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COMMENTARY

P_2 PURINOCEPTORS IN THE BLOOD VESSEL WALL

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Knowledge of physiological mechanisms controlling vascular tone and permeability helps to shed light on pathological processes, notably inflammatory and atherosclerotic vascular diseases. In the last 10 years, since the original proposal by Burnstock [1] that ATP and ADP act at P_2 purinoceptors distinct from those (P_1) that recognize adenosine, it has become clear that extracellular adenine nucleotides have powerful and often divergent actions on the main cellular elements (smooth muscle and endothelium) of the cardiovascular system. Extracellular ATP and ADP have been detected following secretion at the neuromuscular junction [2], secretion from activated blood platelets, or following release from damaged cells [3]. The pathophysiology of purinoceptor-mediated events in the vessel wall may be as important as, though currently less well understood than, the effects of the more generally recognised mediators of vascular tone and permeability, such as kinins and biogenic amines. In this review we have summarized recent research progress concerning the regulation of vascular responsiveness by extracellular adenine nucleotides, and attempted in particular to point out areas where data are sparse or new biochemical or pharmacological tools are needed.

Classification of P_2 purinoceptors

Subtyping of the P_2 receptor was first proposed on the basis of the divergent actions of ATP in visceral smooth muscle preparations, exemplified by its excitatory (constrictor) effect in guinea-pig bladder and relaxant action in guinea-pig taenia coli [4]. The use of a variety of ATP analogues has strengthened the concept that these two responses are mediated by different receptors, designated P_{2X} and P_{2Y}, because they exhibit different potency rankings for these analogues as agonists. For example, 2-chloro- and 2-methylthio-ATP are substantially more potent than ATP at relaxing taenia coli but equiactive at constricting bladder, whereas analogues in which methylene or imido groups replace the α, β or β, γ oxygen bridges in the triphosphate are less potent than ATP at P_{2Y} receptors but often considerably more potent at P_{2X} receptors. Finally, substitution of the natural D-ribose by its L-enantiomer yields L-ATP and its analogues, which are approximately equiactive with the D-isomers at P_{2X} but usually significantly less potent at P_{2Y} receptors [5]. A similar pharmacological profile indicates the presence of P_{2X} and P_{2Y} receptors in blood vessels (Table 1). The P_2 purinoceptors on certain other cell types, notably leukocytes, mast cells and platelets, have features that differentiate them from either P_{2X} or P_{2Y} receptors, and they have therefore at present been assigned further subtypes [5].

A current problem in P_2 receptor classification is the lack of any selective, reversible antagonist. Two compounds have been used that may provide clues for the design of better antagonists, but each has obvious shortcomings. ANAPP3, a photoaffinity ATP analogue, is a highly selective antagonist at P_2 (particularly P_{2X}) receptors after photolysis, but its action is irreversible [6, 7]. Reactive Blue 2, which reversibly antagonises P_{2Y}-mediated responses, has additional non-specific effects at similar or only slightly greater concentrations [8, 9]. Thus, although much indirect evident supports the separate identity of P_{2X} and P_{2Y} receptors, there is, as yet, no proof.

P_2 purinoceptors on endothelial cells

In nearly all intact blood vessels with resting tone the response to added ATP is vasodilatation. Nonetheless, isolated smooth muscle from many of these vessels contracts when ATP is added. This apparent paradox, which applies to several other agonists (notably acetylcholine), was resolved by Furchgott, who first showed that the dilator response to acetylcholine required the presence of intact endothelial cells [10], and this was confirmed subsequently for ATP by De Mey and Vanhoutte [11].

Adenosine is also a powerful dilator in most vascular beds (with the notable exception of the kidney) acting in an endothelium-independent manner directly on smooth muscle [12]. Since endothelial cells possess nucleotidase ectoenzymes that degrade ATP to adenosine (see below), it might be thought that ATP is a dilator only by virtue of its biotransformation to adenosine, and consequent action on P_1 receptors. In nearly all vascular sites studied, however, it is clear that ATP acts at P_2 receptors on endothelium, at which adenosine is inactive. Several observations support this conclusion: for example, in a variety of vascular beds (e.g. the coronary) ATP is a more potent dilator than adenosine [13, 14]; also, selective inhibition of adenosine receptors with methylxanthine antagonists rarely blocks the effect of ATP [5, 11, 12].

The endothelial vasodilator P_2 purinoceptor has

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Table 1. Vascular P_2 purinoceptors

Feature	P_{2X}	P_{2Y}
Location	Smooth muscle	Endothelium, plus some smooth muscle
Action of ATP	Constrictor	Dilator
Potency of analogues:		
(a) 2-Purine substituted	<ATP	>ATP
(b) APCPP, APPCP, APPNP	>ATP	<ATP
Stereoselectivity for D vs L enantiomers	Little or none	High
Selective antagonism	$ANAPP_3$	Reactive blue 2

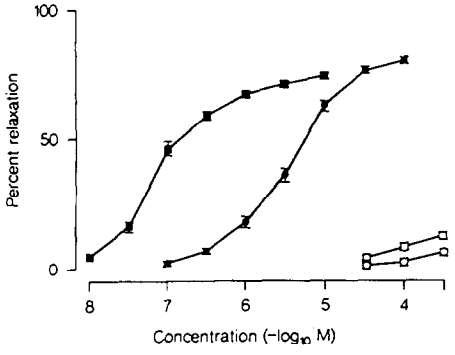

Fig. 1. Endothelium-dependent relaxation of pig aortic strips preconstricted with histamine (20 μM), induced by enantiomers of adenine nucleotides. Key: (●) ATP; (■) 2-methylthio-ATP; (○) L-ATP; and (□) 2-methylthio-L-ATP. Vertical bars show SE of the mean for 4–8 tissues. Reproduced from Ref. 15, with permission.

been subtyped as P_{2Y} on the basis of the rank order of potency of ATP analogues (see Fig. 1). Thus, in several vessels, C2-substituted ATP analogues are more potent than ATP, whereas L-ATP and its analogues are very poorly active [5, 15]. In support of this, Hourani *et al.* [16, 17] who developed L-β,γ-methylene-ATP and adenosine 5′-(2-fluoro-diphosphate) as highly selective P_{2X} and P_{2Y} agonists, respectively, on the basis of their actions in guinea-pig bladder and taenia coli, found that the latter compound was active, and the former inactive, as dilators of guinea-pig aorta.

The synthesis of current data from several species, including human, indicates that endothelial cells in many vascular beds possess P_{2Y} receptors, which are activated by ATP at a threshold concentration of $\simeq 1$ μM. It is to be expected that further studies will clarify differences between species and/or vascular beds—for example, experiments on the vasculature of the brain have established that cerebral, basilar and pial vessels are unusually sensitive to ATP, responding to doses of <1 nM [18, 19].

It has been proposed in a few vessels that the endothelium lacks P_{2Y} receptors (though more detailed studies may be needed in order to clarify the position). For example, when endothelium-dependent vasodilatation induced by ATP was observed in the rabbit central ear artery, it was deduced to be due to the catabolism of ATP to adenosine, despite the fact that endothelium-dependent vasodilatation to adenosine has rarely been reported [20]. Endothelial cells have been shown to possess A_2 adenosine receptors, linked to stimulation of adenylate cyclase [21], but it is not known what the biological relevance of this response may be. There is little evidence of any functional interaction between A_2 and P_{2Y} receptor activation, for which the intracellular signalling pathways are discussed below.

Endothelium-dependent vasoconstriction has also been found in response to a variety of stimuli in certain blood vessels. The mediator(s) involved has not been identified directly, though candidates include superoxide anion, thromboxane A_2 and the peptide endothelin [22]. The first study noting P_2 receptor-mediated endothelium-dependent contraction (in canine basilar artery where thromboxane A_2 was suggested to be the mediator) was published recently [23]. Subclassification of this P_2 receptor has not yet been reported.

In microvessels, exogenous ATP causes increased vascular permeability. Although it has been argued in some cases that this is due to a direct action of ATP to increase cytoplasmic [Ca^{2+}] in the endothelium, the response can also be mediated by histamine, released when ATP binds to local mast cell P_2 purinoceptors [24, 25].

P_2 receptors on vascular smooth mucle cells

As noted above, the response of isolated vascular smooth muscle to ATP is often constriction. This has been well studied in the coronary bed. Removal of endothelium, or the use of P_{2X}-selective agonists, induces endothelium-independent contractions [8, 14]. Thus, the net effect of ATP *in vivo* may depend critically on the extent to which it can act on vascular smooth muscle, i.e. whether the ATP is generated luminally or abluminally, and whether the endothelium is intact. In some perfused vessels (e.g. rat pancreatic and dog carotid arteries) ATP induces only vasoconstriction, apparently acting at smooth muscle P_{2X} receptors, in the presence of endothelium

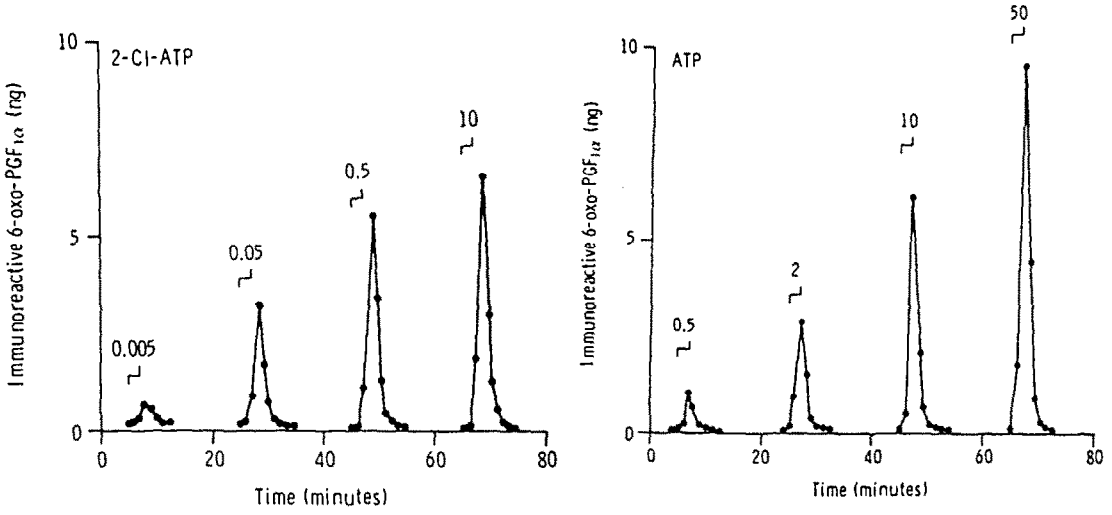

Fig. 2. Dose-dependent stimulation of PGI_2 production by ATP or 2-chloro-ATP. Pig aortic endothelial cells on microcarrier beads were superfused and exposed to agonist for 2-min periods. Reproduced from Ref. 68, with permission.

[26, 27]. Whether the constrictor response in these arteries reflects a relative lack of endothelial P_{2Y} receptors is not clear. There are also two examples of isolated vessels (rabbit mesenteric artery and portal vein) that can contract or dilate to ATP in the absence of endothelium depending on basal tone, where the use of analogues indicates the presence of both P_{2X} and P_{2Y} purinoceptors on the smooth muscle cells [28, 29].

Cellular signalling pathways at endothelial cell P_2 purinoceptors

At least two labile, potent, vasodilators and inhibitors of platelet function are secreted by endothelial cells in response to P_{2Y} receptor activation. Prostacyclin (PGI_2) (see Fig. 2) may contribute to endothelium-dependent vasodilatation in certain beds; but the more important mediator at most sites is endothelium-derived relaxing factor (EDRF), recently identified as nitric oxide (NO), which is synthesised from L-arginine [30, 31]. PGI_2 acts by stimulating adenylate cyclase in smooth muscle cells or platelets [32], whereas NO stimulates guanylate cyclase [33], and interacts synergistically with PGI_2 to inhibit platelet aggregation [34].

Some of the transduction pathways at the endothelial P_{2Y} receptor have now been characterised. ATP and ADP, like other agonists that stimulate receptor-mediated PGI_2 synthesis in endothelium, each induce dose-depedent elevations in cytoplasmic [Ca^{2+}] associated with production of inositol trisphosphate (IP_3), indicating receptor coupling to phospholipase C [35–38]. There are two components to the [Ca^{2+}] rise: a rapid initial peak elevation (up to a few micromolar, from a resting level of about 100 nM), which then falls to a steady-state elevation (up to 300–400 nM) maintained for many minutes in the presence of the agonist. The initial peak is due to release of Ca^{2+} from internal stores, presumably induced by IP_3. The steady-state level is dependent on the presence of extracellular Ca^{2+} and reflects the activity of a receptor-stimulated Ca^{2+} influx mechanism [37]. It should be noted that this description of the changes in cytoplasmic [Ca^{2+}] is derived from studies of populations of cells. Recent work in which cytoplasmic calcium concentrations have been determined in individual endothelial cells indicates that, at least in response to histamine, the observed steady-state [Ca^{2+}] in cell populations is a mean, reflecting the summation of repetitive short-lived spike elevations in single cells [39]. Further changes in ionic conductance are induced by P_2 agonists, apparently as a consequence of raised cytoplasmic [Ca^{2+}]. These include stimulated K^+ efflux [40, 41], and acidification followed by alkalinization of the cytoplasm dependent on Na^+/H^+ exchange [42].

A range of intracellular proteins is phosphorylated following P_{2Y} receptor activation, some of which can also be phosphorylated by activating protein kinase C with exogenous phorbol esters [43]. Since diacylglycerol (an endogenous activator of protein kinase C) is generated concomitantly with IP_3, these results suggest that some of the phosphorylation events triggered by ATP involve protein kinase C, whereas others involve separate, perhaps Ca^{2+}-dependent, kinases. There is evidence in other cell types that a guanine nucleotide binding protein (G_p) couples receptors to phospholipase C. The ability of pertussis toxin to inhibit ATP-stimulated IP_3 production in bovine endothelium, and the demonstration that GTP analogues stimulate phospholipase C in human endothelial cells, support the view that G_p links the endothelial P_{2Y} receptor to phospholipase C [38, 44]. It is not yet known whether the P_{2Y} receptor is coupled directly, or via a G protein, or via soluble cytoplasmic mediators to the activation of Ca^{2+} influx.

PGI_2 synthesis

Under normal conditions, elevation of cytoplasmic

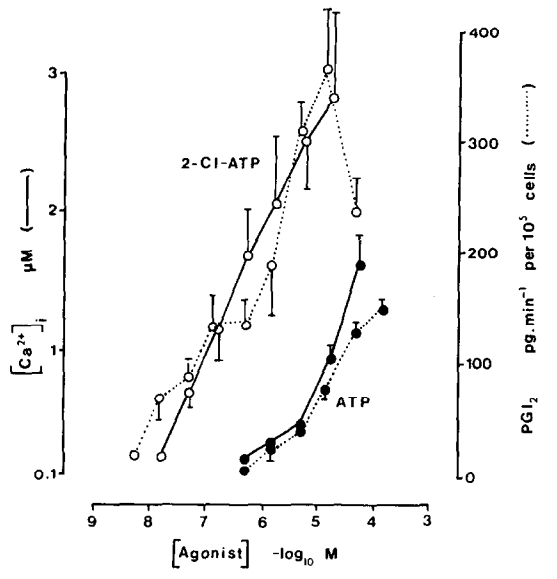

Fig. 3. Dose-dependent elevations of $[Ca^{2+}]_i$ and PGI_2 production by human umbilical vein endothelial cells in response to ATP or 2-chloro-ATP. Based on data in Ref. 37. Values are means ±SE, N = 4–12.

$[Ca^{2+}]$, above a threshold of ≈0.8 μM, is both necessary and sufficient to account quantitatively for PGI_2 production in response to ATP or other agonists such as thrombin [37, 45]. The dose–response relationships for elevation of cytoplasmic $[Ca^{2+}]$ and PGI_2 synthesis by ATP and its more potent analogue, 2-chloro-ATP, are shown in Fig. 3. Raised cytoplasmic $[Ca^{2+}]$ is thought to stimulate phospholipase A_2, known to be Ca^{2+}-sensitive in other cell types, thus activating the main metabolic path for the release of arachidonate. The transience of PGI_2 release, which is complete within a few minutes even in the continued presence of the agonist, reflects the transient elevation of cytoplasmic $[Ca^{2+}]$ above the threshold level. Several features of agonist-stimulated PGI_2 release, however, have not been explained fully at the molecular level. ATP, in common with other receptor-binding agonists, induces rapid and profound homologous tachyphylaxis of PGI_2 release, so that a second challenge is not fully effective until about 60 min after the first [46]. This process is not due to inactivation of cyclooxygenase or PGI_2 synthase, but it has yet to be determined whether release of Ca^{2+} from internal stores to the cytoplasm is similarly desensitized. It could be postulated that diacylglycerol (produced with IP_3 on P_{2Y} receptor activation), though it plays no apparent role in the release of PGI_2, triggers protein kinase C-mediated events that inhibit the ability of ATP to induce PGI_2 release on a subsequent challenge. Activation of protein kinase C by pretreating endothelial cells with phorbol esters, however, substantially enhances PGI_2 release in response to ATP and other agonists [47].

EDRF synthesis

The molecular signals linking P_{2Y} receptor activation to endothelial EDRF (NO) synthesis are poorly understood. Endothelium-dependent vasodilatation and EDRF production, unlike PGI_2 synthesis, are highly sensitive to extracellular $[Ca^{2+}]$ [48, 49]. Furthermore, the release of EDRF in response to ATP (or other agonists that induce transient PGI_2 release) can be maintained for many minutes. These two observations suggest that NO synthesis requires elevations of cytoplasmic Ca^{2+} with a lower threshold than that needed for PGI_2 synthesis, and is related more closely to the effectiveness of the agonist at inducing Ca^{2+} influx to maintain an elevated steady-state cytoplasmic $[Ca^{2+}]$. It is not known where the Ca^{2+} acts, but a recently described enzyme in endothelium, which converts arginine to citrulline and may thus be implicated in NO synthesis, is inhibited by EDTA, suggesting that it may be Ca^{2+}-sensitive [50]. Cumulative dose–response curves for endothelium-dependent vasodilatation to ATP can be produced easily [15], which suggests further that the rapid tachyphylaxis of PGI_2 synthesis on repeated challenge with ATP, though probably associated with molecular events closely coupled to receptor occupation, is unlikely to be due to modulation of the receptor itself.

There is little evidence in favour of any regulatory interaction between PGI_2 and NO synthesis. Blocking prostanoid production does not alter NO synthesis [10, 51]. NO elevates endothelial cGMP levels, as do agonists that induce release of NO and PGI_2, but agonist-stimulated PGI_2 formation can be reduced only slightly by levels of NO much higher than those needed to relax smooth muscle [52].

Cellular signalling pathways at vascular smooth muscle cell P_2 purinoceptors

There is considerable evidence that ATP is a co-transmitter in the cardiovascular system, acting on vascular smooth muscle P_2 receptors. Pre-junctional modulation of transmitter release by purines occurs in mammals predominantly only via P_1 receptors [2, 12]. Electrophysiological studies of isolated vascular smooth muscle have shown that ATP can induce either depolarization of hyperpolarization. Such studies are not often correlated with vascular tone measurement, but it seems likely that depolarization corresponds to contraction mediated by P_{2X} receptors—for example, α,β-methylene-ATP depolarizes and contracts rabbit mesenteric artery smooth muscle, and desensitizes the tissue to depolarization by ATP [53]. Patch clamp studies of smooth muscle cells from the rabbit ear artery demonstrate that ATP induces depolarization and opens Ca^{2+}-selective cation channels, and that the influx of Ca^{2+} is apparently not a consequence of the generation of a soluble intracellular mediator [54]. In addition, ATP induces phosphoinositide turnover in aortic smooth muscle cells [55]; this generates diacylglycerol, which has been implicated in the maintenance of vascular smooth muscle tone in response to other agonists [56].

None of these studies, however, has yet reported results obtained with P_{2X}- or P_{2Y}-selective ATP analogues. It is therefore not certain that these signalling pathways correspond to occupation of P_{2X} receptors or whether P_{2X} and P_{2Y} receptors can co-exist on individual muscle cells. P_{2Y} receptors on vascular

smooth muscle are coupled to relaxation. P_2-mediated relaxation in vascular smooth muscle, as in non-vascular smooth muscle, can be accompanied by hyperpolarization (e.g. guinea-pig coronary artery; Ref. 57), which is though to reflect stimulation of K^+ efflux (itself dependent on Ca^{2+} influx) and may, as in skeletal muscle, be related to relaxation by closing voltage-operated Ca^{2+} channels.

Regulation of extracellular adenine nucleotide concentration by ectonucleotidases

The removal of extracellular ATP and ADP, necessary for the regulation of tissue responsiveness to P_2 agonists, occurs by catabolism to adenosine. Within the blood vessel lumen, this process takes place as a result of sequential dephosphorylation by specific ectonucleotidases (ATPase, ADPase and 5′-nucleotidase) at the endothelial cell surface. Studies with cultured endothelial cells have characterized the kinetics and substrate and inhibitor specificity of these enzymes and demonstrated their capacity to catabolise biologically active concentrations of ATP and ADP very rapidly [58, 59] (see Fig. 4). Parallel studies in isolated perfused organs confirm that extensive inactivation occurs on a single passage through microvascular beds, with sequential dephosphorylation apparently taking place in the same manner as in cultured large vessel endothelial cells [60, 61]. Comparative experiments indicate that soluble enzymes in plasma, or ectoenzymes on blood cells, play little role in this catabolic pathway *in vivo* in flowing blood [62].

P_2 and P_1 receptor-mediated responses are thus coupled functionally by the fact that inactivation of P_2 agonists leads to the generation of the P_1 agonist adenosine, before this is removed by cellular uptake [58]. It is therefore of interest that one of the properties of the ectonucleotidase cascade at the surface of the endothelium—namely, the powerful inhibition of AMP hydrolysis by ADP and ATP—operates to maximize the separation in time between the degradation of P_2 agonists to AMP (which is relatively inactive at purinoceptors) and the subsequent production of adenosine [59]—note the time delay in Fig. 4 before adenosine is formed. In a flowing system, this time separation results in a spatial separation, so that P_2-mediated responses (platelet aggregation, endothelium-dependent vasodilatation, endothelium-dependent vasoconstriction) can occur at different vascular sites from P_1-mediated responses (inhibition of platelet aggregation, endothelium-independent vasodilatation).

Vascular smooth muscle cells possess a similar cascade of ectonucleotidases, though their properties are not identical [63]. In particular, recent experiments provide kinetic evidence consistent with the hypothesis that, unlike on endothelial cells, the individual enzymes of the cascade may be physically associated in clusters on the cell surface. As a consequence, production of adenosine occurs rapidly from ATP or ADP [64]. This, together with the slower uptake of adenosine by smooth mucle cells than endothelial cells [65], increases the opportunities for adenosine to generate local P_1-mediated effects that functionally interact with the initial P_2-mediated response—for example, such as inhibition of further release of ATP or other transmitters at the neuromuscular junction.

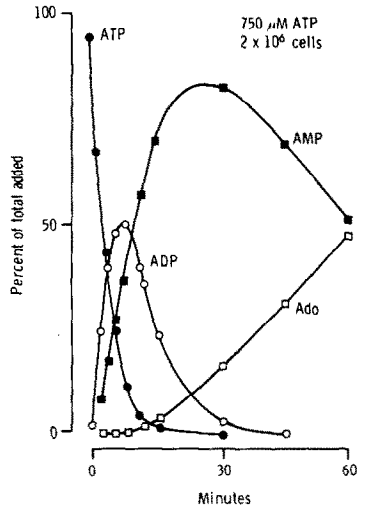

Fig. 4. Rapid sequential dephosphorylation of added ATP when perfusate (0.68 ml) was recirculated over pig aortic endothelial cells on microcarrier beads. Based on data in Ref. 59.

Conclusions

Our knowledge of the pharmacology of P_2 purinoceptors has advanced rapidly in the last decade. The first proposals for the existence of such receptors were greeted by some scepticism that ATP and ADP, ubiquitous and abundant as intracellular molecules with a clearly defined role in energy metabolism, could play any specific extracellular role as signalling molecules, particularly as they do not readily cross cell membranes by conventional mechanisms. Nonetheless it is now evident that many cell types (not just those within the cardiovascular system) possess P_2 purinoceptors that are clearly distinguishable, in terms of their pharmacological profile and in the cellular responses they evoke, from P_1 receptors.

This review has attempted to summarize current knowledge of P_2 purinoceptors within the blood vessel wall, and to identify those research areas where new approaches are required. The most immediate need is potent, selective, reversible antagonists for P_2 receptors. A strategy to clone a gene encoding a P_2 receptor, which may well be analogous to the methods successfully employed in the last 2 years for acetylcholine receptors and adrenoceptors [66, 67], should lead to the definitive identification of P_2 receptor subtypes. Sequence information, particularly of the cytoplasmic domain of the receptors, will complement physiological and biochemical studies to identify the intracellular transduction pathways used by these receptors and thus to classify them in relation to the signalling routes used by the other receptor types. Taken together, this information will allow the rational design of selective pharmacological agents that may provide novel approaches to the treatment of vascular disease.

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