1984; Pearson and Gordon, 1985; Gordon, 1986).

Platelets also possess an ectoenzyme, nucleoside diphosphokinase, capable of phosphorylating ADP to ATP, and this activity actually predominates over the ectonucleotidase when ADP is added to washed platelets (Chambers et al., 1968; Packham et al., 1969). It has been suggested that nucleoside diphosphokinase acts as the ADP receptor and that ADP directs ATP away from some energy-requiring process responsible for maintaining platelets in an inactive state and thereby induces aggregation (Guccione et al., 1971; Packham et al., 1974; Mustard et al., 1975a). However, nucleoside diphosphokinase differs from the ADP receptor in that it requires divalent cations (Agarwal and Parks, 1971) and has a low substrate specificity (Mourad and Parks, 1966) and, in the study by Adler and Handin (1979), could be separated from the ADP-binding protein (for discussion, see Haslam and Cusack, 1981). Also, a study of platelet aggregation and fibrinogen binding showed no correlation for a series of blood donors between these parameters and the transformation of [^{14}C]ADP into either [^{14}C]ATP (by nucleoside diphosphokinase) or [^{14}C]AMP (by ectonucleotidases) (Legrand et al., 1984).

4. Physiological and pathological importance. The importance of released ADP as part of the feedback mechanism for enhancing and propagating platelet activation is clear, although as discussed above ADP is no longer thought to be responsible for primary aggregation induced by other agents (see Haslam and Cusack, 1981). Platelets from patients with storage pool disease lack stored nucleotides and have impaired secondary phase aggregation resulting in a mild bleeding tendency and showing that this release of ADP has physiological importance too (Mustard and Packham, 1977).

The role of ADP in triggering haemostasis or thrombosis in vivo is less clear because of the lack of good selective antagonists; those currently available are nucleotide analogues and, therefore, have a variety of other pharmacological effects on vascular tone (see Gordon, 1986; Burnstock, 1988). ADP was first discovered as the factor in red cells that caused platelet stickiness (Gaarder et al., 1961), and ADP released from red cells by high shear stress at sites of vascular damage was originally envisaged as playing an important role (Born, 1962b), but the forces required to release nucleotides from red cells are probably much higher than those normally encountered in vivo (Born and Kratzer, 1984). However, nucleotides are released in high concentrations when blood vessels are cut, initially from damaged cells and then from aggregating platelets, and therefore could be important in initiating thrombus formation (Born and Kratzer, 1984).

Studies in which rat mesenteric blood vessels were used showed that infusion of ADP-removing enzyme systems can prolong the bleeding time from small blood vessels (Zawilska et al., 1982), but the specificity of these enzyme systems has been questioned (Huang and Detwiler, 1980; Nunn and Chamberlain, 1983). Infusion of stable ATP analogues also prolongs the bleeding time (Born et al., 1986), but although overall circulatory effects were not observed, local effects on vascular tone cannot be ruled out. A methylene analogue of P^1,P^4-diadenosine tetraphosphate has also been shown to reduce the incidence of clots in an in vivo rabbit model of thrombosis, lending further support to the suggestion (Born et al., 1986; McClure et al., 1988) that an ADP antagonist could have therapeutic potential (Kim et al., 1989a).

B. Catecholamines (α-Receptor)

The stimulatory effect of adrenaline on human platelets was discovered by O'Brien (1963) who showed that it induced aggregation without a preceding change in shape. Primary aggregation induced by adrenaline is weak compared with other agonists such as ADP and is not observed in suspensions containing a physiological Ca^{2+} concentration (Glusa and Markwardt, 1980; Lages and Weiss, 1981). Aggregation to adrenaline is species dependent and does not occur in most nonprimate mammals, including rats, guinea pigs, rabbits, and dogs, although in some species adrenaline has a "proaggregatory" effect, potentiating responses to other agonists even though its direct effects are too weak to be detected in the aggregometer (Dodds, 1978; Kerry and Scrutton, 1985).

The observed response to adrenaline seems to depend on the number of stimulatory α-adrenoceptors present

as well as the ratio of these receptors to the inhibitory β-adrenoceptors also present on platelets (Kerry et al., 1984). The response to adrenaline is variable even among normal human donors, and some donors have platelets that consistently fail to aggregate to adrenaline, although their responses to other aggregating agents are normal. These platelets and platelets in a suspension medium containing physiological Ca^{2+} concentrations display a proaggregatory response to adrenaline, showing that they do possess functional stimulatory α-adrenoceptors. These receptors and the inhibitory β-adrenoceptors on platelets have been the subject of several recent reviews (Scrutton and Wallis, 1981; Kerry and Scrutton, 1985; Barnett et al., 1985; Lanza and Cazenave, 1987; Jakobs and Minuth, 1987).

1. Structure-activity relationships. Early studies of the response of platelets to catecholamines showed that the stimulatory receptor is an α-adrenoceptor. Adrenaline and noradrenaline are effective agonists, whereas isoprenaline is not, and aggregation induced by adrenaline is blocked by phentolamine and by dihydroergotamine but not by appropriate concentrations of propranolol (O'Brien, 1964; Mills and Roberts, 1967). α-Adrenoceptors have been subdivided into two types, α_1 and α_2, originally on the basis of differences in anatomical position within the sympathetic nervous system (α_1, postjunctional; α_2, prejunctional) but more recently on the basis of selective agonists and antagonists. It is also clear now that α_2-receptors can mediate postjunctional effects as well as merely presynaptic inhibition of transmitter release, although α_2-receptors may, in general, be located outside the synaptic cleft and therefore respond preferentially to blood-borne catecholamines.

The stimulus-response coupling mechanisms for α_1- and α_2-receptors in general also seem to be different, with α_1 linked to phospholipase C and the phosphoinositide pathway and α_2 linked to inhibition of adenylate cyclase (see Homcy and Graham, 1985; Kerry and Scrutton, 1985 for review). The platelet α-receptor has been characterised pharmacologically as an α_2-receptor (see below), which is in keeping with its access to blood-borne catecholamines and its inhibitory coupling to adenylate cyclase, although this may not be the only cause of aggregation (see "Introduction"). Dopamine has been reported to be a weak platelet activator, but this is via an interaction with the α-receptor rather than through a separate dopamine receptor (Rossi, 1978).

Adrenaline is a more powerful platelet activator than noradrenaline which is consistent with the existence of α_2-receptors (Lasch and Jakobs, 1979), and platelets can be activated by α_2-selective synthetic agonists such as clonidine but not by α_1-selective agonists such as phenylephrine or methoxamine (Hsu et al., 1979; Grant and Scrutton, 1979, 1980). In addition, platelet activation by adrenaline is inhibited more effectively by α_2-selective antagonists such as yohimbine and rauwolscine than by α_1-selective antagonists such as prazosin and indoramin (Grant and Scrutton, 1979, 1980; Glusa et al., 1979; Kerry and Scrutton, 1983c). Some α_2-selective compounds such as clonidine are weak partial agonists in platelets with rather variable efficacy, causing only a proaggregatory response in some donors and also antagonising the effects of adrenaline (Grant and Scrutton, 1979, 1980; Hsu et al., 1979; Glusa and Markwardt, 1981; Lasch and Jakobs, 1979; Jakobs, 1978). The low efficacy of these agonists in platelets has been taken as evidence that the platelet α_2-receptor is unique (Jakobs, 1978; Petrusewicz and Kaliszan, 1986), but α_2-adrenoceptors in some other tissues also show reduced efficacy for these agonists, and these differences could in any case be explained by differences in receptor density or coupling mechanisms between tissues (Grant and Scrutton, 1980; Kerry and Scrutton, 1985).

There may also be α_1-receptors present on the platelets of some donors because in approximately 30% of those individuals studied α_1-agonists, such as methoxamine and phenylephrine, can induce a proaggregatory response that is inhibited more effectively by prazosin than by yohimbine (Grant and Scrutton, 1979, 1980). However, these α_1-receptors are not important in mediating platelet activation by catecholamines, and most binding studies have failed to detect them (see below), although this may be because they do not exist in most donors (Scrutton and Wallis, 1981; Kerry and Scrutton, 1985).

The ability of α-agonists and α-antagonists to affect platelet adenylate cyclase has also been investigated, and these result confirm that the platelet α-adrenoceptor is of the α_2-subtype (Lasch and Jacobs, 1979). It has, however, been suggested, by analogy with the suggestion for the ADP receptor (see above), that two different α_2-receptors may mediate aggregation and the inhibition of adenylate cyclase (Scrutton and Wallis, 1981). Studies in which a range of synthetic analogues were used showed that some agonists could cause aggregation without inhibiting adenylate cyclase and that some antagonists apparently also had selective effects (Clare and Scrutton, 1983; Clare et al., 1984). These discrepancies also confirm the view (see "Introduction") that inhibition of adenylate cyclase is not in itself sufficient to cause platelet aggregation.

2. Binding studies and receptor isolation. α-Receptors have consistently been detected on intact human platelets and platelet lysates by several groups using both the nonselective α-antagonist [^{3}H]dihydroergocryptine and selective α_2-antagonists, mainly [^{3}H]yohimbine (see Kerry and Scrutton, 1985, and Lanza and Cazenave, 1987, for tables of collected results). The presence of α_1-adrenoceptors have been investigated using [^{3}H]prazosin, but, whereas two studies failed to detect any (Motulsky and Insel, 1982; Daiguji et al., 1981), a study in which only those donors whose platelets showed a proaggregatory response to the α_1-agonist methoxamine were

used did detect α_1-receptors at a density of approximately 195 per platelet (Kerry et al., 1982). The results from binding studies were, therefore, in accordance with the pharmacological studies and confirm that the platelet adrenoceptor is of the α_2-subtype but that in a small proportion of the population some α_1-receptors may exist as well.

However, the average number of receptors identified by [^{3}H]dihydroergocryptine (366 sites per platelet) is higher than that identified by [^{3}H]yohimbine (268 sites per platelet) (Kerry and Scrutton, 1985), and this discrepancy has also been found in those studies in which the two ligands were directly compared (Motulsky and Insel, 1982; Daiguji et al., 1981; Boon et al., 1983). The significance of the "extra" sites identified by [^{3}H]dihydroergocryptine is unknown, but in spite of the nonselective nature of the ligand they do not represent α_1-receptors, 5-HT receptors, or dopamine receptors (Hoffman et al., 1979; Daiguji et al., 1981; Motulsky and Insel, 1982). Interestingly, several studies in which [^{3}H]dihydroergocryptine was used detected alterations in the α_2-adrenoceptor density in various pathological conditions, whereas similar studies in which [^{3}H]yohimbine was used failed to demonstrate alterations, and it has been suggested that these "extra" sites may have greater functional significance than those labeled by [^{3}H]yohimbine alone (Barnett et al., 1985). However, such comparisons are not easy to make, and the overall pattern is not so clear (Kerry and Scrutton, 1985; Lanza and Cazenave, 1987). To address some of these problems, the α_{2A}-antagonist 2-methoxy-idazoxan (RX821002), which antagonizes adrenaline-induced human platelet aggregation more potently than yohimbine, has been introduced (Galitzky et al., 1990). [^{3}H]RX821002 binds to human platelet membranes with a higher affinity (K_D 0.92 nM), to a greater number of sites (approximately 150 sites per platelet), and with a fourfold reduction in nonspecific binding compared to that of yohimbine in the same preparation. The increased number of binding sites detected was attributed to binding to an increased number of low affinity sites, because [^{3}H]RX821002 and [^{3}H]yohimbine each labeled a similar number of high affinity sites, amounting to 21 to 34% of the total (Galitzky et al., 1990).

Binding studies in platelets using agonist ligands have consistently shown a considerably lower density of high affinity binding sites than those using antagonists (see Lanza and Cazenave, 1987, for a table), and this binding, as expected for a G protein-linked receptor, is modulated by guanine nucleotides. Binding sites for agonists appear to exist in two interconvertible forms, low and high affinity, and agonist binding is reduced by GTP or its more stable analogue GMP-PNP, showing that agonists bind preferentially to the high affinity state. When labeled antagonists are displaced by agonists, the inhibition curves are shallow or biphasic and can be shifted and made steeper (corresponding to conversion from the high affinity to the low affinity form) by GMP-PNP (Lynch and Steer, 1981; Hoffman et al., 1981, 1982; Tsai and Lefkowitz, 1979; Shattill et al., 1981; Garcia-Sevilla and Fuster, 1986).

The platelet α_2-receptor has been solubilised using digitonin and retains its ability to bind [^{3}H]yohimbine (Smith and Limbird, 1981; Michel et al., 1981). The receptor was partially purified using a lectin column, implying that it is a glycoprotein (Smith and Limbird, 1982), and using affinity columns containing α_2-selective ligands (Regan et al., 1982, 1984, 1986a). The molecular mass of the partially purified receptor was estimated to be 64 kDa (Regan et al., 1986a), whereas radiation inactivation analysis in platelet membranes gave an estimate of 160 kDa for the native receptor complex (Venter et al., 1983). The α_2-adrenoceptor has been reconstituted in lipid vesicles containing G proteins and, in the presence of G_i, possesses GTPase activity which is stimulated by adrenaline and inhibited by phentolamine (Cerione et al., 1986). The partly purified receptor preparation has also been investigated with an α_2-selective photoaffinity reagent which specifically and irreversibly labeled the 64-kDa protein (Regan et al., 1986b). Recently, the gene for the α_2-adrenoceptor on platelets has been cloned, sequenced, and expressed in frog oocytes, where it bound [^{3}H]yohimbine with a K_D of 2.5 nM and was found to have the expected structural characteristics of a G protein-coupled receptor (Kobilka et al., 1987).

3. Uptake and metabolism of catecholamines. Platelets can accumulate various catecholamines including adrenaline, noradrenaline, and dopamine, but the mechanism for this is not clear (see Sneddon, 1973; Drummond, 1976; Stahl, 1985, for reviews). Platelets have an active uptake system for 5-HT which can be inhibited by tricyclic antidepressants such as imipramine (see section IIC), but accumulation of catecholamines, although it is inhibited by high concentrations of such inhibitors and by metabolic poisons, is probably due to passive diffusion rather than active uptake (Stahl, 1985). Most studies have shown that uptake of catecholamines is not saturable but is proportional to the extracellular concentration, and the inhibitory potencies of the tricyclic antidepressants do not match their potencies as inhibitors of amine uptake in neurones (Born and Smith, 1970; Ahtee and Saarnivaara, 1971; Abrams and Solomon, 1969; McLean and Potoczak, 1969; Stahl and Meltzer, 1978). Platelets also contain monoamine oxidase type B, which is capable of metabolising catecholamines (Youdim, 1988), but lack catechol-O-methyltransferase (Lahovaara et al., 1968).

4. Physiological and pathological importance. The concentrations of adrenaline, the most likely physiological agonist, needed to induce platelet aggregation are in the micromolar range, considerably higher than the highest concentrations ever likely to be found in the circulation;

the latter are in the nanomolar range (Roizen et al., 1975). This suggests that aggregation induced by adrenaline is unlikely to have any significance in vivo, especially because it only occurs at reduced extracellular Ca^{2+} concentrations (Glusa and Markwardt, 1980; Lages and Weiss, 1981), and donors whose platelets do not aggregate to adrenaline do not have any obvious haemostatic disorder (Scrutton et al., 1981). Even the proaggregatory response to adrenaline occurs at concentrations unlikely to be reached in vivo, but addition of other proaggregatory agonists, such as 5-HT and vasopressin, brings the required adrenaline concentration within a plausible range and probably reflects the situation in blood more accurately, suggesting that circulating adrenaline may have a role in sensitising platelets (Culliver and Ardlie, 1981).

Many investigators have looked at platelet responsiveness to adrenaline and/or the number and affinity of platelet α_2-adrenoceptors in various physiological and pathological states, but the results are often contradictory and no very clear pattern has emerged (for tables see Kerry and Scrutton, 1985, and Lanza and Cazenave, 1987). In vitro, prolonged exposure to adrenaline results in a specific desensitisation, initially without a change in receptor density but eventually with a significant decrease in receptor *density*, if measured using [^{3}H] dihydroergocryptine (Cooper et al., 1978a), or receptor *affinity*, if measured using [^{3}H]yohimbine (Karliner et al., 1982). In vivo, pathological conditions resulting in altered circulating catecholamine levels, such as pheochromocytoma, have been reported by some workers to result in the appropriate alterations in receptor density (Davies et al., 1982), but other studies have given contradictory results and the reasons for these discrepancies is unknown (Lanza and Cazenave, 1987). Similar studies of cardiovascular and neurological disorders and alterations in steroid hormone levels have given varied and inconsistent results (Lanza and Cazenave, 1987; Kerry and Scrutton, 1985). Any true changes in receptor number would presumably have to occur at the megakaryocyte because platelets are anucleate and, therefore, incapable of synthesizing new protein.

C. 5-Hydroxytryptamine

5-HT (serotonin) aggregates human platelets at concentrations in the micromolar range, but this aggregation is variable and generally reversible, and concentrations greater than approximately 10 μM give a reduced response (Mitchell and Sharp, 1964; Baumgartner and Born, 1968). The response is species dependent and, unlike responses to adrenaline and to ADP, is somewhat reduced, rather than enhanced, by chelation of Ca^{2+} with citrate (Drummond, 1976). 5-HT added together with or soon before other aggregating agents such as ADP potentiates them, but if added more than a few minutes before inhibits aggregation, both effects being receptor-mediated (Baumgartner and Born, 1968; Michal and Motamed, 1975).

The platelet 5-HT receptor is coupled to phospholipase C, and receptor activation results in rapid phosphoinositide breakdown, increases in intracellular Ca^{2+} levels, and appropriate protein phosphorylation (De Chaffoy de Courcelles et al., 1984, 1985, 1987, 1988; Affolter et al., 1984; Erne and Pletscher, 1985a). Desensitization of 5-HT receptors on human platelets, which then fail to aggregate following successive doses of 5-HT, has been shown to correlate with decreased mobilization of intracellular Ca^{2+}, apparently due to a negative feedback mechanism involving protein kinase C because stimulators of this enzyme (such as phorbol 12-myristale 13 acetate) inhibit 5-HT-induced phosphatidylinositol turnover and Ca^{2+} mobilization, and inhibitors of the enzyme (such as H-7) prevent inhibition of these two events (Watson and Lapetina, 1985; Kagaya et al., 1990). 5-HT does not appear either to stimulate or to inhibit adenylate cyclase in platelets (Haslam, 1975; Laubscher and Pletscher, 1980). For recent reviews of 5-HT receptors on platelets, see Pletscher (1987), Stahl (1985), De Clerck and Herman (1983), Leysen (1988), Scrutton and Thompson (1987), and De Chaffoy de Courcelles et al. (1988).

1. Structure-activity relationships. Early studies showed that 5-HT-induced aggregation is inhibited strongly by antagonists such as LSD and methysergide but weakly by drugs such as atropine and morphine, suggesting that the 5-HT receptor was a "D-type" 5-HT receptor rather than an "M-type" as defined by Gaddum and Picarelli (1957) (see Drummond, 1976, for review). Many of the compounds reported to inhibit 5-HT-induced aggregation were not specific and, therefore, not useful for classifying receptors, particularly because the classification itself became unsatisfactory. However, the classification of 5-HT receptors has now become better defined, although more complex with the development of more selective ligands, and currently three major types of 5-HT receptor are recognised, 5-HT_1, 5-HT_2 and 5-HT_3, with 5-HT_1 being subdivided into four classes (see Fozard, 1987). The platelet 5-HT receptor appears to be a 5-HT_2 receptor and aggregation is inhibited specifically by ketanserin, the selective 5-HT_2 antagonist (De Clerck et al., 1982). The potency of a range of antagonists to inhibit 5-HT-induced platelet aggregation correlated well with their ability to inhibit [^{3}H]ketanserin binding to rat brain 5-HT_2 receptors, suggesting that platelet receptors are similar to 5-HT_2 receptors in the CNS (De Clerck and Herman, 1983; De Clerck et al., 1984b). Further evidence for the presence of a 5-HT_2 receptor comes from radioligand-binding studies on platelets (see below). It has also been suggested that platelets may possess 5-HT_{1A}-like receptors, inhibiting 5-HT release in a manner similar to presynaptic inhibition of transmitter release,

because 5-HT_{1A} agonists inhibit ADP- and collagen-induced 5-HT release (Youdim et al., 1990).

2. *Binding studies and receptor isolation.* Early studies in which [^{3}H]5-HT was used as the radioligand demonstrated the existence of three binding sites on rat platelets; the one with the highest affinity was identified as the receptor rather than the uptake site (middle affinity) because there was a good correlation overall between the ability of antagonists to inhibit binding and to inhibit 5-HT-induced shape change (Drummond and Gordon, 1975a,b). The receptor density was estimated to be 2500 per platelet and the K_D was 6.5 nM, but these parameters are hard to measure accurately from a Scatchard plot of three independent binding sites, and observed K_D values for 5-HT and other agonists were at least 100 times lower than their EC_{50} values for inducing aggregation (Drummond, 1976). Other workers using [^{3}H]5-HT have also reported binding to two saturable sites, one with high affinity but low capacity, representing the receptor, and one being the uptake site (Peters and Grahame-Smith, 1980), but others have failed to detect 5-HT receptors on platelets using this ligand which appeared to label only the uptake site (McBride et al., 1983).

Because of its multiple interactions with platelets, its rapid uptake into platelets and its release from platelets, 5-HT itself may not be the best ligand to use. In addition, binding studies in other tissues have shown that 5-HT_2 receptors bind agonists with low affinity (micromolar) and antagonists with high affinity (nanomolar), and the opposite is true of 5-HT_1 receptors (Peroutka and Snyder, 1979; Leysen, 1988). [^{3}H]5-HT is, therefore, a better ligand for 5-HT_1 receptors and, therefore, would not be expected to label platelet 5-HT_2 receptors reliably, and recent studies have used radiolabeled antagonists instead. Although the ^{3}H-labeled 5-HT_{1A}-selective agonist 8-hydroxy-2-(di-n-propylamino)tetralin does bind to human platelets, this binding is to the 5-HT uptake site rather than to a pharmacological receptor (Ieni and Myerson, 1988).

The rather nonselective antagonist [^{3}H]spiperone showed saturable binding to intact human platelets (K_D 2.7 nM), and this binding was displaced by 5-HT (IC_{50} 2.3 μM) and by 5-HT_2 antagonists such as ketanserin (IC_{50} values in the nanomolar range) but not by the 5-HT uptake blocker imipramine or the dopamine D_2 antagonist sulpiride. A good correlation was found between the ability of drugs to displace binding to human platelets and to human frontal cortex, and the binding site resembled a 5-HT_2 site (McBride et al., 1983).

[^{3}H]LSD also binds specifically to human platelet membranes, with a K_D of 0.53 nM, and the structure-activity relationships for displacement of binding were similar to those found in human frontal cortex and are consistent with it being a 5-HT_2 receptor; antagonists have a higher affinity than do agonists. There was a good correlation among the antagonists between inhibition of [^{3}H]LSD binding and 5-HT-induced shape change but not for 5-HT uptake, showing that this binding site does represent the functional receptor. However, the affinity of 5-HT in this study was not affected by the presence of GTP or GMP-PNP, which is rather unexpected (Geaney et al., 1984). An analogue with high specific activity, [^{125}I]2-iodo-LSD, has also been used to label the 5-HT_2 receptor on platelet membranes obtained from a variety of donors, some of whom had psychiatric disorders, and a correlation was found between the receptor density and the sensitivity of the platelets to 5-HT (McBride et al., 1987).

[^{3}H]Ketanserin binds to cat platelet membranes, and the characteristics of this binding appear to be identical with those of 5-HT_2 receptors on rat prefrontal cortex and striatum, and inhibition of this binding correlates well with inhibition of 5-HT-induced cat platelet aggregation and phospholipase C activation (Leysen et al., 1983; De Clerck et al., 1984c; De Chaffoy de Courcelles, 1985; Leysen, 1988). Cat platelets are more sensitive than human ones to 5-HT, and some studies with this ligand using human platelets have been unsuccessful; this finding has been attributed to a high level of nonspecific binding and the apparently low density of receptors detected (approximately 20 per platelet) (Leysen et al., 1983; Leysen, 1988). However, when high platelet concentrations or membrane preparations were used and filters were pretreated to reduce nonspecific binding, [^{3}H]ketanserin-binding sites with the characteristics of 5-HT_2 receptors were detected on human platelets (Biegon et al., 1987). Even in cat platelets and rat striatum, approximately 50% of the binding is nonspecific but saturable, being displaced only by unlabeled ketanserin or close structural analogues, and these nonreceptor ketanserin-binding sites may be involved in the release of biogenic amines by ketanserin from platelets and monoaminergic neurones (Leysen et al., 1988). A high affinity [^{3}H]ketanserin-binding protein complex of 232 kDa has been solubilized from pig platelets, and the affinities of 5-HT antagonists for this protein correlated with their affinities for the receptors in intact membranes. There was also a low affinity-binding site, the significance of which is unknown, but which may be similar to the nonreceptor site detected in other binding studies (Wesemann and Hoffmann, 1985).

Another 5-HT-binding site, which has been called the 5-HT_{1E} site, has been detected on human platelet membranes using [^{3}H]SCH 23390, a dopamine receptor D_1 antagonist which also binds to 5-HT_1 receptors, but the significance of this putative site is unknown (De Keyser et al., 1989). In addition, a 5-HT_{1A}-like binding site has been detected using the selective agonist [^{3}H]8-hydroxy-2-(di-n-propylamino)tetralin as a radioligand, and this site may mediate inhibition of 5-HT release (Youdim et al., 1990).

3. *Uptake and metabolism of 5-hydroxytryptamine.* The

uptake of 5-HT by platelets has been extensively studied because of interest in platelets as an easily obtained model for monoaminergic neurones in the CNS (e.g., Stahl, 1985; Pletscher, 1987, 1988; Langer and Galzin, 1988). 5-HT is taken up by passive diffusion and by a high affinity (K_m approximately 1 μM) energy-dependent carrier that requires Na^+ and Cl^- and is inhibited by ouabain, suggesting that 5-HT may be cotransported with Na^+ down its concentration gradient (Drummond, 1976; Stahl, 1985).

5-HT uptake is inhibited powerfully by tricyclic antidepressants such as imipramine (IC_{50} values in the nM range), and the potencies of these compounds parallel their potencies as inhibitors of 5-HT uptake in brain slices and synaptosomes (see Stahl, 1985, for tables). [^{3}H]Imipramine binds specifically to platelet membranes with an affinity in the nM range, and this binding is displaced by 5-HT uptake blockers, but not by 5-HT antagonists, and is similar to that found with synaptosomes (Briley et al., 1979; Paul et al., 1980; Stahl, 1985). This binding may, however, not be to the 5-HT recognition site but elsewhere on the uptake system (Segonzac et al., 1985a; Langer and Galzin, 1988; Pletscher, 1987), and, indeed, the kinetics of inhibition of 5-HT transport are not always purely competitive (Lingjaerde, 1985).

A detailed study of [^{3}H]imipramine binding to human platelet membranes revealed the presence of high affinity (K_D 1 nM; approximately 1430 sites per platelet) and low affinity (K_D 144 nM; approximately 4900 sites per platelet) binding sites. These were shown by a series of chemical treatments to be heterogeneous and enabled a model of the high affinity imipramine recognition site to be constructed that correlated relative displacement of imipramine binding by various agents with their relative structural and spatial deviations from the aryl and amine portions of the imipramine molecule (Formenko et al., 1990). [^{3}H]Imipramine binding is usually conducted at 0°C, because its high rate of dissociation precludes use of a filtration assay at physiological temperature, and this makes comparison with inhibition of 5-HT uptake conducted at 37°C difficult. However, radiolabeled paroxetine or 6-nitroquipazine binding to the uptake site is feasible at 37°C, and [^{3}H]6-nitroquipazine identified a single population of uptake sites (K_D 0.45 nM; approximately 1630 sites per platelet), and displacement of binding by various uptake inhibitors matched their inhibition of 5-HT uptake into intact human platelets (Hashimoto and Goromaru, 1990). 5-Methoxytryptoline, a competitive inhibitor of [^{3}H]imipramine binding, has been reported to be an endogenous 5-HT uptake blocker, but the significance of this is not clear (Segonzac et al., 1985b; Langer and Galzin, 1988).

5-HT is also actively taken up into the dense granules, probably in exchange for H^+ traveling down its concentration gradient, and uptake is inhibited by drugs like reserpine (Stahl, 1985; Da Prada et al., 1988). 5-HT is stored in the vesicles with nucleotides and divalent cations, and these are released by exocytosis following platelet activation as discussed in the "Introduction." Metabolism of 5-HT in platelets is by mitochondrial monoamine oxidase B, with a greater enzyme susceptibility to inhibition by deprenyl than by clorgyline but a rather low affinity for 5-HT (Murphy and Donnelly, 1974). Metabolism of 5-HT occurs during uptake and release, but only to a small extent (Da Prada et al., 1965), and interest in platelet monoamine oxidase is mainly in its use as an accessible model of the CNS enzyme (e.g., Da Prada et al., 1988; Youdim, 1988). Other metabolising enzymes, such as N-acetyltransferase, have been found in rabbit platelets, which can synthesise melatonin, but the significance of this is unclear (Launay et al., 1982).

4. Physiological and pathological importance. Because platelets take up, store, release, metabolise, and respond to 5-HT, they have been widely used as model systems to study the less accessible CNS serotonergic systems in a variety of psychiatric conditions (Stahl, 1985; Da Prada et al., 1988; Pletscher, 1988; Wirz-Justice, 1988). The role of 5-HT in platelet and vascular physiology and pathology is not clear, although the release of 5-HT during platelet activation is obviously one of the amplification mechanisms by which aggregation is enhanced and circulating platelets recruited to a thrombus. In cats, ketanserin has been shown to inhibit collagen-induced platelet activation in vivo, supporting a role for released 5-HT in this process (De Clerck et al., 1990) Released 5-HT will also affect blood vessel tone in a complex manner, normally causing contractions via 5-HT_2 receptors but also endothelium-dependent relaxations via 5-HT_1 receptors (Houston et al., 1985, 1986). 5-HT is, therefore, one of the factors in platelet-vessel wall interactions that tend to ensure that, if platelets aggregate in intact blood vessels, vasodilation occurs and will limit occlusive damage, whereas if they aggregate in damaged blood vessels, vasoconstriction occurs and will reduce blood loss (see De Clerck et al., 1984a; Vanhoutte, 1988, for reviews). Platelet-derived 5-HT has been implicated in some cardiovascular disorders characterized by impaired tissue perfusion and vasoconstriction, such as Raynaud's phenomenon, hypertension, and diabetes mellitus, although other factors are obviously involved as well (De Clerck et al., 1984a).

D. Vasopressin

Vasopressin was first shown to aggregate platelets by Haslam and Rosson (1972), who showed that the sensitivity of human platelets depended on the anticoagulant used, with little effect seen in citrated plasma, apparently because of a requirement for extracellular Ca^{2+} or Mg^{2+}. More recent work suggests that extracellular Mg^{2+} is more effective than Ca^{2+} in supporting vasopressin-induced shape change and phospholipase C activation (Erne and Pletscher, 1985b; Siess et al., 1986) and that Mg^{2+} enhances binding of vasopressin to platelet recep-

tors (Thibonnier et al., 1987). Vasopressin is capable of inducing irreversible aggregation and the release of dense granule contents but does not inhibit adenylate cyclase in intact platelets and is, therefore, much more powerfully inhibited than ADP by agents such as adenosine and PGE_1 which act by stimulating adenylate cyclase (Haslam and Taylor, 1971; Haslam and Rosson, 1972). In platelet particulate fractions, vasopressin can inhibit adenylate cyclase by a receptor-mediated, GTP-dependent process (Vanderwel et al., 1983), although this inhibition varies widely between donors and has been suggested to involve a G protein distinct from G_i (Vittet et al., 1988b). For a review of vasopressin receptors on platelets, see Scrutton et al. (1987).

1. Structure-activity relationships. The vasoactive peptides bradykinin and angiotensin II are inactive, and oxytocin is a weak aggregating agent (Haslam and Rosson, 1972); the latter compound may be a partial agonist because it has been reported to inhibit selectively vasopressin-induced aggregation (Mills and Macfarlane, 1976). Effective agonists have a basic L-amino acid at the 8 position, and activity is reduced by replacement with a neutral amino acid and abolished by replacement with a basic D-amino acid (Scrutton et al., 1987). [8-Lysine] and [8-ornithine]-vasopressins are as potent as AVP, and [2-phenylalanine, 8-lysine]-vasopressin is rather more potent, suggesting that the effects on platelets are more similar to the vasoconstrictor effect of vasopressin (V_1 receptors) than to its antidiuretic effect (V_2 receptors) (Haslam and Rosson, 1972). That a V_1 receptor is indeed involved was shown by the inhibition of vasopressin-induced aggregation by V_1-selective antagonists such as $d(CH_2)_5[Tyr(Me)^2]AVP$ and dVDAVP, and the failure of V_2 agonists, such as 1-deamino-[8-D-arginine]-vasopressin to induce aggregation and their ability to act as antagonists instead (Vanderwel et al., 1983; Thomas et al., 1983).

The pA_2 values for inhibition of vasopressin-induced aggregation of the V_1 antagonist $d(CH_2)_5[Tyr(Me)^2]AVP$ and the V_2 antagonist $d(CH_2)_5[D\text{-}Ile^2Ala^4]AVP$ are in close agreement with their pA_2 values for inhibition of the pressor action of vasopressin, implying that the V_1 receptors are similar for both effects (Thibonnier and Roberts, 1985). A correlation has been noted for five antagonists between their effect on aggregation and on the pressor effects of vasopressin, although detailed differences may exist (Scrutton et al., 1987). Vasopressin V_1 receptors have recently been subdivided using new synthetic analogues, and platelet V_1 receptors appear to be of the V_{1A} subtype, similar to those on the liver and vasculature rather than the V_{1B} subtype as found in the anterior pituitary (Launay et al., 1987).

The same V_1 receptors appear to be responsible for aggregation and for the weak inhibition of adenylate cyclase in platelet membrane preparations, because the pA_2 values for inhibition of these effects by the V_1 antagonist dVDAVP are similar, in spite of the fact that the EC_{50} values for AVP are different for the two effects (27 nM and 1.2 nM, respectively) (Vanderwel et al., 1983). [The V_1 antagonist $d(CH_2)_5[Tyr(Me)^2]AVP$ also inhibits the stimulation by vasopressin of phosphatidic acid production and Ca^{2+} mobilization, showing that V_1 receptors on platelets, as elsewhere, are coupled to phospholipase C activation (Pollock and MacIntyre, 1986). The phosphorylation of platelet proteins seen in response to vasopressin has also been demonstrated, using selective agonists and antagonists, to be a V_1 effect (Thibonnier et al., 1987). The ability of AVP to stimulate human platelet membrane GTPase activity (as a measure of G protein involvement) is also a V_1 effect and was blocked by V_{1A}-selective antagonists (Vittet et al., 1988a).

2. Binding studies and receptor isolation. Binding sites having a K_D value of approximately 1 nM were detected on human platelets using [^{3}H]AVP, which is somewhat lower than the EC_{50} value for aggregation, which was reported to be 28 nM in the same series of experiments. The K_i values of $d(CH_2)_5[Tyr(Me)^2]AVP$ and $d(CH_2)_5[D\text{-}Ile^2Ala^4]AVP$ correspond to their pA_2 values for inhibition of both aggregation and the pressor effects of vasopressin, and for a series of agonists and antagonists there was a close correlation between their ability to inhibit [^{3}H]AVP binding and their in vivo vascular effect, but not their renal effects, confirming that the platelet-binding site is a V_1 receptor (Thibonnier and Roberts, 1985).

Investigators of the binding of [^{3}H]AVP to intact platelets also found a K_D of approximately 1 nM, a B_{max} corresponding to 95 sites per platelet, and a ligand specificity consistent with the presence of V_1 receptors. The K_D values for a series of analogues correlated with those determined on rat liver membrane V_1 receptors but not with those determined on rat kidney membrane V_2 receptors (Vittet et al., 1986). Binding was reversed by ethylenediaminetetraacetic acid and could be restored by Mg^{2+} or Mn^{2+}, but not by Ca^{2+}, but was not affected by GTP or GMP-PNP, suggesting that the platelet V_1 receptor may not be linked to phospholipase C via a G protein, at least under the experimental conditions used to measure binding (Vittet et al., 1986; Thibonnier et al., 1987); other studies, however, have suggested a role for G proteins in the actions of vasopressin (Vittet et al., 1988a,b).

[^{125}I]AVP has also been used in binding studies and one class of vasopressin-binding sites was detected in platelet lysates at a density of approximately 75 sites per platelet with a K_D of 5.6 nM; these values are similar to the results found with [^{3}H]AVP (Berrettini et al., 1982). The receptor was proposed to be similar to that in the renal medulla (V_2), but appropriate selective ligands were not used and all other available evidence suggests otherwise (Scrutton et al., 1987).

The human platelet vasopressin receptor has been

solubilised and it binds [^{3}H]AVP with a K_D of 11 nM, which is approximately 10 times higher than the K_D in membrane preparations (approximately 1 nM) (Thibonnier, 1987a). The K_D values of the V_1 and V_2 antagonists, d(CH$_2$)$_5$[Tyr(Me)^2]AVP and d(CH$_2$)$_5$[D-Ileu^{2}Ala^4]AVP, were also somewhat higher than might be expected in the membrane preparation but were consistent with the solubilised protein being a V_1 receptor (Thibonnier, 1987a). The receptor has also been photoaffinity labeled using [^{3}H]AVP itself, and the labeled receptor was solubilised and identified as a 125-kDa protein; labeling could be suppressed by an excess of cold vasopressin but not by angiotensin II (Thibonnier, 1987b).

3. Physiological and pathological importance. The significance of vasopressin-induced platelet aggregation remains unclear, because the concentration required is far higher than that found in human blood, even in conditions in which the blood vasopressin levels are elevated (Haslam and Rosson, 1972; Scrutton et al., 1987). ADP and adrenaline can potentiate the effects of vasopressin; therefore, vasopressin might have subtle effects on platelet responsiveness in combination with other weak agonists (Launay et al., 1987).

E. Prostaglandins and Thromboxane

Platelets metabolise free arachidonic acid largely via the aggregating agents, PGG_2 and PGH_2 (the prostaglandin endoperoxides), to the potent TXA_2. Platelet arachidonic acid release and metabolism are triggered by strong aggregating agents such as thrombin and collagen and by the act of aggregation itself (see Longenecker, 1985b, and Roth, 1986, for reviews of platelet arachidonate metabolism). The study of the receptors mediating aggregation induced by the endoperoxides and by TXA_2 is complicated by their instability. PGH_2 has a half-life of approximately 5 min and is rapidly converted, mainly by thromboxane synthetase to TXA_2 (which breaks down to the inactive TXB_2 with a half-life of <1 min), but it can also be converted into the inhibitory PGD_2 (see section IIIB) as well as the essentially inactive PGE_2 and $PGF_{2\alpha}$. It is, however, generally accepted that PGH_2 is an aggregating agent in its own right, although less potent than TXA_2, because it can still induce aggregation in the presence of dazoxiben, a thromboxane synthetase inhibitor (see Jones et al., 1985, for discussion). Because of the problems with using the natural agonists, most investigators of the stimulatory prostaglandin receptor on platelets have used stable synthetic analogues.

TXA_2 causes shape change, aggregation, and release of granule contents and is now thought to act via membrane receptors coupled by a G protein to phospholipase C (see MacIntyre et al., 1987, for review), although it has also been proposed to act as an intracellular ionophore releasing Ca^{2+} directly (Gerrard et al., 1981). A new TXA_2 receptor antagonist, [1r-[1α(Z),2β,3β,5α]-(+)-7-[5[[1,1′-biphenyl) -4-yl]methoxy] -3-hydroxy-2- (1-piperidinyl)cyclopentyl]-4-heptenoic acid HCl (GR32191), appears to bind to a single site where it irreversibly inhibits receptor-mediated phospholipase C activation, platelet aggregation, and secretion but reversibly inhibits shape change and Ca^{2+} mobilization; these are actions consistent with the existence of a single TXA_2 receptor coupled to two effector systems (Takahara et al., 1990). TXA_2 also inhibits platelet adenylate cyclase via G_i (Avdonin et al., 1985), presumably via the same receptor as that inducing aggregation, because both effects are inhibited by 13-APA, a TXA_2 antagonist (Bonne et al., 1980), although this effect has not been thoroughly investigated. Although some stable analogues of PGH_2 were originally reported to be without effect on adenylate cyclase (Mills and Macfarlane, 1977), more recent studies have shown that they, like the natural agonists, can inhibit this enzyme (Bonne et al., 1980; Avdonin et al., 1985).

Because TXA_2 is the most potent natural agonist, we will refer to the platelet receptor as the TXA_2 receptor, although some workers refer to it as the TXA_2/PGH_2 or TXA_2/endoperoxide receptor. Several recent reviews have been published concerning various aspects of this topic (Jones et al., 1987; Myers et al., 1985; Lagarde, 1987; MacIntyre et al., 1987; MacIntyre and Armstrong, 1987; Halushka et al., 1987, 1989).

1. Structure-activity relationships. Prostanoid receptors have been classified recently into five types (with some potential subtypes), mainly on the basis of agonist potencies but with some supporting evidence from antagonists. The receptors have been called P receptors, with the natural prostanoid to which they are most sensitive indicated by a preceding letter, so that there are EP, FP, TP, IP, and DP receptors sensitive to PGE_2, $PGF_{2\alpha}$, TXA_2, PGI_2 and PGD_2, respectively (see Coleman et al., 1984; Halushka et al., 1989). The platelet TXA_2 receptor is, therefore, by definition a TP receptor, and platelets also possess inhibitory IP and DP receptors (see section IIIB) which can complicate the study of structure-activity relationships.

Several studies of the structure-activity relationships for agonists have been carried out (see fig. 3 for some examples of structures), and detailed accounts have been published, although no clear picture of the structural requirements has emerged [Smith et al., 1977; MacIntyre et al., 1978; MacIntyre, 1981; Jones et al., 1985 (unfortunately, in this paper the diagrams of the structures of the analogues appear to have been rotated so that they do not match their correct names); Myers et al., 1985; MacIntyre and Armstrong, 1987].

Although arachidonic acid (20:4ω6) is the major substrate of cyclooxygenase, prostanoids can be formed from the related fatty acids dihomo-γ-linolenic acid (20:3ω6) and eicosapentaenoic acid (20:5ω3), giving rise to the 1 and 3 series of prostanoids, respectively. TXA_1, TXA_3, PGH_1, and PGH_3 have little aggregating activity, however (Willis, 1974; Raz et al., 1977), and this finding has been invoked to explain the apparent antithrombotic

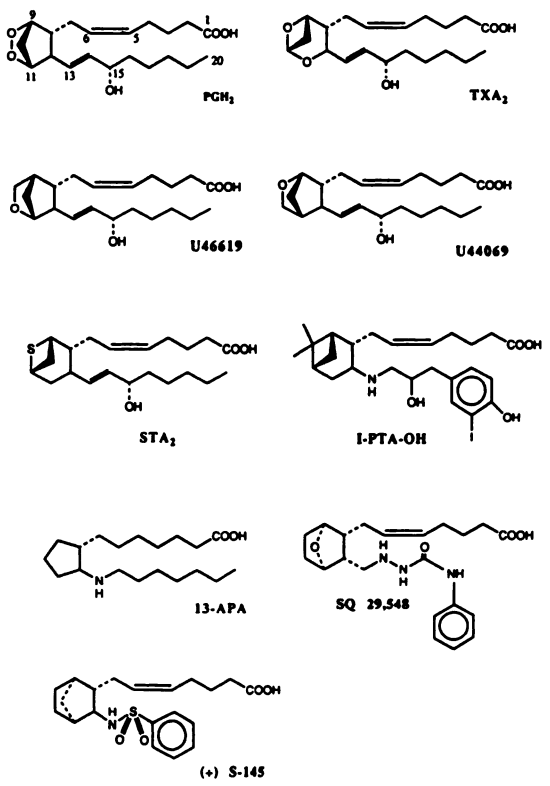


FIG. 3. Some examples of platelet TXA_2 receptor agonists and antagonists.

effects of dietary fish, which contain high concentrations of eicosapentaenoic acid (Dyerberg and Bang, 1979). Early work suggested that the minimum structure needed to induce aggregation was the monocyclic 9,11-dideoxy-PGH_2, although this compound is approximately 25 times less active than PGH_2 itself. Activity is much enhanced by a 9,11 linkage to form a bicyclic compound, as in 11,9-epoxymethano-PGH_2 (U46619) and 9,11-epoxymethano-PGH_2 (U44069), in which one of the ring oxygens has been replaced by a methylene group, thus forming potent analogues that have become widely used as stable reference agonists (Smith et al., 1977). In a detailed study (MacIntyre et al., 1978; MacIntyre, 1981), it was found that, although the monocyclic PGE_2 and $PGF_{2\alpha}$ are themselves inactive, some of their analogues can induce aggregation, this activity being enhanced by C_{11} deoxygenation and C_{15} or C_{16} alkylation. Some stereoselectivity is observed toward the C_{15} position, because the *S* isomers (the natural configuration) are more potent than the *R* isomers. All the bicyclic PGH_2 analogues investigated were active as long as they retained a C_{15} hydroxy residue, the nature of the linkage between C_9 and C_{11} of the ring being apparently not very important. MacIntyre et al. found that their results were consistent with the idea that a "hairpin" conformation with multiple points of contact with the receptor was necessary for activity, because substitutions that disturbed this conformation resulted in loss of activity (MacIntyre et al., 1978; MacIntyre, 1981).

Elongation of the α-chain or shortening of either α- or side chains eliminates activity, whereas the ω-chain can be elongated with retention of activity (LeDuc et al., 1981). Replacement of the terminal butyl group on the ω-chain by a *p*-fluorophenoxy group greatly increases activity, and indeed EP 171, the 16-*p*-fluorophenoxy derivative of 9,11-oxy-10α-homo-PGH_2, is the most potent analogue yet found, although responses are rather slow in onset (Jones et al., 1985, 1987).

Stable analogues of TXA_2 rather than of PGH_2 have also been synthesized, but CTA_2, in which the two linking oxygens in the six-membered ring have been replaced by methylene groups, and the related analogue PTA_2, in which two methyl groups are substituted on to one of these methylene groups, are only weakly active and also have an inhibitory effect and, therefore, appear to be partial agonists, although because they also increase cyclic AMP levels in platelets, this may contribute to their inhibitory activity (Lefer et al., 1980; Nicolaou et al., 1979; Armstrong et al., 1985; Jones et al., 1985). STA_2 (ONO 11113), a stable analogue in which only one oxygen is replaced by a methylene group and the other by sulphur to form 9,11-epithio-11,12-methano-TXA_2, is a very potent aggregating agent (Katsura et al., 1983) and has also been shown to increase cytoplasmic Ca^{2+} levels and cause phosphorylation of platelet proteins (Kawahara et al., 1983).

There are some differences between the structure-activity requirements for platelet aggregation and for contraction of various smooth muscle preparations; for example, STA_2 has been reported to be more potent than U46619 in the human and canine saphenous vein but less potent in human platelets (Mais et al., 1985d). In particular, CTA_2 is an effective vasoconstrictor that has little agonist activity on platelets, suggesting that the TP receptors mediating these effects may be different (Lefer et al., 1980), although because CTA_2 was reported to be an antagonist in platelets only differences in efficacy may be involved. Some stable difluoro-TXA_2 analogues have also been reported to be agonists in the canine saphenous vein while acting as antagonists at the human platelet TXA_2 receptor (Morinelli et al., 1989b). A study of 9,11-aza analogues of PGH_2 also showed differences in structure-activity relationships between platelets and blood vessels and concluded that the receptors were different, but because these compounds were thromboxane synthetase inhibitors these conclusions are not clear-cut (Gorman et al., 1981).

The suggested difference in receptors has been challenged and CTA_2 has been shown in other studies to have partial agonist activity for both effects and also to increase cyclic AMP levels in platelets, which complicates interpretation of its action (Armstrong et al., 1985). Indeed, a study of the potencies on various tissues of the TP agonists EP171, STA_2, and U46619, as well as of the FP agonists $PGF_{2\alpha}$ and ICI 81008, led to the conclusion

that, based on agonist potencies at least, there is no evidence that subtypes of the TP receptor exist, as well as confirming the absence of FP receptors on platelets (Jones et al., 1987). This study found STA_2 to be more potent than U46619 on all preparations tested, including the dog saphenous vein; these results differ from those reported by Mais et al. (1985d).

As well as CTA_2 and PTA_2 which have inhibitory effects, other synthetic analogues (see fig. 3 for some examples of structures) act as competitive antagonists at the platelet TXA_2 receptor. The first one reported was 13-APA, which inhibits primary aggregation induced by arachidonic acid, PGH_2, and U44069 but not aggregation produced by ADP or by thrombin; 13-APA is not an inhibitor of cyclooxygenase or of thromboxane synthetase (Le Breton et al., 1979). A study of a series of analogues of 13-APA as inhibitors of the effects of arachidonic acid and of U46619 on human platelets and on rat aorta showed that antagonistic activity was retained when the five-membered ring was expanded to a six-membered ring as occurs in 11α-homo-13-APA (which is more similar to the structure of PGH_2 and TXA_2) and that the *trans* isomer of 13-APA was approximately twice as effective as the *cis* isomer; no stereoselectivity was observed for the isomers of 11α-homo-13-APA. The authors concluded that the receptors in platelets and aorta are similar, although in the aorta the *cis* and *trans* isomers of 13-APA were equally effective and the rank orders of potency were not identical; however, because only IC_{50} values were quoted for inhibition of aggregation, comparisons are not easy to make (Huzoor-Akbar et al., 1985).

A study of five related 13-aza analogues of PTA_2 having phenyl rings in the side chain as inhibitors of the effects of U46619 in human and canine platelets and in saphenous vein found that the rank orders of potency in platelets was different from that in veins in both species. It was concluded that the receptors in these tissues were different and the authors called those in platelets (TXA_2/PHG_2)α and those in veins (TXA_2/PGH_2)τ (Mais et al., 1985d). Again, however, only IC_{50} values were quoted, and no evidence was presented that the antagonists were truly competitive. An extension of this study in which three of these compounds were resolved into their 15-hydroxy epimers found that the orientation of the group did not influence their activity on platelets but did in veins, confirming the authors' belief in the proposed receptor subdivision (Mais et al., 1985b). These authors have recently studied 16 of these 13-aza analogues of PTA_2, calculating K_B values for inhibition of the effect of U46619 on human platelets and canine saphenous veins and checking competitivity by the use of Schild plots. All 16 were antagonists on platelets, and 12 were antagonists and 4 partial agonists on veins. Of the 12 that were antagonists in both systems, there was no correlation between their K_B values for each effect, thus providing good evidence for the existence of different receptor subtypes (Mais et al., 1988). This study also showed that in platelets antagonist potency increased with increasing length of the linking group replacing C-16 on the ω-chain and that substitution on the phenyl group affected activity, para-substituted analogues being more potent than meta-substituted ones and halogen analogues being more potent than amino derivatives (Mais et al., 1988). More recently, further evidence for heterogeneity of TXA_2 receptors has been provided by comparing pA_2 values (derived from Schild plots) of a number of TXA_2 antagonists on rabbit, rat, and human platelets and on guinea pig trachea and on rat and rabbit aorta (Tymkewycz et al., 1991). Although excellent agreement was found for agonist potencies between these six preparations, only weak or not significant correlations was seen between any pair of smooth muscle preparations and between any pair of platelet/smooth muscle preparations. This finding indicates that, although the agonist recognition sites may be similar or identical, the receptors were not homogeneous.

Another series of antagonists are the analogues EP 092 and EP 045, which have a bicyclic heptane headgroup (similar to PGH_2 but with the endoperoxide group replaced by methylenes) and a modified ω-chain ending in a phenyl group. EP 045 appeared to be a competitive antagonist, but the mode of action of EP 092 may be more complex because agonist dose-response curves were flattened rather than simply displaced to the right, although this observation has been explained in terms of a slow dissociation from the receptor. The K_B values obtained were similar to those found in various smooth muscle preparations including the dog saphenous vein, implying either that the receptors are similar or that if there are receptor subtypes these analogues cannot distinguish between them (Armstrong et al., 1985; Jones et al., 1985). EP 045 has also been reported to inhibit U44069-induced phosphatidate formation and the elevation of cytoplasmic Ca^{2+} levels in platelets (Pollock et al., 1984). Some other compounds in this series, such as EP 035 and EP 157, as well as being TXA_2 antagonists also act as agonists at the PGI_2 receptor linked to adenylate cyclase, so, although of potential therapeutic interest, they are of little use in characterizing platelet receptors (Armstrong et al., 1986).

A series of analogues of PGH_2 containing a stable 7-oxabicycloheptane ring and aza-substituted modified ω-side chains led to the development of SQ 29,548 [the active (+)-enantiomer of the racemic mixture SQ 29,528, also known as SQ 28,053] which has a phenyl group at the end of the ω-chain and was reported to be a potent, selective TXA_2 antagonist with no effect on cyclooxygenase, thromboxane synthetase, or platelet adenylate cyclase, unlike other compounds in the series such as SQ 27,427 (Nakane et al., 1985; Ogletree et al., 1985; Harris et al., 1984). However, the inhibition was not completely

surmountable by high concentrations of the aggregating agent used, 9,11-azo-PGH_2, making it impossible to calculate a definite K_B value and suggesting that SQ 29,548 is not purely a competitive antagonist in human platelets, although it appears to be one in other tissues such as guinea pig trachea and rat aorta (Ogletree et al., 1985). Another study on human platelets, using U46619 as an agonist, did report a pA_2 value for SQ 29,548 derived from a Schild plot. The Schild plot appears to be somewhat steep, no value for the slope was reported, and from the shape of the dose-response curves shown it is not clear whether or not the inhibitor is truly competitive (Bush and Smith, 1986). In guinea pig platelets, however, SQ 29,548 was a competitive antagonist of the effects of U46619, as shown by a Schild plot, and its pA_2 value was very close to that observed in various guinea pig smooth muscle preparations, leading the authors to suggest that the receptors in these tissues were similar (Eglen and Whiting, 1988). Another PGH_2 analogue with a stable bicycloheptane headgroup and a substituted ω-chain with an aromatic ring at the end is the compound S-145, which is a selective inhibitor of the effects of U46619 on platelets, although its competitivity has not been established. It may be a weak partial agonist because it induces transient shape change and increases intracellular Ca^{2+} (Hanasaki and Arita, 1988a; Nakano et al., 1988).

There also exist several TXA_2 antagonists that are not obviously structurally related to TXA_2 or PGH_2, such as AH 23,848, AH 19,437, and BM 13,177 (Coleman et al., 1984; Brittain et al., 1985; Patscheke and Stegmeier, 1984; Bush and Smith, 1986; Jones et al., 1987). Because of the obvious therapeutic potential of a TXA_2 antagonist, many of the major pharmaceutical companies have been active in this field, and so we can expect many more compounds to be developed and pharmacologically defined, and the question of whether subclasses of TP receptors exist may finally be resolved.

2. Binding studies and receptor isolation. The stable agonist [^{3}H]9,11-epoxymethano-PGH_2 ([^{3}H]U44069) has been used as a ligand in studies of binding to human platelets and was shown to bind to two sites, one of which appears to be the TXA_2 receptor. It binds to approximately 1700 sites per platelet with a K_D of approximately 70 nM and can be displaced by agonists and antagonists but not by prostanoids known to be inactive on TXA_2 receptors. Calculation of occupancies suggested that 9,11-epoxymethano-PGH_2 must occupy all the receptors to induce irreversible aggregation, whereas 11,9-epoxymethano-PGH_2 (U46619) need only occupy approximately 40% of them (Armstrong et al., 1983a; Pollock et al., 1984; Jones et al., 1985). A single binding site was found (K_D 108 nM) at a density of 2200 sites/platelet using [^{3}H]U46619 as ligand, and binding was displaced by the antagonists SQ 29,548, ONO 3708, BM 13,177, and 13-APA with the same order of potency as for inhibiting aggregation (Kattelman et al., 1986). The authors also found that U46619 appeared to be accumulated by platelets in a competitive, but nondisplaceable manner, and suggested that platelets might contain an uptake system for prostaglandins as has been suggested for some other tissues (Kattelman et al., 1986). In another study in which [^{3}H]U46619 was used as radioligand showed two binding sites, occupancy of the one with the higher affinity (K_D 41 nM, 1166 sites per platelet) correlating with the concentrations required for shape change and protein phosphorylation but not the release reaction, which instead was related to the low affinity component (K_D 1.46 μM). Binding to both sites was inhibited by TXA_2 antagonists, although the K_D values differed from those previously reported (Morinelli et al., 1987). No nondisplaceable binding was observed in this study. In another study of the binding of [^{3}H]U46619 to intact rat platelets, only one site (K_D 37 nM, approximately 1000 sites per platelet) was found; binding to rat and human platelet membranes was studied and Mg^{2+} was found to enhance the affinity of the ligand. Binding was displaced by a series of antagonists (BM 13,177, ONO 11120, EP 045, ONO 3708, and SQ 29,548), and there was a good correlation between their inhibition of binding and their inhibition of collagen-induced aggregation; the latter effect has been suggested to be due largely to released PGH_2 and TXA_2. The rank order of the antagonists in the study was comparable with their ability to displace [^{3}H]SQ 29,548 binding in rat cultured aortic smooth muscle cells, suggesting that the TXA_2 receptors in these tissues are similar (Hanasaki and Arita, 1988b).

The binding of [^{3}H]U46619 to rat, rabbit, and human platelets was also found to be inhibited by the antagonist S-145 with a K_i value consistent with its ability to inhibit collagen-induced aggregation (Hanasaki and Arita, 1988a). More recently an ^{125}I-labeled TXA_2 agonist, I-BOP, a 7-oxabicycloheptane derivative, has been described as a radioligand for human platelet TXA_2 receptors, binding to one class of high affinity sites with a K_D of 2.2 nM and a B_{max} of 1699 sites per platelet and being displaced by a series of agonists and antagonists with a rank order of potency similar to their pharmacological activities (Morinelli et al., 1989a).

Antagonist binding studies using [^{3}H]13-APA and isolated human platelet membranes detected two classes of binding sites, with the higher affinity site (K_D 100 nM) having the characteristics of the receptor because binding was displaced by U46619 but not by TXB_2 and 6-keto $PGF_{1\alpha}$ which are inactive (Hung et al., 1983). However, binding was displaced by $PGF_{2\alpha}$ which Hung et al. (1982) claimed to be a TXA_2 antagonist, although it did not displace the binding of U44069 (Armstrong et al., 1983a); Armstrong et al. (1983a) found $PGF_{2\alpha}$ to have no action on TXA_2 receptors but did inhibit aggregation by increasing platelet cyclic AMP levels (Armstrong et al., 1983b; Jones et al., 1985). It has been pointed out that determination of the high affinity site binding [^{3}H]13-APA

appeared to rely on only two data points (Mais et al., 1985a), and doubt has been cast on the specificity of [^{3}H] 13-APA as a radioligand (Saussy et al., 1986).

Two ^{125}I-labeled antagonist ligands have been used in which ^{125}I has been substituted onto the phenyl ring at the end of the chain. One of these, an analogue of 13-APA, proved to have relatively low affinity (K_D 0.92 mM), although it did bind to human platelet membranes and revealed a single class of binding sites (Halushka et al., 1985). The other, a ^{125}I-labeled derivative of 13-aza PTA_2 ([^{125}I]-PTA-OH), has a higher affinity and has been used in binding studies with intact canine, guinea pig, and human platelets and human platelet membranes (Mais et al., 1985a,c; Saussy et al., 1986; Halushka et al., 1986). [^{125}I]-PTA-OH competitively inhibited aggregation induced by U46619 in all three species, and its K_B value determined pharmacologically matched its K_D value determined in the binding studies (approximately 10 to 20 nM). In canine platelets there were 4400 sites per platelet and low affinity binding was also detected (K_D 2.1 μM). The rank order of antagonists in displacing binding correlated with their potency in inhibiting aggregation induced by U46619; $PGF_{2\alpha}$ and PGE_2 only displaced the ligand at high concentrations (Mais et al., 1985c). In human platelets only one class of sites was detected with a B_{max} of 2530 sites per platelet, and again the structure-activity relationships for a series of antagonists was consistent with this site being the TXA_2 receptor (Mais et al., 1985a).

Similar results were found in human platelet membranes, although it was noted that, although the rank order of a series of agonists in displacing binding agreed with their order of potency as aggregating agents, in some cases the IC_{50} value for displacement was greater than the EC_{50} value for aggregation, which can be explained if these agonists have high efficacy so that spare receptors exist (Saussy et al., 1986). Indeed, Saussy et al. pointed out a similar discrepancy in the earlier binding studies by Armstrong et al. (1983a) who used [^{3}H]U44069 as a ligand. In guinea pig platelets, again only one class of binding site was detected with a B_{max} of 1927 sites per platelet, and again a series of antagonists displaced binding in the expected manner, whereas for a series of agonists differences were observed between their potency in causing aggregation and their ability to displace [^{125}I]-PTA-OH, the agonist U44069 in particular being much less potent as an aggregating agent than would be expected from the binding data (Halushka et al., 1986).

Comparison of the binding affinities of agonists (but not of antagonists) in intact human platelets and in membrane preparations showed that affinity was decreased in the membrane preparations, and this decrease in affinity was shown to be caused by a protein component (M_r approximately 100 kDa) that is released from platelets during sonication. It has been suggested that this protein may play a role in regulating platelet responses to PGH_2 and TXA_2 and may be a kinase responsible for causing receptor desensitization in intact platelets (Dorn et al., 1987).

[^{3}H]SQ 29,548 has also been used as an antagonist radioligand in intact human platelets and in membrane preparations; it binds to a single class of receptors (1394 sites per platelet) with high affinity (K_D approximately 5 nM) and a high proportion (>90%) of specific binding. Binding was inhibited by agonists and by antagonists, but for the agonists only the displacement curves were biphasic, indicating the existence of two sites as had previously been found when the antagonist ligand [^{125}I]-*p*-hydroxy-SQ 28,668 was used (Hedberg et al., 1985, 1988). The use of [^{3}H]S-145 as an antagonist radioligand has also been reported in pig platelets and aorta, and an excellent correlation was observed for inhibition of binding in each tissue by a range of agonists and antagonists, suggesting that the receptors are similar (Mihara et al., 1989).

Racemic [^{3}H]trimetoquinol (also called trimethoquinol), which is structurally unrelated to the prostaglandins, has been shown to bind to two sites on intact human platelets with K_D values of 2.8 nM and 1.4 μM. Binding to the low affinity site was displaced stereospecifically by unlabeled trimetoquinol and 13-APA and by U46619, at concentrations consistent with their potencies as antagonists or agonists, respectively, but only weakly by arachidonic acid; this binding site was suggested to be the platelet TXA_2 receptor (Ahn et al., 1988). However, although trimetoquinol was the first proposed TXA_2 antagonist (MacIntyre, 1981), earlier binding studies using [^{3}H]U44069 had indicated that trimetoquinol did not bind to the TXA_2 receptor (Armstrong et al., 1983a), and it has been suggested that it might instead act by blocking a signal transduction pathway (MacIntyre and Pollock, 1983a). In view of these doubts as to the action of trimetoquinol, the limited number of displacing ligands tested, and the use of the racemic mixture as the radioligand, these preliminary results must be treated with caution until confirmed by more extensive work.

The binding site for [^{125}I]-PTA-OH has been solubilised from human platelet membranes, and binding to the solubilized site could be displaced by several agonists and antagonists at concentrations similar to those at which they can affect platelet aggregation. The K_D value for [^{125}I]-PTA-OH from equilibrium binding studies (66 nM) was a little higher than that observed for binding to intact platelets (21 nM) and considerably higher than the K_B calculated from pharmacological studies (8 nM), although the authors point out that they are in the same range. Two other antagonists, ONO 11120 and PTA-(ω-1), were also less potent in displacing binding than would be expected from their pharmacological activity, and it was suggested that this might be due to disruption of the platelets, thereby releasing some substance that can alter

the binding site (Burch et al., 1985b). From a study of its hydrodynamic properties the molecular weight of the solubilized protein was calculated to be 140 kDa; the protein appeared to be asymmetrical with an axial ratio of 7:1 (Burch et al., 1985a). Subcellular fractionation of platelets and localization of the binding site showed that it was present in the plasma membrane and/or dense tubular system (which are not easily separated) but not in the cytoplasm, mitochondria, or granules (Saussy et al., 1988).

The [^{125}I]-PTA-OH-binding site has been photoaffinity labeled using the diazonium salt of the structurally similar photoaffinity ligand PTA-POA. Photolysis of platelet membranes in the presence of the diazonium salt resulted in a loss of binding sites for [^{125}I]-PTA-OH, and this loss could be prevented by the presence during photolysis of SQ 26655, a TXA_2 receptor agonist (Mais et al., 1986). More recent studies with a related radioiodinated photoaffinity label, [^{125}I]-PTA-azido, showed incorporation into three proteins of 43, 39, and 27 kDa, and labeling of all of these bands could be inhibited stereoselectively by TXA_2 receptor agonists and antagonists, suggesting that these proteins may represent different forms of the TXA_2-binding site (Mais et al., 1989, 1990). Preparative isoelectric focussing of platelet membrane proteins concentrated two photoaffinity-labeled proteins of 49 and 27 kDa that could bind [^{125}I]-PTA-OH, suggesting that the receptor could exist as a dimer (Dorn, 1989).

A related newer photolysable analogue of TXA_2, the arylazido derivative I-PTA-PON_3, is a potent antagonist of human platelet aggregation induced by U46619 (K_B 9.5 nM) and of [^{125}I]-PTA-OH binding to intact platelets (IC_{50} 36 nM). Photolysis of successive amounts of I-PTA-PON_3 with platelets increased the EC_{50} value for induction of aggregation by U46619 and decreased the B_{max} value without changing the K_D value for binding, an observation consistent with irreversible occupation of TXA_2 receptor sites (Mais et al., 1990). An arylazido analogue of 13-APA has also been used as a photoaffinity ligand and has been shown to inhibit selectively and irreversibly aggregation induced by U46619 and to block irreversibly the binding of [^{3}H]U46619 (Kattelman et al., 1987). Another photoaffinity ligand that has been tested is the azido derivative of the antagonist BM 13,177 ("sulotroban"). In the dark the ligand behaved as a competitive antagonist, but after photolysis it inhibited irreversibly and selectively U46619-induced platelet activation, and this blockade could be prevented by the addition of sulotroban before photolysis (Zehender et al., 1988).

Affinity chromatography using immobilied S-145 resulted in the purification of a 57-kDa protein that bound [^{3}H]S-145; this binding could be inhibited by TXA_2 receptor agonists and antagonists but not by inactive prostanoids (Ushikubi et al., 1989). This protein was partially sequenced and the sequence used to design an oligonucleotide probe with which a cDNA clone coding for the TXA_2 receptor protein was isolated from human placenta. The receptor was expressed in the COS-7 cell line and was shown to bind [^{3}H]S-145 with a K_D value of 1.2 nM, which is comparable to that observed with platelet membranes, and this binding was displaced in an appropriate manner by TXA_2 agonists and antagonists. The receptor was also expressed in *Xenopus* oocytes and application of STA_2 specifically evoked a response consistent with increases in intracellular Ca^{2+}. The receptor protein was sequenced and had a molecular weight of 37 kDa, smaller than that isolated by affinity chromatography, and hydrophobicity analysis revealed seven hydrophobic stretches that could represent transmembrane regions consistent with a G protein-coupled receptor (Hirata et al., 1991).

3. Physiological and pathological importance. The importance of TXA_2 and PGH_2 in platelet function is firmly established, and the number of potential antagonists developed by different pharmaceutical companies testifies to the believed pathological significance of the stimulatory prostanoids in thrombotic and other disorders. For recent reviews concerning this subject, see Lefer and Darius (1987) and Ogletree (1987). PGH_2 and TXA_2 produced by aggregating platelets play an important role in causing secondary aggregation and secretion and are part of the amplification mechanisms enhancing aggregation; they also have a host of other biological activities including vasoconstriction and bronchoconstriction. The antithrombotic effects of low dose aspirin and its tendency to increase bleeding time have been attributed mainly to its blockade of PGH_2 and TXA_2 synthesis, low doses enhancing selectivity toward platelets without excessive inhibition of PGI_2 production by the vessel wall (Fitzgerald et al., 1985, 1987).

The recent interest in fish-rich diets as antithrombotic prophylaxis also rests partly on the belief that ω-3 fatty acids such as eicosapentaenoic acid give rise to inactive thromboxanes but active prostacyclins, although other mechanisms also may be involved (Dyerberg and Bang, 1979; Sanders, 1985). Clinical use of TXA_2 synthetase inhibitors has been disappointing, probably because they do not block the synthesis of PGG_2 and PGH_2, which are also very potent aggregating agents (Fitzgerald et al., 1985). TXA_2 antagonists that block the platelet effects of all of the stimulatory prostaglandins are likely to be more effective (Lefer and Darius, 1987; Ogletree, 1987).

F. Platelet-activating Factor

PAF was first described as a PAF released from sensitized rabbit basophils following stimulation with antigen (Benveniste et al., 1972), although it is now clear that other cells, including platelets, can release PAF and that it has numerous other effects. PAF has the structure 1-O-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine, where the alkyl group on the C_1 position is usually

hexadecyl or octadecyl (Benveniste et al., 1979; Demopoulos et al., 1979), and is sometime referred to as PAF-acether or as AGEPC (acetylglyceryl ether phosphorylcholine). It is formed from a membrane ether phospholipid, 1-O-alkyl-2-acyl-*sn*-glyceryl-3-phosphorylcholine, by the action of phospholipase A_2 which cleaves the fatty acid at the C_2 position (often arachidonic acid) to yield lyso-PAF; the latter compound is then acetylated by acetyltransferase using acetyl coenzyme A as substrate. PAF is destroyed and 1-O-alkyl-2-acyl-*sn*-glyceryl-3-phosphorylcholine is regenerated by the actions of acetylhydrolase which yields lyso-PAF, followed by acyltransferase which uses acyl-coenzyme A as substrate (for review see Braquet et al., 1987).

PAF is a potent aggregating agent with an EC_{50} value in washed rabbit platelets in the pM range (Demopoulos et al., 1979). There is considerable variation among species, with human platelets being less sensitive to PAF than rabbit or guinea pig platelets, whereas rat and mouse platelets are unresponsive (Chignard et al., 1987). PAF is released from platelets following stimulation by A23187, thrombin, or collagen but not by ADP, arachidonic acid, or PAF itself (Chignard et al., 1980), and PAF release has been suggested to be the "third pathway," in addition to ADP release and arachidonic acid metabolism, by which thrombin and collagen activate platelets (Chignard et al., 1979). As well as its potential role in platelet function, PAF has also been implicated in numerous physiological and pathological processes including pregnancy, asthma, inflammation, transplant rejection, and gastrointestinal ulceration (Braquet et al., 1987).

PAF causes shape change, aggregation and release of granule contents from platelets. Whereas in rabbit platelets secretion occurs independently of arachidonate metabolism, in human platelets secretion to PAF, like ADP, appears to be dependent on arachidonate metabolism and is enhanced by a low extracellular Ca^{2+} concentration (Chignard et al., 1987). PAF induces inositol phospholipid metabolism and Ca^{2+} mobilization (MacIntyre and Pollock, 1983b; Hallam et al., 1984a) and inhibits adenylate cyclase in platelet membranes and in intact platelets by a GTP-dependent process (Haslam and Vanderwel, 1982; Williams and Haslam, 1984). Several recent reviews have been published dealing with various aspects of PAF and its effects on platelets (Hanahan, 1986; Braquet et al., 1987; Chesney et al., 1987; Chignard et al., 1987; Valone, 1988).

1. Structure-activity relationships. Although the phospholipid nature of PAF raised the possibility that its actions are due to some nonspecific perturbation of membranes, the clearly defined structure-activity relationships, the existence of competitive antagonists, and the ability to detect saturable binding sites shows that the actions of PAF are receptor-mediated. Analogues of PAF have been synthesised (see fig. 4 for some examples of

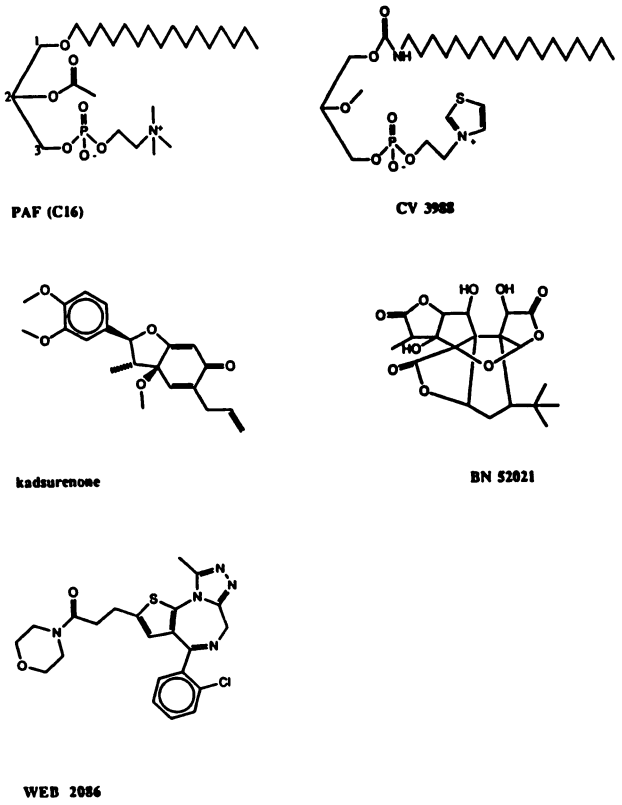


FIG. 4. Some examples of platelet PAF receptor agonists and antagonists.

structures) that vary in the nature and orientation of the substituents on the three-carbon glycerol backbone, and a model of the binding site has been proposed (Godfroid and Braquet, 1986; Braquet et al., 1987). It should be borne in mind, however, that such synthetic analogues, unlike natural PAF, are racemic, and that it is assumed that the unnatural S isomers have no pharmacological effect.

A study of the effect of alterations to the alkyl chain in the C_1 position showed that replacement of the ether oxygen by sulphur, methylene, or an ester linkage dramatically decreases platelet-aggregating activity and that for an ether-linked chain potency was maximal at a chain length of 16 carbons and correlated well with the lipophilicity of the group (Godfroid et al., 1987). Potency to aggregate platelets was also found to correlate well with potency to cause hypotension and bronchoconstriction, which indicates that similar receptors may mediate these effects, although maximal potency for bronchoconstriction was found with the slightly shorter 14-carbon chain (Godfroid et al., 1987). The presence of one or two double bonds slightly enhanced potency with 18:2 > 18:1 > 18:0 (Surles et al., 1985), whereas multiple oxygen substitution reduced potency (Wissner et al., 1986a). For 1-alkylphenoxy analogues the position of the fatty chain in relation to the glycerol backbone is also important for activity, with the *meta* analogue being the most active, the *para* weakly active, and the *ortho* form totally inactive (Wissner et al., 1984).

At the C_2 position the *R* configuration is important for activity, with the *S* isomer being much less potent (Heymans et al., 1981). The length of the chain on the C_2 position is important, lyso-PAF being inactive and maximal potency being observed with a two-carbon chain (Hanahan, 1986; Braquet et al., 1987). The ester linkage is not essential for activity, and analogues with ether or methylcarbamate linkages retain activity, as does the C_2-nitrate analogue (Hadvary et al., 1983).

At the C_3 position, deletion or replacement of the phosphate group by groups other than phosphonate greatly reduced activity, and the distance between the phosphate and the ammonium group is critical (Moshidis et al., 1983; Wissner et al., 1985a, 1986b). The charged trimethylammonium headgroup of choline can be replaced by other nitrogen-containing moieties including cyclic derivatives, some of which, such as N-methyl piperidinium, are even more potent than PAF itself (Coeffier et al., 1986).

Analogues in which the positions of the groups on the glycerol backbone have been changed are rather less potent than PAF, but as long as the chirality of C_2 has been respected they effectively differ only in the lengths of the chains, and the potency of these positional isomers is, therefore, not dramatically less than that of PAF (Hirth and Barner, 1982; Hirth et al., 1983; Godfroid and Braquet, 1986). Analogues in which an extra methylene or acetoxymethylene group is inserted into the glycerol backbone itself have low activity however, showing that the length of this backbone is important (Wissner et al., 1985b).

Based on these structure-activity relationships, a model of the binding site has been developed in which the fatty chain on the C_1 position penetrates deep into a hydrophobic region, anchored by the short two-carbon chain, with electronic transfer from the ether oxygen to the receptor (for discussion, see Godfroid and Braquet, 1986, and Braquet et al., 1987).

Several analogues of PAF have been found to act as antagonists rather than agonists (see fig. 4 for some examples of representative structures) (Braquet and Godfroid, 1986; Braquet et al., 1987). The first of these to be discovered was CV 3988, in which an octadecyl chain is attached to the C_1 position by a carbamate group, the C_2 position has a methyl ether, and the C_3 position has a thiazolium ring instead of the trimethylammonium headgroup of choline (Terashita et al., 1983). Other antagonists with a triazolium ring, but linked by alkyl ether chains rather than by phosphate to C_3, include the potent antagonists Ro 19–3704 and ONO 6240 (Burri et al., 1985; Miyamoto et al., 1985). The structure-activity relationships for antagonist analogues of PAF with a glycerol backbone, but different substituents on the three carbons have been described overall as being: C_1, -O-CONH- > -NHCO-O- > -O-CO- > -O-; C_2, -O-COOMe > -O-CONHMe > -O-COMe > OMe; C_3, -O-CO- > -O-CO-O- > -NHCO- > $-NHSO_2-$ > $-O-PO_2-$ ≃ $-PO_2-O-$ > $O-PO_2-O-$ (Braquet et al., 1987). Analogues of PAF with a constrained backbone produced by replacement of glycerol with a ring system, such as dioxanone, piperidine, or tetrahydrofuran, also give rise to antagonists such as SRI 63–073 and SRI 63–441, although these latter compounds are not very potent (Lee et al., 1985; Burri et al., 1985; Morrison and Shukla, 1989).

Various natural compounds with no obvious structural similarity to PAF also act as antagonists, including terpenes such as BN 52021 and ligands such as kadsurenone; these compounds are extracts derived from Chinese medicinal plants (for review see Braquet et al., 1987). Some drugs with other known pharmacological effects also exhibit PAF antagonism, such as the psychotropic triazolobenzodiazepines alprazolam and triazolam (Kornecki et al., 1984), some Na^+ channel blockers including amiloride (Hwang, 1989), some Ca^{2+} channel blockers including verapamil and diltiazem (Kornecki et al., 1984; Tuffin and Wade, 1985), and some 1,4-dihydropyridines that are not Ca^{2+} channel blockers (Ortega *et at.*, 1990).

There have been reports of differential inhibition by PAF antagonists of the actions of PAF on different cell types, and suggestions that different receptors may be involved (e.g., Voekel et al., 1986; Braquet et al., 1987), but as yet no clear classification of PAF receptors has emerged.

2. Binding studies and receptor isolation. [^{3}H]PAF has been shown by several authors to bind specifically and with high affinity to human platelets, although estimates of the K_D vary from 0.053 to 37 nM and estimates of the number of receptors vary from 150 to 1983 sites per platelet (for review and tables of results see Hanahan, 1986; Chignard et al., 1987; Godfroid and Braquet, 1987; Braquet et al., 1987; Chesney, 1987; Valone, 1988). Some of the differences observed may be attributable to the use of different methods of preparing washed platelets and differing sources of [^{3}H]PAF (Chesney, 1987). Although some investigators have reported more than one binding site, the other sites detected have been unsaturable or of low affinity, and there is no convincing evidence that two classes of PAF receptor exist on platelets (Chignard et al., 1987; Valone, 1988). In a study comparing different species, no high affinity PAF-binding sites were detected on rat platelets, which are known to be unresponsive to PAF, whereas rabbit platelets, which are very sensitive to PAF, were shown to have approximately 10 times as many sites per platelet as human platelets, with a rather similar K_D value (Inarrea et al., 1984). PAF-binding sites have also been detected in human platelet membrane preparations, and their properties correlate well with those found in intact platelets (Tahraoui et al., 1990).

PAF agonists and antagonists inhibit the binding of [^{3}H]PAF to the high affinity sites on platelets, and a correlation has been shown to exist for five structurally

diverse antagonists (two triazolobenzodiezepines, BN 52021, CV 3988, and kadsurenone) for inhibition of PAF-induced aggregation and of [^{3}H]PAF binding (Chignard et al., 1987; Valone, 1988). Antagonists, including [^{3}H] dihydrokadsurenone and the triazolobenzodiazepine [^{3}H] WEB 2086, have also been used as radioligands and have been shown to interact with the same site as [^{3}H]PAF (Hwang et al., 1986a; Ukena et al., 1988), but another antagonist, the pyrrolothiazole derivative [^{3}H]52770 RP, could not be displaced by PAF from human platelets and labeled >10 times as many sites as [^{3}H]PAF (Ukena et al., 1988), although [^{3}H]52770 RP appeared to be a satisfactory ligand on rabbit platelets (Robaut et al., 1987).

Displacement of [^{3}H]PAF binding to canine platelets has been used to refine the design of a series of PAF antagonists that are based on N-[4-(3-pyridinyl)butyl]3-substituted propenyl carboxamides, some of which have K_i values in the nanomolar range (Guthrie et al., 1990). [^{3}H]PAF binding has been reported to be inhibited by sulphydryl reagents, suggesting that SH groups are involved in this binding (Ng and Wong, 1988), and to be enhanced by Mg^{2+}, Ca^{2+}, Mn^{2+}, K^+, Cs^+, and Rb^+ but inhibited by Na^+ and Zn^{2+} (Hwang et al., 1986b; Kumar and Hanahan, 1989). GTP also inhibits PAF binding and PAF stimulates membrane GTPase, as expected for a G protein-coupled receptor (Hwang et al., 1986b). The PAF receptor may exist in multiple conformations dependent on the concentration of GTP and the local ionic conditions (Hwang et al., 1989).

Attempts to solubilise and isolate the PAF receptors have been complicated by several problems associated with the phospholipid nature of PAF, which makes it difficult to detect PAF binding to isolated receptors. PAF interacts with detergents used for solubilisation and with the membrane phospholipids released in the process, and these phospholipids will also compete with PAF for binding (Valone, 1988). By affinity chromatography using a column of PAF bound noncovalently to albumin-Sepharose, Valone (1984) isolated a human platelet membrane protein of 180 kDa that was eluted by PAF, but no evidence was presented that this protein had the characteristic structure-activity requirements of the PAF receptor. More recently, binding of [^{3}H]PAF to a solubilised membrane protein was demonstrated, but the K_D was found to be 20 μM which is far higher than expected from binding studies to the receptor in intact platelets and membranes (Valone, 1987). Nishihira et al. (1985) also identified a PAF-binding protein of 160 kDa by binding [^{3}H]PAF to platelets and solubilising and purifying the labeled protein using standard nonaffinity techniques. A similar approach was taken by Chau and Jii (1988), who identified a protein with an apparent molecular weight of 220 kDa. GTP and Na^+ were found to promote the dissociation of the PAF-protein complex, suggesting that a G protein was present in addition to the receptor, and labeling was reduced by preincubation of the platelets with excess unlabeled PAF or the antagonist L-652,731.

A protein with a much lower molecular weight (52 kDa), which may represent the binding subunit of the PAF receptor complex, was identified using a photoaffinity analogue of PAF to label the putative receptor; the labeling could be prevented by excess PAF and PAF antagonists but not by lyso-PAF (Chau et al., 1989). Another photoaffinity ligand, an azido *trans*-2,5-aryltetrahydrofuran, L-662,025, irreversibly inhibited [^{3}H] PAF binding to human platelet membranes after photolysis and could be suitable for identification of the PAF receptor, although the compound is not very potent, having an IC_{50} value of 1 μM before photolysis (Hussaini and Shen, 1989).

3. Uptake and metabolism of platelet-activating factor. PAF is metabolised by platelets via lyso-PAF to alkylacylGPC by the actions of an acetylhydrolase and an acyltransferase, which preferentially places arachidonate at the 2 position (see Braquet et al., 1987, for a review of PAF metabolism). Incubation of PAF with human platelets results in its complete destruction within 1 h (Tonqui et al., 1985), but significant metabolism is unlikely to occur during aggregation or binding studies (Chignard et al., 1987; Valone, 1988). However, it has been suggested that PAF must bind to its receptor and be internalised before metabolism can occur because metabolism is much slower in intact platelets than it is in platelet lysates and is blocked by the receptor antagonist BN 52021 in intact platelets but not in platelet lysates (Lachachi et al., 1985). Studies by Homma et al. (1987) also showed internalisation of PAF but showed that this was enhanced not only by PAF itself (and was therefore inhibited by PAF antagonists) but also by thrombin, suggesting that it is platelet activation rather than simply PAF-receptor complex formation that stimulates uptake and metabolism of PAF. It was suggested that PAF internalisation may be related to the reorganisation of the plasma membrane that occurs during platelet activation. This study also showed nonspecific binding of PAF and lyso-PAF, probably to the membrane lipids (Homma et al., 1987).

4. Physiological and pathological importance. Although it was originally suggested that PAF might represent the third pathway mediating platelet aggregation and secretion produced by thrombin and collagen, independently of ADP release or arachidonate metabolism (Chignard et al., 1979), in human platelets at least this seems unlikely. PAF is unable to cause secretion without arachidonate metabolism (Chignard et al., 1987), and PAF antagonists do not block thrombin-induced aggregation of platelets that have been treated with aspirin (to block cyclooxygenase) and the snake venom convulxin (to deplete ADP stores), and in which this putative third pathway might therefore be expected to be the major

mediator of aggregation (Adnot et al., 1987). PAF antagonists have also been stated not to prevent rabbit platelet deposition in a model expected to demonstrate antithrombotic activity (Hadvary and Baumgartner, quoted in Braquet et al., 1987). Although these results and the species variation found in the response to PAF cast doubt on there being a major role for PAF in thrombosis and haemostasis, it is clear that in humans PAF is released from activated platelets (Chignard et al., 1980) and activates them at low concentrations and, therefore, must play its role in the complex system of synergy and amplification that is characteristic of platelet responses. Platelet-derived PAF may also act as a local hormone and influence endothelial integrity, thereby leading to an increase in vascular permeability and oedema (Braquet et al., 1987).

Now that selective PAF antagonists are available, the precise role of PAF in both platelet function and in all of the other pathological and physiological processes in which it has been implicated can be resolved.

G. Thrombin

Thrombin is a clotting factor (factor IIa) that has an important role in blood coagulation, in cleaving fibrinogen (factor I) to yield fibrin which then forms the fibrillar basis of the clot, and in activating factors V, VIII, and XIII. Thrombin is formed during coagulation by the action of factor Xa on the plasma precursor, prothrombin, and is a serine protease with a molecular weight of 36 kDa; it consists of two chains, A and B, linked by disulphide bonds. Thrombin is the only clotting factor to activate platelets and, at physiological concentrations (low nM), causes aggregation and secretion from dense granules, α-granules, and lysosomes, and therefore acts as a very powerful stimulus.

Although thrombin is an enzyme, it is not clear what role this enzymic activity plays in platelet aggregation. In favour of a receptor-mediated process are the findings that thrombin acts via a G protein to activate phospholipase C, resulting in increases in DAG and Ca^{2+} levels. This phospholipase C activation is similar to that produced by those aggregating agents without any enzymic activity and is not due to secondary effects of released ADP or TXA_2 because it is not blocked by ADP-scavenging enzyme systems or by indomethacin (Agranoff et al., 1983; Rink et al., 1982; Huang and Detwiler, 1987; Brass et al., 1986; Baldassare et al., 1989; Hrbolich et al., 1987). In addition, the kinetics of activation by thrombin are consistent with a receptor-mediated effect because the extent of the response is dependent on the thrombin concentration, whereas, if an enzymic reaction were involved, it would be expected that only the rate of response would vary and that even low doses would eventually cause a full response (see Detwiler and McGowan, 1985, for discussion). Enzymically active thrombin is required for platelet stimulation and pretreatment with inhibitors such as hirudin or diisopropyl fluorophosphate blocks platelet activation as well as coagulation. On the other hand, many proteases clot fibrinogen but do not aggregate platelets, and vice versa, and a congenitally abnormal thrombin (thrombin Quick 1), which is enzymically active but shows impaired fibrinogen binding, has a reduced effect on platelets, indicating that the relationship between platelet activation and enzymic activity is not simple (White and Lapetina, 1987; Henriksen and Brotherton, 1983). Thrombin does cleave an 82-kDa platelet membrane glycoprotein, GPV, and liberates a 70-kDa product. Although this cleavage does, in general, correlate with platelet activation, its role in this process is not clear (McGowan et al., 1983a). The somewhat obscure relationship among thrombin binding, enzymic activity, and platelet activation, and some models that have been proposed to explain this relationship, have been discussed in several recent reviews (Detwiler and McGowan, 1985; Shuman and Greenberg, 1986; Berndt et al., 1986; White and Lapetina, 1987; Jamieson, 1988).

1. Structure-activity relationships. As well as the normal form of thrombin, α-thrombin, two partially proteolysed forms, γ-thrombin and β-thrombin, also activate platelets, although they are less potent (Mohammed et al., 1976). Covalent modification of the active site (as with diisopropyl fluorophosphate) abolishes activity. Other inhibitors of the proteolytic activity of thrombin such as hirudin, heparin, or thrombomodulin also inhibit thrombin-induced platelet aggregation. Catalytically active proteases whose specificities overlap with that of thrombin, such as trypsin, papain, and thrombocytin, also activate platelets, whereas those proteases with dissimilar specificities, such as plasmin and chymotrypsin, do not cause activation, although the effects of proteases on platelets do not exactly parallel their ability to clot fibrinogen (Davey and Luscher, 1967; Tollefsen et al., 1974; Workman et al., 1977a,b; Martin et al., 1975; White et al., 1981; see White and Lapetina, 1987, for tables).

Although most substances that "antagonise" platelet activation by thrombin do so by interacting with the active site of thrombin rather than with any platelet receptor, two synthetic dipeptide analogues, SC40476 (N-(3-methyl-1-S{[2-S-(methylamino)-carbonyl]-1- pyrrolidinyl}carbonyl)butyl-D-alanine) and SC42619 (N-[3-methyl- S -(1-pyrrolidinylcarbonyl)butyl]-D-alanine), have been proposed to act as true receptor antagonists with no effect on the proteolytic activity of thrombin. These compounds selectively inhibit aggregation induced by thrombin but not by ADP, collagen, vasopressin, or 11,9-epoxymethano PGH_2, and they cause parallel shifts to the right of the dose-response curves to thrombin, indicating competitive antagonism. These compounds also inhibited the increases in intracellular Ca^{2+} and the activation of phospholipase C caused by thrombin. However, mM concentrations were required and a pA_2 value of approximately 3 was estimated from a Schild plot for SC42619. These dipeptide analogues also showed some

agonist activity; aggregation was observed as a small decrease in the single platelet count of platelet suspensions. SC40476 and SC42619 could provide a starting point for the design of more potent thrombin antagonists and agonists, and their use may support the idea that thrombin acts on a receptor rather than simply as a proteolytic enzyme (Ruda et al., 1986, 1988). Indeed, more recent studies have attempted to define the structure-activity requirements for such analogues and have shown that SC42619 also inhibited the effect of thrombin on vascular endothelial cells, although this inhibition did not appear to be a simple competitive antagonism (Ruda et al., 1990).

Thrombin with the active site inhibited by treatment with D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone, unlike other active site-modified thrombins, was also reported to act as a thrombin antagonist inhibiting the release of [^{3}H]5-HT caused by 2 nM thrombin with an EC_{50} of 230 nM (Harmon and Jamieson, 1986). This report was later withdrawn however, and it was stated that thrombin treated with D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone has no inhibitory effect (Jamieson, 1988).

2. Binding studies. Although many studies of the binding of ^{125}I-labeled thrombin to platelets and to platelet membranes have been carried out, no clear picture of the relationship between any component of this binding and platelet activation has emerged (for reviews see Detwiler and McGowan, 1985; Berndt et al., 1986; Shuman and Greenberg, 1986; White and Lapetina, 1987; Jamieson, 1988). In general, such studies have shown that binding is rapid, reversible, and saturable, with two classes of binding sites, one of high affinity (K_D approximately 1 nM; B_{max} 500 sites per platelet) and one of lower affinity (K_D approximately 100 nM; 5,000 to 50,000 sites per platelet,) plus a large amount of nonsaturable binding (Ganguly, 1974; Tollefsen et al., 1974; Martin et al., 1976; Tollefsen and Majerus, 1976; Alexander et al., 1983; Detwiler and McGowan, 1985). With time a component of the bound thrombin becomes more slowly dissociable until, after approximately 30 min, the binding is irreversible. This observation suggests that the thrombin-receptor complex undergoes some change that may be related to proteolysis because it is not observed with active site-inhibited thrombin (Tam et al., 1979; Yeo and Detwiler, 1985). Computer-assisted analysis has detected two comparable binding sites but with rather different parameters (high affinity: K_D 0.3 nM; 50 sites per platelet; low affinity: K_D 11 nM; 1700 sites per platelet), as well as a large number of other low affinity sites (K_D 2900 nM; 590,000 sites per platelet). The latter sites probably correspond to the nonspecific binding reported in the earlier studies (Harmon and Jamieson, 1985; Jamieson, 1988).

Binding to the high affinity site occurs at the same range of thrombin concentrations as platelet activation, and the extent of binding correlates well with the extent of activation when this is altered by changes in experimental conditions (Shuman and Majerus, 1975; Martin et al., 1976). The binding is specific in that prothrombin or prothrombin activation intermediates do not bind (Tollefsen et al., 1975). However, a major stumbling block to the acceptance of this binding site as representing the thrombin receptor is that some structural variants, such as γ-thrombin, can activate platelets but do not bind (Alexander et al., 1983) and that active site-inhibited thrombins bind to platelets in exactly the same way as native thrombin but do not induce or inhibit platelet activation (Tollefsen et al., 1974; Ganguly, 1974; Workman et al., 1977a; Martin et al., 1976). Even thrombin treated with D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone, which was originally reported to inhibit dense granule secretion (Harmon and Jamieson, 1985), does not appear to affect platelet activation, although it blocks binding (Jamieson, 1988). The functional significance of the observed thrombin binding, therefore, remains unclear.

There is evidence that the high affinity-binding site for thrombin may be the platelet membrane 180-kDa dimeric glycoprotein GPIb and that this binding site is involved in platelet activation (see Berndt et al., 1986; White and Lapetina, 1987; Jamieson, 1988). GPIb and a proteolytic product, glycocalicin, can block both thrombin binding and platelet activation, and thrombin will bind to free GPIb (Okumura and Jamieson, 1976; Okumura et al., 1978; Ganguly and Gould, 1979). Antibodies directed against GPIb selectively inhibit thrombin-induced platelet activation (Hagen et al., 1982; Yamamoto et al., 1985; Jenkins et al., 1983; McGregor et al., 1983), and thrombin binding, GPIb content, and platelet responsiveness are all reduced when platelets are treated with certain proteases or are taken from patients with Bernard-Soulier syndrome (Yoshida et al., 1983; McGowan et al., 1983b; Ganguly, 1977; Jamieson and Okumura, 1978). Other platelet proteins, including thrombospondin and a platelet protease, nexin, have also been shown to interact with thrombin, but the role of these proteins in activation and the binding of thrombin to intact platelets is unclear (Shuman and Greenberg, 1986; White and Lapetina, 1987; Gronke et al., 1987).

3. Physiological and pathological importance. The central role of thrombin in blood clotting and in the interaction between platelets and the coagulation cascade is not in doubt; thrombin does activate platelets powerfully at physiological concentrations (Davey and Luscher, 1967; Shuman and Majerus, 1976). However, patients with clotting factor disorders who might, therefore, be expected to have a deficiency of active thrombin have a normal bleeding time (indicating normal platelet function), as do 50% of patients given heparin (Kaneshiro et al., 1969; Heiden et al., 1977), suggesting that thrombin is not obligatory in haemostatic platelet activation in

vivo, presumably because other mechanisms for activating platelets exist. Early studies comparing heparin and citrate as anticoagulants had suggested a role for thrombin in platelet secretion induced by ADP. More detailed work showed that thrombin was not required and that the observed enhancement of release seen in citrated plasma was due to Ca^{2+} chelation (Macfarlane et al., 1975).

Because it is so difficult to disentangle the proteolytic activity of thrombin from its activation of platelets, it is hard to say how important its effects on platelets are in isolation from its role in the coagulation cascade. However, selective antagonists of its effect on platelets, such as the peptide analogues described by Ruda et al. (1986, 1988, 1990), may help to clarify this matter.

H. Collagen

Platelets adhere to the connective tissue protein collagen, and this adhesion results in platelet activation, arachidonic acid metabolism, release of dense granule contents, and aggregation; the latter process is largely dependent on the released ADP and PGH_2/TXA_2. When platelet-collagen adhesion is studied in the combined presence of antagonists of TXA_2, 5-HT, and PAF receptors, a fibrinogen/fibronectin inhibitor, and an ADP-scavenging system, adhesion of human platelets to collagen can be shown to be associated with increases in levels of intracellular Ca^{2+} and phosphatidic acid, presumeably as a consequence of phosphatidylinositol 4,5-biphosphate cleavage (Smith and Dangelmaier, 1990).

The interaction of platelets with collagen is rather more complex than a simple receptor-ligand interaction, but because collagen, unlike other connective tissue proteins, does act as a platelet activator and because this activation is clearly of physiological and pathological importance, this interaction will be considered briefly here. Just as platelet-platelet interaction (via fibrinogen) causes activation and release, so does platelet-collagen interaction. Thus, the collagen "receptor" on platelets is clearly more than just a nonspecific adhesive site. Some reviews of platelet-collagen interactions have been published (Jaffe, 1976; Meyer, 1980; Santoro and Cunningham, 1981; Santoro, 1988).

1. Structure-activity relationships. The structure-activity relationships for the activation of platelets by collagen is complex, because, not only is the chemical structure of the protein important, its physical shape and organisation is also critical. Collagen fibres consist of a staggered array of the rod-shaped collagen monomer, tropocollagen, stabilized by cross-links to a variable extent. Monomeric collagen is a triple helix of three polypeptide chains each consisting of approximately 1000 amino acids; five different types exist that differ in their tissue distribution and amino acid composition, although these are highly homologous with a high proline content and almost every third amino acid residue is glycine.

The quaternary structure of collagen is crucial in activating platelets, because the purified polypeptide chains do not induce aggregation (Puett et al., 1973; see Jaffe, 1976, for review). Studies suggesting that, unlike other collagen peptides, a peptide derived from the $\alpha 1$ chain of chick skin collagen can induce aggregation (Katzman et al., 1973; Kang et al., 1974) may reflect the ability of this peptide to reform fibrils rapidly (Jaffe, 1976). Not only is the triple helical structure necessary for platelet aggregation but the monomers must first polymerise into fibres before collagen is active (Muggli and Baumgartner, 1973; Jaffe and Deakin, 1974; Brass and Bensusan, 1974). Reported differences in the ability of different monomeric collagens to induce aggregation seem to be explained in terms of their different tendencies to reform fibrils in solution (Barnes et al., 1976; Santoro and Cunningham, 1977). Indeed, the surface curvature of the collagen molecule may be a critical determinant of its thrombogenicity, because platelets adhere to glass plates coated with monomeric collagen but do not aggregate, whereas similar collagen-coated glass fibres induce aggregation presumably because the surface is curved, the critical diameter of curvature being <2 μm or approximately the diameter of a platelet (Meyer and Weisman, 1981).

It has been suggested that a combination of favourable morphology and surface chemistry is required to trigger aggregation, so that if only one of these conditions is met platelets may adhere but are not activated. Thus, collagen-coated plates possess only favourable surface chemistry, whereas uncoated or albumin-coated glass fibres possess only favourable geometry and, although platelets adhere to these surfaces, they adhere more strongly and are activated only if these properties are combined as they are in the native collagen polymer (Meyer, 1980). Neither the polypeptide chains nor monomeric collagen inhibit collagen-induced aggregation (Puett et al., 1973; Santoro and Cunningham, 1977), suggesting that they have lower affinity for the platelet surface, possibly because native collagen binds to more than one site and that binding to subsequent sites is enhanced by the initial binding which brings the surfaces into closer proximity (Santoro and Cunningham, 1981). Attempts to define those chemical features of collagen that interact with platelets are complicated by the requirement for fibrillar collagen. Modifications that alter the quaternary structure or prevent fibril formation from subunits will reduce platelet aggregation whether or not these modifications also affect the binding of collagen to the platelet surface. A different approach has been to measure the adhesion of ^{51}Cr-labeled platelets to collagens and peptides adsorbed to flat dishes (Santoro, 1986). Experiments with synthetic peptides and collagen fragments indicate, not surprisingly, that the high proline and hydroxyproline content of collagen may be recognised by platelets, because peptides rich in these residues inhibit platelet adhesion to collagen (Meyer, 1980).

Various peptides derived from the different types of collagen by treatment with cyanogen bromide have been shown to support platelet adhesion and to induce aggregation, and some conclusions have been drawn from these studies regarding the structural requirements for these processes. It has been suggested that there may be just one site on collagen type III that is responsible for causing platelet aggregation, whereas on collagen type I several sites are distributed along the length of the molecule (Fitzsimmons and Barnes, 1985; Fitzsimmons et al., 1986; Morton et al., 1987). Lysine and arginine residues appear to be involved, and it has been proposed that platelet aggregation involves the amino acid sequence GK [or R]PG(EY)GPK[or R]G(EY) or, less favourably, GPK [or R]G(EY)G(XY)GK [or R] PG(EY) (Morton et al., 1987). Two peptides from type I and type III collagen have been shown to support adhesion but do not induce aggregation, suggesting that these processes may be mediated by different sites (Morton et al., 1989).

Platelet adhesion to collagen has been shown to be a complex process involving not only direct interaction between the platelet and collagen but also secondary, reinforcing interactions involving adhesive proteins such as von Willebrand factor and fibronectin which link the two surfaces together. The primary direct interaction occurs in the absence of divalent cations but is greatly enhanced by Mg^{2+}, an effect that is inhibited by Ca^{2+}. It is not clear whether the Mg^{2+}-dependent adhesion is different in mechanism from the divalent cation-independent adhesion or just greater in extent (Santoro, 1986, 1988). The adhesion of rabbit platelets, unlike that of human platelets, has been reported not to be enhanced by Mg^{2+}, which may reflect a species difference in the collagen-binding site(s) on platelets (Morton et al., 1989).

2. Identification of the collagen receptor. Various platelet membrane proteins have been proposed as the platelet collagen-binding site, but the evidence in favour of any of these is not conclusive (for review see Santoro, 1988). A platelet membrane protein of 160 kDa that appears to be identical with GPIa has been shown to bind reversibly to collagen in a Mg^{2+}-dependent manner, suggesting that this may be the collagen receptor (Santoro, 1986). Patients whose platelets are deficient in GPIa have a mild bleeding disorder, and their platelets show impaired adhesion and aggregation to collagen but not to other agonists, which supports the possible role of this glycoprotein in collagen-induced activation (Nieuwenhuis et al., 1985, 1986). Attempts to purify the receptor by affinity chromatography on a collagen column resulted, however, in isolation of a heterodimeric complex of GPIa/IIa. This finding suggests that this complex may act as the collagen-binding site and may be one of a family of heterodimeric adhesive proteins found in cell membranes of which the platelet GPIIb/IIIa complex, the fibrinogen receptor, is another (Santoro, 1988). Indeed, studies with a monoclonal antibody to the GPIa/IIa complex and with an antibody to the GPIIb/IIIa complex suggest that, in blood, collagen binds directly to GPIa/IIa and indirectly to the GPIIb/IIIa via adhesive proteins supplied by the plasma (Coller et al., 1989). Incorporation of a solubilized and purified platelet membrane GPIa/GPIIa into liposomes causes them to adhere to collagen specifically and in a Mg^{2+}-dependent manner, providing additional evidence that the GPIa/IIa complex is the direct mediator of platelet adhesion to collagen (Staatz et al., 1989).

Another candidate for the collagen receptor is a 65-kDa protein solubilized from platelet membranes and purified by affinity chromatography on collagen or on the α1(I)-polypeptide chain of collagen (Chiang and Kang, 1982). Polyclonal and monoclonal antibodies raised against this protein inhibited aggregation induced by collagen but not by other agonists, and the purified IgG and isolated Fab fragments competitively inhibited binding of the radiolabeled α1(I)-chain to washed platelets (Chiang et al., 1984, 1987, 1989). A collagen-binding protein with a similar molecular weight (61 kDa) was identified by Kotite and Cunningham (1986), but this does not appear to be identical with the 65-kDa protein of Chiang and Kang (1982).

GPIV, an 88-kDa membrane component, has been suggested to play a role in collagen-platelet interactions, because purified GPIV inhibits collagen-induced platelet aggregation and Fab fragments from a polyclonal antiserum specific for GPIV inhibits collagen-induced platelet aggregation (Tandon et al., 1989). Platelet GPIV has also been implicated as the thrombospondin receptor, however. Therefore, it may have a wider role in cell adhesion (Asch et al., 1987).

Platelet factor XIII, the catalytic subunit of transglutaminase which stabilizes fibrin, has been shown to bind to collagen, and it has been proposed as the collagen receptor because antibodies raised against it induced aggregation (Saito et al., 1986). Although platelet factor XIII is thought to be largely cytoplasmic, when the Fab fragment of the antibody directed against it is used, factor XIII has been detected on the surface of intact bovine platelets. Some additional evidence for a receptor role has been provided by the ability of this antibody fragment to inhibit aggregation induced by collagen but not by ADP, arachidonic acid, or 5-HT (Kasahara et al., 1988). That the intact antibody stimulated aggregation and the Fab fragment inhibited it was taken by these workers to suggest that multiple receptor interactions or clustering may be necessary for aggregation.

Various other platelet proteins, including collagen glycosyl transferase, fibronectin, the GPIIb/IIIa complex, and an unidentified 80-kDa protein, have been proposed as being involved in the interaction of collagen with platelets, but the evidence for any of them being the collagen receptor involved in primary adhesion is not complete (for review see Santoro, 1988).

3. Physiological and pathological importance. The adhesion of platelets to exposed subendothelial surfaces and the resulting aggregation and thrombus formation is accepted as a vital part of the haemostatic process, and the collagen (largely types I and III) content of these surfaces accounts for their platelet-activating ability (see Baumgartner and Muggli, 1976; Jaffe, 1976; Santoro and Cunningham, 1981, for reviews). Similar processes are likely to be involved in thrombosis if subendothelial surfaces are exposed following sudden rupture of an atherosclerotic plaque (Davies and Thomas, 1981).

I. Other Stimulatory Agonists

Various other agonists have been reported to activate platelets, but in some cases these effects are highly species dependent and, in general, have been little studied.

Acetylcholine and some of its analogues cause aggregation and release in dog platelets but not in human, rat, or rabbit platelets. Activation can be blocked by the muscarinic antagonists atropine and scopolamine, but not the nicotinic antagonist α-bungarotoxin, suggesting that the platelet receptor is muscarinic (Shermer and Chuang, 1973; Chuang et al., 1974). Acetylcholine and carbachol can also increase cyclic GMP levels in platelets (Haslam, 1975), but because very high concentrations (1 mM) of these compounds are required to activate the receptors, cholinergic agonists are unlikely to have any physiological significance.

Dopamine has been reported both to induce human platelet aggregation, probably via an interaction with the platelet α_2-adrenoceptors, and to inhibit adrenaline-induced aggregation (Braunstein et al., 1977; Boullin and Glenton, 1978; Sharmaa et al., 1975). Although binding studies of intact platelets using [^{3}H]haloperidol revealed only nonspecific binding (Boullin et al., 1978), more recent experiments using platelet membrane preparations have demonstrated saturable specific binding of the rather nonspecific D_2 antagonist [^{3}H]spiperone. This binding appeared to be to dopamine receptors rather than to 5-HT receptors, although the affinity of this site for dopaminergic agents was consistently less than was their affinities for striatal dopamine receptors (Khanna et al., 1987). Specific binding of the D_1 antagonist [^{3}H] SCH 23390 has also been reported, and although these receptors, unlike striatal D_1 receptors, were not coupled to adenylate cyclase, it was originally suggested that they might mediate the weak inhibitory effect of dopamine on adrenaline-induced aggregation (De Keyser et al., 1988). Further investigation, however, suggested that [^{3}H]SCH 23390 labeled a novel 5-HT-binding site ("5-HT_{1E}") in platelet membranes instead (De Keyser et al., 1989); therefore, the status of dopamine receptors on platelets is still uncertain.

Specific binding sites for atrial natriuretic factor and for angiotensin II have been reported on platelets, but because the number of receptors per platelet is very low in each case (<20), and because neither peptide appears to influence platelet activity, the significance of these sites is unclear (Schiffrin et al., 1986; Strom et al., 1987; Moore and Williams, 1981; Moore et al., 1984; Ding et al., 1984; Mann et al., 1985). Human platelets do not appear to possess endothelin receptors or opioid receptors (Battistini et al., 1990; Reches et al., 1980).

The protease cathepsin G, derived from human neutrophils, has been shown to activate platelets in a manner similar to that of thrombin (Selak et al., 1988). It exhibits reversible, saturable binding to platelets consistent with binding to a single receptor type (K_D approximately 60 μM; 2.32×10^7 sites per platelet) (Selak and Smith, 1990). The relationship between this binding site and the thrombin receptor(s) is unknown.

III. Receptors for Inhibitory Agonists

As mentioned in the "Introduction," the inhibition of platelet aggregation by the receptor agonists discussed below is mediated by increases in intracellular cyclic AMP concentration as a result of G_s protein-coupled adenylate cyclase activation. In each case there is a nonspecific, noncompetitive inhibition of platelet responses, and the sensitivity of platelets to stimulatory agonists is subsequently reduced.

A. Adenosine

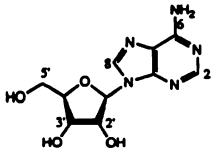
Adenosine was the first inhibitory agonist shown to inhibit platelet aggregation induced in vitro by ADP (Born and Cross, 1963), thrombin (Clayton and Cross, 1963), adrenaline (Clayton and Cross, 1963), 5-HT (O'Brien, 1964), and vasopressin (Haslam and Rosson, 1975). Adenosine also inhibits platelet shape change (Born, 1970) and the release reaction (Zucker and Jerushalmy, 1967). Considerable species variation exists toward adenosine, which is a potent inhibitor of aggregation of human, sheep, dog, and rabbit platelets (Born et al., 1965; Sinakos and Caen, 1967; Philp and Bishop, 1970; Michal et al., 1969) but is a much less effective inhibitor of aggregation of horse, guinea pig, cat, rat, and mouse platelets (Sinakos and Caen, 1967; Philp and Bishop, 1970; Nunn, 1981).

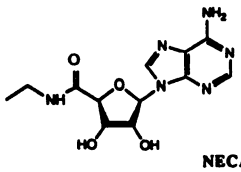
The inhibitory actions of adenosine on platelets have been shown to be mediated by an adenosine receptor that is coupled to adenylate cyclase via a G_s regulatory protein. Receptor occupation stimulates adenylate cyclase, which then results in an increase in the intracellular concentration of cyclic AMP (Haslam and Rosson, 1975; Edwards et al., 1987). Increasing inhibition of platelet aggregation by adenosine is matched by parallel increases in cyclic AMP content (Mills and Smith, 1971; Haslam and Rosson, 1975; Cusack and Hourani, 1981a) and, as expected, the inhibitory action of adenosine is potentiated by inhibitors of its uptake into platelets (see section III.A.3) and by inhibitors of cyclic AMP phosphodiesterase (Haslam, 1973; Haslam and Rosson, 1975). The failure of adenosine to inhibit aggregation of rat

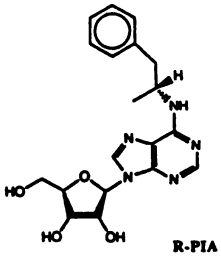
platelets was correlated with an insufficient production of cyclic AMP compared with that produced by PGE_1, which does inhibit rat platelet aggregation (Michel et al., 1976).

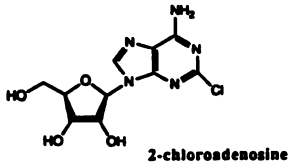
1. Structure-activity relationships. A large number of analogues of adenosine have been synthesised (see fig. 5 for some examples of structures) and tested for their ability to inhibit activation of platelets. Usually human or rabbit platelets have been used, with adenosine analogues being assessed by their inhibition of ADP-induced aggregation. Comparisons of inhibitory potency among analogues is complicated by their relative, and usually unknown, susceptibility to metabolism by adenosine deaminase or to their uptake into platelets (see Haslam and Cusack, 1981). Nonetheless, some general conclusions regarding the structural requirements of the adenosine receptor can be made and, overall, this receptor is clearly of the A_2 subtype (Haslam and Cusack, 1981; Hourani and Cusack, 1985; Cusack and Hourani, 1991).

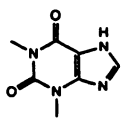
A high degree of structural specificity for agonist activity exists, and the other naturally occurring nucleosides, guanosine, inosine, cytosine, and uridine, are inactive (Born et al., 1965). The adenine base is required for maximal activity, and of the five nitrogen atoms, the N^3, N^6, and N^9 members are essential, but the N^1 may be replaced by methylene as occurs in 1-deazaadenosine (Born et al., 1965; Antonini et al., 1984); the N^7 nitrogen can be replaced by carboxamidomethylene, as in sangivamycin, or by cyanomethylene provided the C^8 position has an amino substituent, as found in 6-aminotoyocamycin (Agarwal et al., 1979). Substitutions on the N^1 atom lead to loss of activity (Born et al., 1965; Kikugawa et al., 1977).











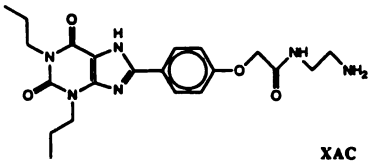


FIG. 5. Some examples of platelet A_2 receptor agonists and antagonists.

Some C^2-modified analogues, such as 2-azaadenosine, 2-methylthioadenosine, and 2-trifluoromethyladenosine, are virtually inactive (Born et al., 1965; Gough and Maguire, 1965), whereas others, including 2-fluoroadenosine, 2-chloroadenosine, 2-bromoadenosine, certain S-derivatives of 2-thioadenosine such as 2-cycloheptylthioadenosine, 2-alkynyladenosines, and the photolysable 2-azidoadenosine, are potent inhibitors of human platelet aggregation (Born et al., 1965; Kikugawa et al., 1973b; Agarwal and Parks, 1975; Cusack and Born, 1976, 1977; Agarwal et al., 1979; Cristalli et al., 1990).

The N^6 nitrogen may be monosubstituted by a variety of aryl groups as in *R*-PIA, and N^6-phenyladenosine, by cycloalkyl groups as in CPA and CHA, and by unsaturated alkyl groups as in N^6-allyladenosine. However, disubstitution on the N^6 nitrogen leads to loss of activity (Dietman et al., 1970; Kikugawa et al., 1973a). There is an absolute requirement for the D-ribose, because replacement by other pentose sugars, as occurs in 9-β-D-arabinofuranosyladenine, abolishes activity. The nature and placement of the ribose linkage to the adenine base is also crucial, because 9-α-D-ribofuranosyladenine (α-adenosine) and 7-β-D-ribofuranosyladenine are inactive (Born et al., 1965). Agonist activity is retained, however, if the ring oxygen of ribose is replaced by a methylene group, e.g., carbocyclic adenosine (Agarwal et al., 1979).

The platelet adenosine receptor exhibits absolute stereoselectivity toward the natural enantiomer of adenosine. Its unnatural L-enantiomer, 9-β-L-ribofuranosyladenine (L-adenosine), as well as its related L-enantiomers, 2-chloro-L-adenosine and 2-azido-L-adenosine, are all devoid of activity (Cusack et al., 1979). The 2′ and 3′ ribose hydroxyls are both required, but the 5′-hydroxyl may be replaced by an ethylamine group provided the 5′-methylene group is converted to a carbonyl. The latter compound, NECA, is the most potent adenosine analogue and is up to 10-fold more potent than adenosine at inhibiting ADP-induced aggregation. Again, the L-enantiomer, L-NECA, is inactive, confirming the absolute stereoselectivity of the adenosine receptor (Cusack and Hourani, 1981a). The related compounds, CPCA and MECA, are both more potent than adenosine, but the unsubstituted 5′-carboxamidoadenosine is less potent (Ukena et al., 1984). The 2-alkynyl-NECA derivatives are also potent inhibitors (Cristalli et al., 1990). Replacement of the N^1-nitrogen of NECA by a methylene group, as in 1-deaza-NECA, results in a 10-fold reduction in potency (Cristalli et al., 1988).

In general, the effects of structural modifications of adenosine on its potency as an inhibitor of platelet aggregation parallel the effects on its potency as a stim-

ulator of adenylate cyclase, where adenosine itself has an EC_{50} value of approximately 1 μM and can generate up to a 10-fold increase in the content of cyclic AMP (Mills and Smith, 1971; Haslam and Rosson, 1975). In intact platelets, in which relatively few analogues have been tested for their effects on adenylate cyclase, 2-chloroadenosine and 2-azidoadenosine are as potent as adenosine and generate similar maximal increases in cyclic AMP (Mills and Smith, 1971; Haslam and Rosson, 1975; Cusack and Born, 1977). NECA is approximately 1.3-fold more potent than adenosine and again generates a 10-fold increase in cyclic AMP content (Cusack and Hourani, 1981a; Hourani and Cusack, 1985). Stimulation of cyclic AMP production by adenosine and by these analogues is stereospecific, because their L-enantiomers are all inactive (Cusack et al., 1979; Cusack and Hourani, 1981a).

Some ribose-modified analogues, such as 2′,5′-dideoxyadenosine as well as high concentrations of adenosine itself, inhibit the formation of cyclic AMP; inhibition apparently occurs following their transport into the platelet and a subsequent action at an intracellular "P" site on the catalytic subunit of adenylate cyclase (Haslam and Rosson, 1975; Haslam et al., 1978). Most analogues of adenosine are not taken up by platelets (see section III.A.3). However, in membranes prepared from homogenates of platelets the actions of adenosine analogues on adenylate cyclase can be complicated by their possible inhibitory actions at the P site to which they now have unrestricted access. Thus, 2-chloroadenosine has no inhibitory actions on cyclic AMP production in intact platelets, whereas in membranes low concentrations stimulate, and high concentrations inhibit, adenylate cyclase (Haslam et al., 1978). 2-Benzylthioadenosine at high concentrations also has an inhibitory action at the internal P site in membrane preparations, but 2-aminoadenosine, 2-hydrazinoadenosine, 2-methyladenosine, and 2-azidoadenosine do not. These analogues, as well as some 2-alkynyladenosines, stimulate adenylate cyclase in membrane preparations approximately as well as does adenosine at low concentrations (Haslam et al., 1978; Londos and Wolff, 1977; Cristalli et al., 1990).

In platelet membranes, the N^6 analogues CHA, CPA, 2-chloro-CPA, and *R*-PIA are approximately as potent as adenosine as activators of adenylate cyclase, but the N^1-deaza versions, 1-deaza-CHA, 1-deaza-CPA, 1-deaza-*R*-PIA, and 1-deaza-2-chloro-CPA, have only one-tenth their potency (Londos and Wolff, 1977; Haslam et al., 1978; Hüttemann et al., 1984; Cristalli et al., 1988; Lohse et al., 1988b). There is an absolute requirement for D-ribose, because adenine coupled to L-ribose, various deoxyriboses, or to other sugars fails to stimulate adenylate cyclase in membrane preparations (Londos and Wolff, 1977; Haslam et al., 1978). NECA and CPCA are the most active analogues, being up to fourfold more potent than adenosine, whereas MECA and 5′-carboxamidoadenosine are less potent than adenosine (Hüttemann et al., 1984; Ukena et al., 1984). 1-Deaza-NECA has only one-tenth the potency of NECA and, because the maximal extent of activation of adenylate cyclase produced in membrane preparations by 1-deaza-NECA, CPA, *R*-PIA, and the 1-deaza versions of CPA, CHA, and of *R*-PIA, is approximately 50% that of NECA, these analogues have been described as partial agonists (Cristalli et al., 1988). 2-Alkynyl derivatives of NECA are also active (Cristalli et al., 1990), and the tolerance allowed at the C^2 position to very large substitutents is illustrated well by the agonist potency of the rather baroque analogue, 2-[4-(2-{2-(4-aminophenyl)methylcarbonylamino)ethylaminocarbonyl}ethyl)phenyl]ethylamino-NECA, which is fully as active on adenylate cyclase as NECA itself (Barrington et al., 1989). N^6-substituted analogues of NECA generally have potency values for activation of adenylate cyclase between that of NECA and the N^6-substituted adenosines (Olsson et al., 1986).

Most of the analogues of adenosine that fail to exhibit agonist activity, i.e., inhibition of platelet aggregation and/or stimulation of adenylate cyclase, have not been tested as antagonists of these actions of adenosine (Haslam and Cusack, 1981). Prevention by 5′-deoxy-5′-methylthioadenosine of inhibition caused by adenosine or its analogues of platelet aggregation was originally interpreted as being due to transmethylation of membrane phospholipids (Agarwal and Parks, 1980). However, this effect was later shown to be a consequence of adenosine receptor antagonism (Agarwal and Parks, 1982).

Methylxanthines, including the naturally occurring caffeine and theophylline (fig. 5), are adenosine receptor antagonists, but elucidation of their action has been complicated by their concurrent ability to inhibit cyclic AMP phosphodiesterase activity, which in itself causes inhibition of platelet aggregation (Mills and Smith, 1971). Antagonism of adenosine receptor-mediated accumulation of cyclic AMP in intact platelets can be demonstrated in the presence of concentrations of papaverine that are sufficient to completely inhibit phosphodiesterase activity. Using this method, Haslam and Rosson (1975) found caffeine and theophylline to be competitive antagonists of adenosine with K_i values in the μM range; theophylline is an antagonist of NECA and has similar μM K_i value (Cusack and Hourani, 1981a). Similarly, in platelet membrane preparations, theophylline and caffeine antagonize stimulation of adenylate cyclase by NECA with K_i values similar to those derived from intact platelets (Ukena et al., 1986b).

Replacement of one, two, or three of the N-methyl groups of caffeine by propyl, or substitution of the N^1-methyl by propargyl, improves caffeine's antagonist potency up to 10-fold in the case of 7-methyl-1,3-dipropylxanthine (Ukena et al., 1986a). The theophylline analogue, N^3-isobutyl,N^1-methylxanthine, antagonises

NECA-stimulated adenylate cyclase activity in platelet membranes (Edwards et al., 1987; Martinson et al., 1987). Substitution at C^8 of theophylline by phenyl or cycloalkyl groups increases antagonist potency up to 100-fold, and a systematic study found 8-cycloalkyl analogues of caffeine and of 1,3-dipropyl-7-methylxanthine to have similar potencies, with 8-phenyl-1-propylxanthine being the most potent (Shamim et al., 1989). Substitutions on the C^8-phenyl group can increase antagonist potency still further, as occurs in 8-(2-amino-4-chlorophenyl)-xanthine, whose potency is 600-fold greater than that of theophylline (Martinson et al. 1987), and led to the development of XAC (fig. 5) which is also approximately 600-fold more potent that theophylline, having a K_i value of 0.024 μM (Ukena et al., 1986a). Substitutions on the cycloalkyl moiety did not improve the potency of 8-cyclopropyl-1,3-dipropylxanthine or of 8-cyclohexyl-1,3-dipropylxanthine, except in the case of 8-(*trans*-4-acetamidomethylcyclohexyl)-1,3-dipropylxanthine, which is as potent as 8-(2-amino-4-chlorophenyl)xanthine and XAC (Katsushima et al., 1990).

Stripping D-ribose from adenosine leaves adenine, which does not antagonise inhibition by adenosine of platelet aggregation and inhibits only weakly stimulation of adenylate cyclase by NECA. Replacement of ribose by a methyl group, as in 9-methyladenine, increases antagonist potency 32-fold, and an additional substitution at N^6 allows further increases, N^6-cyclopentyl-9-methyladenine being threefold more potent than theophylline (Ukena et al., 1987). The importance of the N^6 nitrogen is apparent from the complete lack of activity of the corresponding O^6-substituted 9-methylhypoxanthines (Ukena et al., 1987).

A medley of structurally more remote fused planar ring systems are also adenosine antagonists. These include a series of 1,3-dimethylbenzodipyrazoles, of which the most potent is 1,7-dihydro-3,5,8-trimethylbenzo[1,2-c:5,4-c′]dipyrazole which is threefold more potent than theophylline (Peet et al., 1988). Certain pyrazolopyridines such as cartazolate, imidazopyridines such as 6-bromo-5,7-dimethyl-2-phenylimidazopyridine, the pryrazoloquinolinone CGS 8216, some mesionic benzothiazolopyrimidines, and the benzopteridone alloxazine, are two- to 10-fold more potent than theophylline, and the triazoloquinazoline CGS 15943a is 7000-fold more potent than theophylline at antagonizing stimulation by NECA of adenylate cyclase (Daly et al., 1988).

2. Binding studies and receptor isolation. In a study of the effect of analogues of NECA on platelets, their relative potencies as stimulants of adenylate cyclase matched their abilities to displace [^{3}H]NECA from platelet membranes, with NECA, MECA, and CPCA being the most potent with K_i values of 0.34, 0.38, and 0.68 μM, respectively (Ukena et al., 1984). The number of high affinity [^{3}H]NECA-binding sites in platelet membranes prepared from intact human platelets that had been incubated for 24 h with 2-chloroadenosine was reduced by up to 60% with no change in the K_D value, a finding consistent with the homologous desensitization of adenosine receptor-mediated stimulation of adenylate cyclase in membranes similarly prepared (Edwards et al., 1987).

Early experiments using [^{3}H]PIA and [^{3}H]NECA to determine their suitability as radioligands for the human platelet adenosine receptor demonstrated that binding was consistent with that expected for the A_2 subclass (Hüttemann et al., 1984). Specific binding of [^{3}H]NECA was threefold greater than that of [^{3}H]PIA, and nonspecific binding of [^{3}H]NECA was 6% of total binding compared to 45% of total in the case of [^{3}H]PIA. Unlabeled NECA was 1000-fold more potent as a displacer of [^{3}H]NECA than of [^{3}H]PIA, but *R*-PIA only weakly displaced [^{3}H]PIA and did not displace [^{3}H]NECA binding. In view of this, [^{3}H]NECA was selected for further studies of its binding to human platelet membranes. In these studies, saturation experiments revealed the existence of high affinity sites (K_D 0.16 μM; B_{max} approximately 5500 sites per platelet) and low affinity sites (K_D 2.9 μM; B_{max} approximately 22,000 sites per platelet) (Hüttemann et al., 1984). [^{3}H]NECA binding was not displaced by ADP or by dipyridamole, but binding was displaced potently by 2′,5′-dideoxyadenosine and by adenine (Hüttemann et al., 1984); these and other reservations about the identity of these [^{3}H]NECA-binding sites on platelet membranes led to subsequent studies using solubilised A_2 adenosine receptors (Lohse et al., 1988a).

Human platelet membranes solubilised in 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid and then subjected to gel filtration yielded two [^{3}H]NECA-binding fractions, the first eluting in the void volume and representing 10 to 25% of the total NECA binding and the second well separated and having the remainder of the binding activity. Saturation experiments with the first fraction gave a K_D value of 46 nM and a B_{max} of approximately 330 sites per platelet, and equilibrium was achieved after 90 min at 0°C with a half-life for displacement by XAC of 35 min. Similar experiments with the second fraction gave a K_D value of 230 nM and a B_{max} of approximately 58,000 sites per platelet, and equilibrium was very rapid with a half-life for association and displacement of 40 and 20 s, respectively. Comparison of the results of competition experiments with the first and second fractions and with unsolubilised membranes showed that displacements of [^{3}H]NECA from the first fraction by a variety of adenosine receptor agonists and antagonists were consistent with their pharmacological profile at the adenosine receptor, and displacements from the second fraction and from membranes were similar to each other and not consistent with activity at an adenosine receptor (Lohse et al., 1988a). In particular, K_i values for the displacement of [^{3}H]-NECA from the first fraction by theophylline, 1,3-die-

thyl-8-phenylxanthine, and XAC closely matched their K_i values for antagonism of NECA-stimulated adenylate cyclase; these antagonists failed to displace radioligand binding from either the second fraction or from membranes. The first fraction would appear, therefore, to be a soluble preparation of the platelet adenosine A_2 receptor. Because [^{3}H]NECA binding was found to be inhibited by GTP, the receptor seemed to remain partly coupled to the G_s protein after solubilization and implied the existence of a low affinity state which, however, was too low to be detected by this radioligand assay. In any event, a good correlation existed between the agonist potencies in the high affinity state and the adenylate cyclase assay (Lohse et al., 1988a), unlike the findings in other tissues with the A_1 receptor with which agonist potencies were correlated with its low affinity state (Lohse et al., 1986).

The radiolabeled antagonist XAC has also been used in binding studies with human platelet membranes (Ukena et al. 1986a). [^{3}H]XAC binds at 37°C in a saturable fashion to a homogenous population of noncooperative sites with a K_D value of 12 nM and B_{max} of approximately 700 sites per platelet. Nonspecific binding, defined by excess theophylline or NECA, is very high at 75%, occurring mostly to the filters used, and was diminished only a little to 60% by conducting the filtration at 4°C. Despite this, the profile of displacement of [^{3}H]XAC by competing ligands is in accord with their pharmacological profile on intact platelets and on adenylate cyclase activity in platelet membranes (Ukena et al., 1986a). In particular, [^{3}H]XAC is displaced by theophylline, caffeine, and unlabeled XAC, with K_i values similar to their K_i values for antagonism of those actions of NECA on platelets and, importantly, was not displaced by the ADP receptor antagonist, ATP (Ukena et al., 1986a).

A photoaffinity analogue of PIA, (*R*)-2-azido-N^6-*p*-hydroxyphenylisopropyladenosine has recently been shown to become irreversibly incorporated into human platelet membranes. This incorporation can be blocked by theophylline and reduces the ability of NECA to stimulate adenylate cyclase, indicating that the adenosine receptor has been covalently modified (Lohse et al., 1991). Based on the finding that the reduction in the number of adenosine receptors led to a less than proportional decrease in the extent and rate of adenylate cyclase activation, a new model, involving restricted collision coupling, was proposed for the adenosine receptor-effector interactions (Gross and Lohse, 1991).

3. Uptake and metabolism of adenosine. The uptake of adenosine by platelets has been studied extensively because of its intrinsic interest as a process in the synthesis of intracellular nucleotides and because of its possible relationship to inhibition by adenosine of platelet aggregation (for review, see Haslam and Cusack, 1981). Adenosine is transported into human platelets by both a high affinity and a low affinity uptake system (Sixma et al., 1976). The high affinity system has a K_m value of 9.8 μM and a V_{max} of 0.8 nmol/min per 10^9 platelets and is inhibited powerfully by papaverine, N^6-(4-nitrobenzyl)thioguanosine, N^6-(4-nitrobenzyl)thioinosine, and dipyridamole, with K_i values in the μM range (Born and Mills, 1969; Haslam and Rosson, 1975; Sixma et al., 1976; Lips et al., 1980). Adenosine transported by the high affinity uptake system is immediately phosphorylated by an adenosine kinase to AMP, which is further phosphorylated to ADP and ATP. Because the normal ATP to ADP ratio in platelets is 1.5:1 and because [^{14}C] adenosine labels ATP to ADP in the ratio 7:1, this means that the destination of adenosine is largely the metabolic nucleotide pool rather than to the dense granule pool; subsequent nucleotide exchange is very slow from the metabolic pool (Rozenberg and Holmsen, 1968; Haslam and Rosson, 1975; Reimers et al., 1977; Agarwal and Parks, 1975). Evidence in support of this is provided by studies of the uptake of 2-fluoroadenosine and of carbocyclic adenosine, which are rapidly taken up and phosphorylated by platelets to 2-fluoro-ATP and carbocyclic-ATP, respectively. These phosphorylated derivatives are separable from ATP by high performance liquid chromatography and are found in the metabolic pools and not in the dense granules (Agarwal et al., 1979). Not surprisingly, studies of the actions of a wide range of adenosine analogues found no direct relationship between their susceptibilities to uptake and phosphorylation and their potencies as inhibitors of platelet aggregation (Agarwal et al., 1979; Lips et al., 1980). [^{3}H]NECA has been shown not to be taken up in platelets under conditions in which [^{3}H]adenosine is rapidly transported (Hourani and Cusack, 1985).

The nature of the uptake process when high concentrations of adenosine are present is controversial. A low affinity uptake system has been described, having a K_m value of 9.4 mM and a V_{max} of 106 nmol/min per 10^9 platelets, that is not inhibited by N^6-(4-nitrobenzyl)thioguanosine but is inhibited by adenine and by 8-bromoadenine at μM concentrations (Sixma et al., 1976; Lips et al., 1980). Transport in this case could also be one of passive diffusion (Haslam and Rosson, 1975), but in any event adenosine appears to arrive inside the platelet to be either deaminated to inosine or phosphorylated to adenine nucleotides (Haslam and Rosson, 1975; Sixma et al., 1976). The metabolism of adenosine by platelets from different mammalian species varies, and the rate of uptake is faster in human platelets than it is in those of rats and guinea pigs (Doni, 1981).

4. Physiological and pathological importance. Because adenosine inhibits platelet activation by all agents, it has essentially an antiplatelet action and, therefore, possibly assists in the prevention of atherogenesis and thrombogenesis arising from platelet adhesion and aggregation. Adenosine concentration in vivo is normally very low (20

to 200 nM), and adenosine given exogenously has a half-life measured in seconds (Moser et al., 1989). However, the concentration locally of adenosine is greatly increased during periods of hypoxia and ischaemia (Belardinelli et al., 1983; for reviews, see Belardinelli et al., 1989; Pelleg and Porter, 1990), and, as well as causing vasodilation, adenosine could serve to render platelets less responsive to the effects of activating agents released by hypoxic cells.

ATP released from damaged blood vessel walls and from platelets that adhere to them is readily dephosphorylated initially to ADP by ectonucleotidases present on endothelial cells and on vascular smooth muscle. ADP recruits circulating platelets to the site of injury to form a haemostatic plug (Born and Kratzer, 1984). Further dephosphorylation of ADP generates adenosine, which again serves to render platelets less responsive, perhaps to limit the size of the haemostatic platelet plug and to minimize the formation of thrombi (for review of cardiovascular actions of adenosine and adenine nucleotides, see Olsson and Pearson, 1990).

Extensive research has been performed by the pharmaceutical industry aimed at synthesizing adenosine A_2 receptor agonists as antiplatelet drugs thereby producing compounds with therapeutic potential as antithrombotics, but so far the use of adenosine analogues in such a role would appear to be fraught with cardiovascular complications (for review, see Williams, 1990).

B. Prostaglandins

It has now been established from several lines of investigation that there are two inhibitory prostanoid receptors on human platelets which, in the nomenclature of Coleman et al. (1984), would be called an IP receptor (recognising PGI_2) and a DP receptor (recognising PGD_2). Of the naturally occurring cyclooxygenase metabolites of arachidonic acid (the bisenoic prostaglandins), PGI_2 and PGD_2 (and some of their active metabolites) are the most potent platelet inhibitors, whereas PGE_2 has weaker, but more complex, actions and can inhibit or potentiate platelet activation according to the circumstances. PGE_1, a cyclooxygenase metabolite of dihomo-γ-linolenic acid, is also a potent platelet inhibitor, although somewhat less potent than PGI_2 and PGD_2, and it is believed to act through the IP platelet receptor. Although it is generally accepted that the weak inhibitory effects of PGE_2 are also mediated via the IP receptor, this interpretation has been questioned and a separate PGE_2 receptor ("EP") has been proposed (Eggerman et al., 1986). For general reviews concerning inhibitory prostaglandin receptors on platelets, see MacIntyre (1981), Myers et al., (1985), and Oliva and Nicosia (1987).

Several lines of evidence support the idea that there are separate receptors for PGD_2 and for PGI_2 and PGE_1 on platelets (for reviews see MacIntyre, 1981, and Whittle et al., 1985). Whereas PGI_2 is active in all mammalian species tested, as is PGE_1 whose potency generally parallels that of PGI_2, the potency of PGD_2 is highly species dependent; dog, rabbit, guinea pig, cat, and monkey platelets are much less sensitive to PGD_2 than are human platelets, whereas rat platelets do not respond (Mills and Macfarlane, 1977; Whittle et al., 1978, 1985). In guinea pig platelets the dose-response curve to PGD_2 is bell shaped, with high concentrations having less inhibitory effect than expected and even inhibiting the effects of PGI_2; these actions appear to be due to a weak agonist interaction with the platelet TXA_2 receptor (Hamid-Bloomfield and Whittle, 1986). The potencies of PGD_2 and PGE_1 in human platelets also vary independently from donor to donor (Mills and Macfarlane, 1974), and, indeed, platelets from patients with myeloproliferative disorders fail to respond to PGD_2 but do retain sensititivty to PGI_2 (Cooper et al., 1978b).

Desensitisation studies also support the existence of two inhibitory prostaglandin receptors on platelets, because cross-desensitisation is observed between PGE_1 and PGI_2 but not between these agonists and PGD_2 (Miller and Gorman, 1979). The PGD_2 antagonist N-0164 (a phloretin derivative) inhibits the effect of PGD_2 on platelets but not that of PGE_1 or PGI_2 (MacIntyre and Gordon, 1977; Whittle et al., 1978) and, although at similar concentrations N-0164 also has antagonist effects at the platelet TXA_2 receptor (Hamid-Bloomfield and Whittle, 1986), this differential effect strengthens the suggestion that PGD_2 does not act via the same receptor as PGI_2. Similar differential effects have been reported with AH 6809, a PGD_2 antagonist which, like N-0164, also has antagonist actions at the TXA_2 receptor (Keery and Lumley, 1985, 1988). The lack of effect of AH 6809 on the actions of PGE_1 also confirms the belief that these actions are mediated via the PGI_2 receptor and not via a separate EP_1 receptor, because AH 6809 is also a potent EP_1 antagonist (Coleman et al., 1985; Eglen and Whiting, 1988). Binding studies using [^{3}H]PGI_2 and [^{3}H]PGD_2 also confirm the existence of two inhibitory prostaglandin receptors on human platelets and suggest that PGE_1 interacts with the PGI_2 receptor (Schafer et al., 1979; Siegl et al., 1979a,b; Siegl, 1982).

1. Structure-activity relationships. Because PGI_2 is such a powerful platelet inhibitor, hundreds of analogues have been synthesised (see fig. 6 for some examples of structures) in an attempt to find a potent, stable, orally active compound that could be of therapeutic value as an antiplatelet drug. Studies of structure-activity relationships are complicated by the existence on platelets of two separate inhibitory prostaglandin receptors as well as by the presence of a stimulatory TXA_2 receptor and the lack of specific antagonists to define clearly on which receptor a compound is acting. In spite of this, several very detailed reviews have appeared concerning this subject, some of which support the pharmacological data with evidence derived from binding studies (MacIntyre,

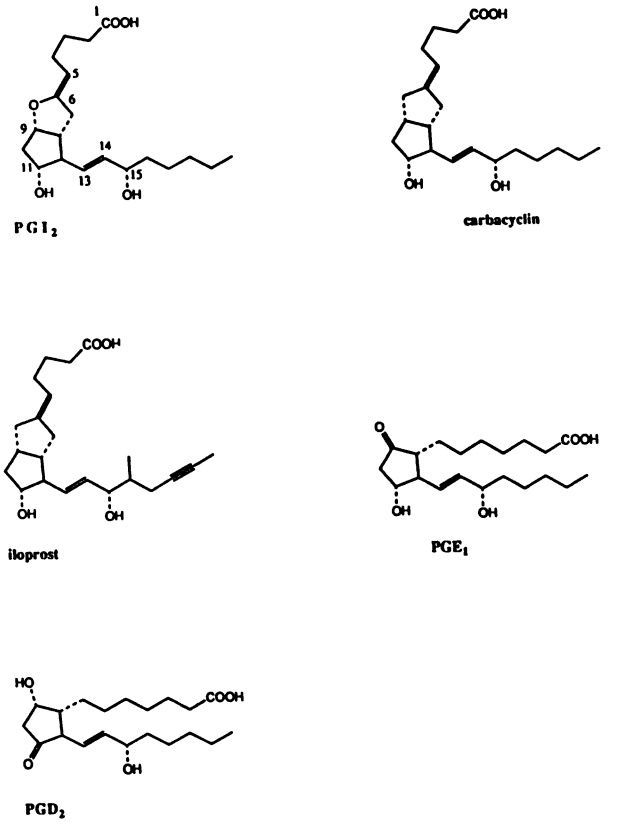


FIG. 6. Some examples of platelet IP and DP receptor agonists.

1981; Myers et al., 1985; Whittle et al., 1985; MacIntyre et al., 1987; Oliva and Nicosia, 1987; Tsai and Wu, 1989).

PGI_2 analogues with modifications of the bicyclic ring system in which the oxygen link between C_6 and C_9 has been replaced by a more stable methylene (to give carbacyclin), thio, or nitrilo linkage are active on platelets, although not all are as potent as PGI_2 itself (Morton et al., 1979). Hydrolysis of the C_6,C_9 oxygen linkage, which occurs rapidly in aqueous solutions, results in the formation of 6-keto-$PGF_{1\alpha}$ which is inactive. Some derivatives of carbacyclin, such as the ω-chain-modified analogue iloprost (ZK 36374) or the 9-substituted analogue 9-ethynylcarbacyclin, are even more potent than PGI_2 and, like carbacyclin, have the advantage of stability (Schror et al., 1981; Skuballa and Vorbrüggen, 1983; Aristoff et al., 1983b).

Saturation of the C_5,C_6 double bond yields the two isomers of PGI_1, of which the $C_{6\beta}$ isomer is more similar in configuration to PGI_2 and more potent than the $C_{6\alpha}$ isomer. However, both isomers are less potent than PGI_2, as is the C_5,C_6(E) isomer of PGI_2 in which the orientation of the α-chain about the 5-carbon is inverted (Crane et al., 1978; Johnson et al., 1979). PGI_2 does not adopt a "hairpin" configuration like the other prostaglandins, but instead forms a Z-shaped molecule, and the activity of phenylene analogues with constrained α-chains is maximal when they can achieve this configuration (Aristoff et al., 1983a). Another feature of the α-chain that is important for activity is the terminal carboxyl group, as, indeed, it is for most prostaglandins (Kawasaki et al., 1980).

The hydroxyl groups on carbons C_{11} and C_{15} are important for activity, and replacement or removal of these groups greatly reduces potency, as does inversion of the configuration of the C_{15}-OH from the normal *S* isomer to *R* (Anderson et al., 1981b; Flohé et al., 1983). Most other modifications to the ω-chain of PGI_2 also reduce activity, although elongation by one carbon to give C_{20}-methyl PGI_2 enhances potency; desaturation of the C_{17},C_{18} bond to give PGI_3 does not appear to reduce potency (Anderson et al., 1981a; Van Dorp et al. 1978; Dyerberg and Bang, 1979; Needleman et al., 1979). Desaturation of the C_{13},C_{14} double bond reduces activity, whereas conversion of this bond to a triple bond enhances activity (Johnson et al., 1979, Fried and Barton, 1977), as does addition of a triple bond between C_{18} and C_{19} and addition of a methyl group on C_{16} of carbacyclin to give iloprost (Schror et al., 1981; Skuballa and Vorbrüggen, 1983). The orientation of this C_{16} methyl group in iloprost is important, the *S* isomer being approximately 20 times more potent than the *R* (Tsai et al., 1988).

Overall, the structural features that are thought to be important for the activity of PGI_2 are the α-carboxylic group, the hydroxyls on C_{11} and C_{15}, the $C_{6\alpha}$ ring oxygen, and the relative positions between these groups. The structure-activity relationships of PGE_1 analogues, in general, mirrors those of PGI_2, and the modifications that reduce the ability of PGE_1 to adopt a PGI_2-like conformation reduce activity, as would be expected if these two prostaglandins interact with a common receptor (MacIntyre, 1981; Myers et al., 1985; Tsai and Wu, 1989).

The structure-activity relationships for PGD_2 have not been investigated to the same extent as those for PGI_2, but again the C_{15}-OH is important for activity, as are the C_5,C_6 and C_{13},C_{14} double bonds (Bundy et al., 1983). PGD_1 (5,6-dihydro-PGD_2) is, therefore, less potent than PGD_2, but the potency of PGD_3, which has an extra double bond between C_{17} and C_{18}, has been reported to be both higher (Whitaker et al., 1979) and lower (Bundy et al., 1983) than that of PGD_2. Elongation of the ω-chain reduces rather than enhances activity (Bundy et al., 1983), as does replacement of the four carbons from the end by a *p*-fluorophenoxy substituent (Jones et al., 1979). Although ω-chain modifications seem to reduce activity, some modifications to the ring, such as loss of the 9-OH or inversion of its configuration, result in compounds that are surprisingly more potent than PGD_2 (Bundy et al., 1983). Indeed, a hydantoin prostaglandin analogue, BW 245C, which has a carbonyl group rather than a hydroxyl on C_9, nitrogens instead of carbons at C_{10} and C_{12} of the ring, and a cyclohexyl ring on C_{15} replacing the ω-terminal pentyl chain, is approximately 10 times more potent than PGD_2 in human platelets, and

TABLE 1
Platelet receptors

Agonist	Antagonists	Receptor type	Coupling systems	Radioligands	Structural information
ADP	ATP	P_{2T} (unique)	? Ca^{2+} influx ↓ cyclic AMP	[^{3}H]ADP [^{32}P]2-MeSADP	100-kDa protein "aggregin" isolated, but significance unclear
Adrenaline (α-receptor)	Yohimbine	$alpha_{2A}$	? ↓ cyclic AMP	[^{3}H]Yohimbine [^{3}H]Dihydroergocryptine	64-kDa protein isolated, cloned, sequenced and expressed in frog oocytes
5-HT	Ketanserin	5-HT_2	PL-C	[^{3}H]Ketanserin [^{3}H]LSD	232-kDa complex
Vasopressin	dVDAVP	V_{1A}	PL-C ? ↓ cyclic AMP	[^{3}H]AVP [^{125}I]AVP	125-kDa protein isolated
TXA_2/PGH_2	13-APA, SQ 29,548, S-145	TP (TXA_2/PGH_2)α	PL-C ↓ cyclic-AMP	[^{3}H]U44069/U46619 [^{125}I]PTA-OH [^{3}H]SQ 29,548	37-kDa protein cloned, sequenced and expressed in COS 7 cells and in frog oocytes
PAF	BN 52021 Kadsurenone		PL-C ↓ cyclic AMP	[^{3}H]PAF	180 kDa?, 160 kDa?, 220 kDa?, 52 kDa?
Thrombin	SC42619		PL-C ↓ cyclic AMP	[^{125}I]-Thrombin	GPIb (180 kDa, 165 kDa + 15 kDa subunits); N.B. Enzymic action too (substrate GPV)
Collagen			? Adhesion-induced release		GPIa (160 kDa)?, 65 kDa? GPIV (88 kDa)? Platelet factor XIII?
Adenosine	Theophylline	A_2	↑ cyclic AMP	[^{3}H]NECA [^{3}H]XAC	
PGI_2/PGE_1		IP	↑ cyclic AMP	[^{3}H]PGI_2 [^{3}H]PGE_2 [^{3}H]Iloprost	180 kDa (85 kDa + 95 kDa subunits)
PGD_2	AH6809 N-0164	DP	↑ cyclic AMP	[^{3}H]PGD_2	
Adrenaline (β-receptor)	Butoxamine Propranolol	β_2	? ↑ cyclic AMP	[^{125}I]Iodopinodolol	

is thought to act on the PGD_2 receptor (Whittle et al., 1985).

A number of nonprostanoid compounds such as AH 6809 and N-0164 have rather weak and nonselective antagonist activity at DP receptors (see above and Giles and Leff, 1988), but no PGI_2 antagonists are known. 5Z-Carbacyclin, which has the opposite configuration to the natural 5Z-PGI_2, has been reported to be a partial agonist for PGI_2 receptors in blood vessels, but it is a full agonist on platelets, albeit less potent than the 5E isomer (Corsini et al., 1987). Similar results have been obtained with the related analogue FCE-22176 [(5Z)-13,14-didehydro-20-methylcarbocyclin] which is an antagonist in guinea pig trachea and atria (Fassina et al., 1985) but a full agonist in human platelets (Wilkins and MacDermot, 1986). Although these findings have been interpreted as providing evidence for different receptor types on platelets and blood vessels (Oliva and Nicosia, 1987; Corsini et al., 1987), they could equally easily be explained in terms of different numbers of receptors, with vascular tissues having a lower receptor number and, therefore, converting low efficacy agonists into partial agonists or apparent antagonists. Although some selectivity for platelets is clinically desirable and may be achievable (Whittle and Moncada, 1984), there does not seem at the moment to be any firm basis for concluding that platelet

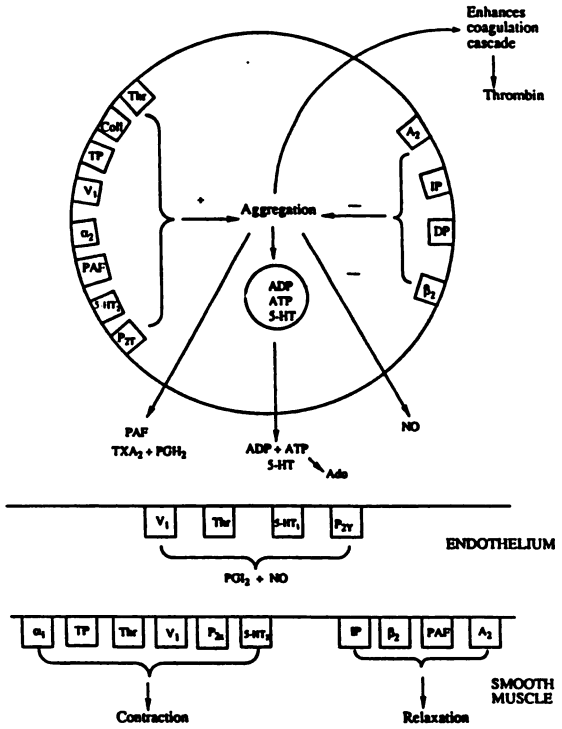


FIG. 7. Cartoon illustrating interactions of pharmacological receptors on blood platelets and related receptors on blood vessels and some of the mediators released by platelets and by endothelial cells. In the presence of intact endothelium these mediators cause vasodilation and inhibition of aggregation via release of PGI_2 and of nitric oxide (NO), whereas in the absence of an endothelial layer the overall response is vasoconstriction and enhancement of aggregation. Thr, thrombin; Coll, collagen; Ado, adenosine; PL-C, phospholipase C. See text for details and abbreviations.

PGI_2 receptors are different from those in smooth muscle.

2. Binding studies and receptor isolation. Binding studies using radiolabeled prostaglandins that have been carried out on intact platelets and in platelet membrane preparations confirm the existence of separate receptors for PGI_2/PGE_1 and PGD_2 on platelets. In spite of the lability of PGI_2, binding studies using [^{3}H]PGI_2 as a ligand have been successful even in platelet-rich plasma, because only a few minutes are required for incubation; breakdown of PGI_2 can be either slowed by reducing the temperature or estimated by bioassay and a correction made for any loss (Siegl, 1982). Most groups have reported the existence of two binding sites, one with high affinity (K_D approximately 10 nM) and a small number of binding sites (100 to 3000 per platelet) and the other with low affinity (K_D approximately 1 μM) but a larger capacity (3000 or more sites per platelet) (see MacIntyre and Armstrong, 1987, for a table). Binding was displaced by PGE_1 and some active analogues of PGI_2 but not by PGD_2 or inactive compounds, confirming that PGD_2 acts at a different receptor from PGI_2/PGE_1 (Siegl et al., 1979a; Shepherd et al., 1983; Schillinger and Prior, 1980; Lombroso et al., 1984).

Binding studies using [^{3}H]PGE_1 as radioligand agree with studies using [^{3}H]PGI_2 and also demonstrate the presence of low and high affinity sites with binding being inhibited by PGI_2 but not by PGD_2 (Schafer et al., 1979).

Although some authors have suggested that the high affinity site corresponds to the receptor through which PGI_2 and PGE_1 act to inhibit platelet aggregation (Siegl et al., 1979a; Shepherd et al., 1983), others have suggested that both sites are involved (Lombroso et al., 1984, Nicosia et al., 1984). It has even been proposed that the low affinity site may be the relevant one (Siegl, 1982). In a study using gel-filtered platelets, however, only one binding site was detected for [^{3}H]PGI_2, and the antiaggregating potency for a number of analogues correlated with their ability to displace the binding at this site. The K_D for this binding site was 60 nM with a B_{max} of 1410 sites per platelet (corresponding to the high affinity site reported by other authors), but this K_D value doubled when the platelets were resuspended in plasma, suggesting that binding to plasma proteins reduced the effective free concentration of ligand (Eggerman et al., 1987). Even in platelet-rich plasma, these authors detected only one binding site, identical with that seen in gel-filtered platelets resuspended in plasma, and they suggested that the low affinity site observed by others is equivalent to nonspecific binding. However, similar studies using [^{3}H]PGE_1 and [^{3}H]PGE_2 as radioligands appeared to show separate binding sites for PGE_2 at which PGE_1 also bound (Eggerman et al., 1986). This finding is contrary to the generally accepted view that PGE_1 acts at the PGI_2 receptor.

[^{3}H]Iloprost has also been used as a radioligand to label PGI_2 receptors and might be expected to be more reliable than [^{3}H]PGI_2 because of its stability (Hall and Strange, 1984; Steurer et al., 1987). Indeed, in a number of studies of a series of analogues, a correlation was observed among inhibition of [^{3}H]iloprost binding, inhibition of aggregation, and increases in cyclic AMP formation (Oliva and Nicosia, 1987). Even when a mixture of isomers was used, only one binding site was observed, and when binding studies were performed using the separated radiolabeled isomers the 16(S) isomer had a K_D of 13.4 nM compared to 288 nM for the 16(R) isomer; the latter isomer was also approximately 20 times less potent at inhibiting aggregation than the 16(S) isomer. When a mixture of isomers was used, the binding or activity of the 16(S) isomer, therefore, dominates, and the 16(R) isomer contributes little and does not interfere with the action of the more active isomer (Tsai et al., 1988).

A PGI_2/PGE_1-binding protein that appears to have the characteristics of the receptor has been isolated and purified from human platelet membranes. It consists of two subunits of 85 and 95 kDa, giving a combined value of 180 kDa (Dutta-Roy and Sinha, 1987).

Binding studies have identified a separate binding site for [^{3}H]PGD_2 at which PGE_1 and PGI_2 compete with

low affinity. Only one class of binding site has been detected by several groups of workers, with a K_D value for PGD_2 of approximately 10 nM and a B_{max} of 200 to 800 sites per platelet (Cooper and Ahern, 1979; Bonne et al., 1981; Siegl et al., 1979b; Siegl, 1982). The platelets of patients with myeloproliferative disorders, which are insensitive to PGD_2 but not to PGI_2, show a reduced number of binding sites for $[^3H]PGD_2$, which suggests that this binding does mediate the antiaggregating activity of PGD_2 (Cooper and Ahern, 1979).

3. Physiological and pathological importance. PGI_2 is the major prostaglandin formed by endothelial cells. It is the most potent endogenous inhibitor of platelet aggregation known and as such is thought to be an important regulator of platelet function (see Moncada, 1982, for a review). Although PGI_2 is no longer thought to be a continuously circulating hormone, and basal levels in blood are too low to affect platelet function, local formation of PGI_2 is thought to be important both in limiting the extent of thrombus formation and in dispersing small platelet aggregates (Haslam and McClenaghan, 1981). The clinical effectiveness of low dose, as opposed to high dose, aspirin as an antithrombotic drug is attributed to the different sensitivities of the platelet and endothelial cyclooxygenase activity (partly due to the inability of platelets to synthesise new enzyme); low dose aspirin blocks TXA_2 formation but spares PGI_2 and, therefore, retains its antiaggregating activity (Moncada, 1982; Fuster et al., 1989). Similarly, the reported beneficial effect of high intakes of eicosapentaenoic acid is thought to be due partly to its substituting for arachidonic acid in the prostaglandin pathway, resulting in the synthesis of the inactive TXA_3 and the active PGI_3, again shifting the balance toward more inhibitory prostanoids and retaining the antiaggregating effect of PGI_2 (Dyerberg and Bang, 1979; Sanders, 1985). Use of PGI_2 itself as an antiplatelet drug is limited by its instability and pronounced vasodilator activity, although some analogues achieve a measure of selectivity in this respect and may lead to more useful compounds (Whittle and Moncada, 1984).

The role of PGD_2 in platelet function is unclear, but because it is formed by aggregating platelets it may act as a feedback inhibitor to limit thrombus formation. Increased production of PGD_2 caused by redirection of the metabolism of PGG_2/PGH_2 may also play a role in the antiplatelet effects of TXA_2 synthetase inhibitors (for review see Giles and Leff, 1988).

C. Catecholamines (β-Receptor)

Although in humans the overall platelet response to adrenaline is aggregation mediated via α_2-adrenoceptors (see section II.B), platelets also possess inhibitory β-receptors that can be revealed by α_2-blockade, and the ratio of the α- to β-receptor population determines the extent of platelet activation (Kerry et al., 1984). In rat platelets, for example, the ratio is close to 1 and no proaggregatory response to adrenaline is observed in the absence of β_2 blockade. It is generally accepted that the platelet β-receptor, as expected, mediates platelet inhibition by stimulating adenylate cyclase via a G_s protein, although this has been questioned and the small observed increases in cyclic AMP caused by β-agonists have been attributed to effects on contaminating white cells (Cook et al., 1988). The platelet β-receptor has not been studied extensively and is unlikely to have any physiological or pathological significance, but there have been some reviews of this field (Scrutton and Wallis, 1981; Kerry and Scruttton, 1985; Barnett et al., 1985; Jakobs and Minuth, 1987). For discussion of the uptake and metabolism of catecholamines by platelets, see section II.B.

1. Structure-activity relationships. Although early studies gave confusing results as to the presence and nature of β-adrenoceptors on human platelets (Scrutton and Wallis, 1981), it is now accepted that the platelet β-adrenoceptor is of the β_2-subtype (Kerry and Scrutton, 1985; Barnett et al., 1985; Jakobs and Minuth, 1987). In the presence of an α-blocker, adrenaline inhibits platelet aggregation, whereas noradrenaline does not, indicating the presence of a β-receptor. Selective β_2-agonists such as salbutamol are inhibitors (although partial agonists), whereas β_1-agonists such as dobutamine are not. The inhibitory effects of isoprenaline are blocked by nonselective β-blockers such as propanolol or by β_2-selective antagonists such as butoxamine but not by β_1-selective antagonists such as atenolol (Kerry and Scrutton, 1983a). Similar agonist and antagonist selectivities are observed if the effects on adenylate cyclase are studied, confirming the existence of a β_2-receptor coupled to adenylate cyclase (Kerry and Scrutton, 1983a; Winther et al., 1985). Platelets from rats and rabbits also possess β_2-receptors that can mediate inhibition of aggregation and, in the case of rats, have been shown to stimulate adenylate cyclase, whereas in guinea pig platelets no responses can be detected probably because the receptor density is very low (Kerry et al., 1984; Jakobs and Minuth, 1987).

Some β-antagonists can themselves inhibit aggregation induced by adrenaline and other aggregating agents. This is not a result of an interaction with the β_2-adrenoceptors but is a nonspecific effect related to the lipophilicity of these compounds, an observation that can cause problems in the interpretation of results (Kerry and Scrutton, 1985).

2. Binding studies. Binding studies using the high specific activity β-antagonist radioligand $[^{125}I]$iodopindolol and related compounds have also detected a small number (<100) of binding sites on intact platelets and on platelet membranes with the characteristics of β_2-adrenoceptors (Steer and Atlas, 1982; Kerry et al., 1984; Cook et al., 1985; Wang and Brodde, 1985). In a study comparing platelets from different mammalian species, the B_{max} per platelet was found to be 66 for humans, 41

for rats, 14 for rabbits, and <5 for guinea pigs, which explains the lack of effect of β-agonists on guinea pig platelets (Kerry et al., 1984). The ability of other β-adrenoceptor ligands to displace [^{125}I]iodopindolol binding confirms that this receptor is of the β_2-subtype, because nonselective or β_2-selective agonists or antagonists inhibit binding more effectively than do β_1-selective compounds (Kerry et al., 1984; Wang and Brodde, 1985; Cook et al., 1985; Jakobs and Minuth, 1987). The same conclusion was reached in a binding study using low concentrations of the antagonist ligand [^{3}H]dihydroalprenolol, although this ligand fails to label β-adrenoceptors specifically at higher concentrations (Winther et al., 1985; Kerry and Scrutton, 1983b).

Binding studies have also been used to investigate whether, as expected, the β_2-adrenoceptor on platelets is coupled to a G protein. Studies using (−)-[^{125}I]iodopindolol as radioligand failed to show any effect of GMP-PNP on the displacement of binding by isoprenaline, suggesting that the human platelet β_2-adrenoceptor is poorly coupled to adenylate cyclase (Cook et al., 1985). This group also failed to detect any stimulation by isoprenaline of adenylate cyclase activity in highly purified human platelets and attributed the stimulation observed by others to contamination of platelet suspensions by small numbers of leukocytes (Cook et al., 1988). In contrast, using [^{125}I]iodocyanopindolol as radioligand and rat platelets, Jakobs and Minuth (1987) reported that GTP caused the characteristic shift to the right and steepening of the displacement curve for an agonist, isoprenaline, but not for an antagonist, propranolol, as would be expected for a G protein-coupled receptor.

IV. Conclusions

From a pharmacologist's point of view, platelets in many ways combine the more interesting features of smooth muscles and nerves. As with smooth muscles, they are activated by agents that increase the amounts of intracellular Ca^{2+} and are inhibited by those that increase intracellular concentrations of cyclic AMP and cyclic GMP, and their responses are contractile in nature, resulting in a physical change that can be easily measured. Like nerves, they also release active agents from granules following activation and possess uptake systems for some agonists, which has led to their use as models for the less accessible CNS neurotransmitter systems.

Platelets possess a unique array of both stimulatory and inhibitory receptors (see table 1 for summary) and are, perhaps, best viewed in the context of their haemostatic role. They have complex and important interactions with blood vessels, in particular with the endothelial cells that normally line blood vessels and shield the underlying vascular smooth muscle from mediators released from platelets. Most of the agonists acting on (and released from) platelets that are likely to have any physiological significance also have effects on blood vessels and in general tend to inhibit aggregation and vasoconstriction in intact blood vessels while promoting both in damaged ones that lack endothelium (fig. 7). Indeed, aggregating platelets cause marked constriction of blood vessel preparations that lack endothelium but much less constriction or even vasodilation in vessels if the endothelium is present. ADP, 5-HT, vasopressin, and thrombin all cause endothelium-dependent vasodilation but directly contract exposed vascular smooth muscle, whereas PAF is a vasodilator, TXA_2 is a vasoconstrictor, and adrenaline causes vasoconstriction via α-receptors and vasodilation via β-receptors. Endothelial cells produce PGI_2 and nitric oxide, both of which inhibit aggregation as well as causing vasodilation. Damage to the blood vessels as well as removing this endothelial protection may also reveal subendothelial collagen which is a powerful platelet stimulus (see Vanhoutte, 1988, for a review of platelet-blood vessel interactions).

Because platelets are so readily accessible and technically easy to work with, they are one of the few cell types in which it should be relatively simple to correlate ligand binding, activation of intracellular second-messenger systems, and physiological responses. Whereas much is indeed known for some agonists such as 5-HT, vasopressin, TXA_2, PAF, adenosine, and PGI_2, for others such as ADP and adrenaline there are still significant gaps in our understanding of the train of events by which receptor binding results in platelet activation. In particular, relatively little is known about the action of the two proteins, collagen and thrombin, that are probably of major importance in vivo.

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