REVIEW ARTICLE

P_2 receptor subtypes in the cardiovascular system

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Extracellular nucleotides have been implicated in a number of physiological functions. Nucleotides act on cell-surface receptors known as P_2 receptors, of which several subtypes have been cloned. Both ATP and ADP are stored in platelets and are released upon platelet activation. Furthermore, nucleotides are also released from damaged or broken cells. Thus during vascular injury nucleotides play an important role in haemostasis through activation of platelets, modulation of vascular tone, recruitment of neutrophils and monocytes to the site of injury, and facilitation of adhesion of leucocytes to the endothelium. Nucleotides also moderate these functions by generating nitric oxide and prostaglandin I_2 through activation of endothelial cells, and by activating different receptor subtypes on vascular smooth muscle cells. In the heart, P_2 receptors regulate contractility through modulation of L-type Ca^{2+} channels, although the molecular mechanisms involved are still under investigation. Classical pharmacological studies have identified several P_2 receptor subtypes in the cardiovascular system. Molecular pharmacological studies have clarified the nature of some of these receptors, but have complicated the picture with others. In platelets, the classical P_{2T} receptor has now been resolved into three P_2 receptor subtypes: the P_{2Y1}, P_{2X1} and P_{2TAC} receptors (the last of these, which is coupled to the inhibition of adenylate cyclase, is yet to be cloned). In peripheral blood leucocytes, endothelial cells, vascular smooth muscle cells and cardiomyocytes, the effects of classical P_{2X}, P_{2Y} and P_{2U} receptors have been found to be mediated by more than one P_2 receptor subtype. However, the exact functions of these multiple receptor subtypes remain to be understood, as P_2-receptor-selective agonists and antagonists are still under development.

INTRODUCTION

Extracellular nucleotides are known to regulate several physiological responses, including vascular tone, cardiac function and haemostasis [1,2]. These effects are mediated by cell-surface receptors called P_2 receptors. The P_2 receptors were classically divided into six subtypes, i.e. P_{2X}, P_{2Y}, P_{2U}, P_{2D}, P_{2Z} and P_{2T} [1,2]. Molecular cloning of the P_2 receptor subtypes has led to modification of the nomenclature [3,4]. According to the new nomenclature, approved by IUPHAR, the P_2 receptors are

Table 1 Pharmacological characteristics of cloned P_{2X} receptor subtypes

BzATP, 2′,3′-*O*-(4-benzoylbenzoyl)ATP; *I*, current; see Abbreviations footnote for other definitions. Data are selected from [181].

Subtype	Species	Pharmacological profile	Properties	GenBank no.	Ref.
P_{2X1}	Rat	2-MeSATP > ATP > pp[CH_2]pA	$I_{Na/K/Ca}$	X80477	[149]
	Human	ATP > pp[CH_2]pA	$I_{Na/K/Ca}$	X83688	[150]
P_{2X2}	Rat	2-MeSATP > ATP; pp[CH_2]pA inactive	$I_{Na/K}$	U14414	[151]
P_{2X3}	Rat	2-MeSATP > ATP > pp[CH_2]pA	$I_{Na/K}$	X90651	[152]
	Rat	ATP > 2-MeSATP > pp[CH_2]pA	$I_{Na/K/Ca}$	X91167	[153]
	Human	2-MeSATP > ATP > pp[CH_2]pA	$I_{Na/K/Ca}$	Y07683	[154]
P_{2X4}	Rat	ATP > 2-MeSATP ≫ pp[CH_2]pA	$I_{Na/K}$	X91200	[155]
	Rat	ATP > 2-MeSATP ≫ pp[CH_2]pA	$I_{Na/K}$	U32497	[156]
	Human	ATP > 2-MeSATP > CTP ≫ pp[CH_2]pA	$I_{Na/K/Ca}$	Y07684	[157]
P_{2X5}	Rat	ATP > 2-MeSATP > ADP	$I_{Na/K/Ca}$	X92069	[158]
	Rat	ATP > 2-MeSATP ≫ pp[CH_2]pA	$I_{Na/K}$	X97328	[137]
P_{2X6}	Rat	ATP > 2-MeSATP > ADP	$I_{Na/K/Ca}$	X92070	[158]
P_{2X7}	Rat	BzATP > ATP > 2-MeSATP > ADP; UTP inactive	$I_{Na/K}$, then pore formation	X95882	[79]
	Human	BzATP > ATP	$I_{Na/K}$, then pore formation	Y09561	[159]

Abbreviations used: A2P5P, adenosine 2′-phosphate 5′-phosphate; A3P5P, adenosine 3′-phosphate 5′-phosphate; A3P5PS, adenosine 3′-phosphate 5′-phosphosulphate; ARL 66096, 2-propylthio-D-β,γ-difluoromethylene-ATP; 2-Cl-ATP, 2-chloro-ATP; MAPK, mitogen-activated protein kinase; 2-MeSADP, 2-methylthio-ADP; 2-MeSATP, 2-methylthio-ATP; PKC, protein kinase C; PLC, phospholipase C; pp[CH_2]pA, adenosine 5′-[α,β-methylene]triphosphate; P_{2TAC}, platelet ADP receptor coupled to inhibition of adenylate cyclase; P_{2TPLC}, platelet ADP receptor coupled to activation of PLC; RT-PCR, reverse transcription–PCR; VSMC, vascular smooth muscle cells.

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Table 2 Pharmacological characteristics of cloned P_{2Y} receptor subtypes

AC, adenylate cyclase; see Abbreviations footnote for other definitions. Data are selected from [181].

Subtype	Species	Pharmacological profile	Coupling	GenBank no.	Ref.
P_{2Y1}	Chick	2-MeSATP > ATP > ADP; UTP inactive	PLC-β/InsP_3/Ca^{2+}	X73268	[160]
	Turkey	2-MeSATP > ATP > ADP; UTP inactive	PLC-β/InsP_3/Ca^{2+}	U09842	[161]
	Rat	2-MeSATP > 2Cl-ATP > ATP; pp[CH_2]pA inactive	PLC-β/InsP_3/Ca^{2+}	U22830	[162]
	Human	2-MeSADP > ADP; A3P5PS antagonist	PLC-β/InsP_3/Ca^{2+}	AF018284	[30]
	Human	2-MeSADP > ADP; ATP antagonist	PLC-β/InsP_3/Ca^{2+}	Z49205	[163,164]
	Human	2-MeSATP > ATP = ADP	PLC-β/InsP_3/Ca^{2+}	–	[165]
	Bovine	2-MeSATP > ATP $\gg$ UTP	PLC-β/InsP_3/Ca^{2+}	X87628	[166]
P_{2Y2}	Mouse	ATP = UTP $\gg$ 2-MeSATP	PLC-β/InsP_3/Ca^{2+}	L14751	[167]
	Human	ATP = UTP $\gg$ 2-MeSATP	PLC-β/InsP_3/Ca^{2+}	U07225	[168]
	Rat	ATP = UTP	PLC-β/InsP_3/Ca^{2+}	U09402	[169]
	Rat	ATP = UTP > ADP = UDP	PLC-β/InsP_3/Ca^{2+}	L46865	[170]
P_{2Y3}	Chick	ADP > UTP > ATP = UDP	PLC-β/InsP_3/Ca^{2+}	X98283	[171]
P_{2Y4}	Human	UTP = UDP > ATP = ADP	PLC-β/InsP_3/Ca^{2+}	X91852	[172]
	Human	UTP > UDP	PLC-β/InsP_3/Ca^{2+}	U40223	[173]
	Rat	ATP = UTP = ITP = AP_4A	PLC-β/InsP_3/Ca^{2+}	Y14705	[144]
	Rat	ATP = UTP; AP_4A inactive	PLC-β/InsP_3/Ca^{2+}	Y11433	[148]
	Turkey	ATP = UTP > AP_4A	PLC-β/InsP_3/Ca^{2+}	AF031897	[174]
P_{2Y5}	Chick	ATP > ADP > 2-MeSATP $\gg$ UTP = pp[CH_2]pA (binding)	Not determined	P32250	[74]
P_{2Y6}	Rat	UTP > ADP = 2-MeSATP > ATP	PLC-β/InsP_3/Ca^{2+}	D63665	[175]
	Rat	UDP > UTP > ADP > 2-MeSATP > ATP	PLC-β/InsP_3/Ca^{2+}	X97058	[176]
P_{2Y7}*	Human	Leukotriene B4		U41070	[177,178]
P_{2Y8}	*Xenopus*	ATP = UTP = ITP = CTP = GTP	PLC-β/InsP_3/Ca^{2+}	X99953	[179]
P_{2Y9}†	Human	Not determined	Not PLC; not AC	AF005419	[180]
P_{2Y10}†	Human	Not determined	Not determined	AF000545	
P_{2Y11}	Human	ATP > 2-MeSATP $\gg$ ADP	PLC-β/InsP_3/Ca^{2+} and AC stimulation	AF030335	[66]

* This receptor subtype should be deleted from the list, as it was identified as a leukotriene B_4 receptor.

† These receptor subtypes are included in the list based on their sequence identity with P_{2Y} receptors (direct submission to GenBank; they have yet to be shown to respond functionally to nucleotides).

separated into ligand-gated intrinsic ion channels, designated P_{2X}, and G-protein-coupled P_{2Y} receptor subtypes [3,4]. The subtypes are then numbered in the order of their molecular cloning from mammalian sources. To date, seven subtypes of P_{2X} and ten subtypes of P_{2Y} have been identified. However some of these subtypes, identified by sequence identity, still need to be demonstrated to be functional nucleotide receptors (Tables 1 and 2) [5,6]. Although some of the classical pharmacological P_2 receptor subtypes can be identified as a specific molecular subtype, most appear to be a mixture of molecular P_2 receptor subtypes. For example, the P_{2Z} subtype is now identified as P_{2X7} and the human P_{2U} subtype as P_{2Y2} [3,4]. On the other hand, the classical P_{2T} receptor has been resolved into three subtypes [7]. In this review article, we focus on the molecular subtypes of P_2 receptors in the cardiovascular system and their role in physiological function. Certain aspects of this subject have recently been reviewed [8].

SOURCES OF NUCLEOTIDES IN THE CARDIOVASCULAR SYSTEM

Nucleotides can be released from five different sources in the body and subsequently reach the target organs [9]. ATP is released together with noradrenaline (norepinephrine) from sympathetic neurons. Both ATP and ADP are stored in the dense granules of platelets and are released into the bloodstream upon platelet activation. Adrenal chromaffin cells also release nucleotides. Both vascular smooth muscle cells (VSMC) and endothelial cells transport nucleotides from the cytosol across the membrane. Finally, disruption of cells releases cytosolic nucleotides that have various effects on platelets, endothelial cells, leucocytes and VSMC during vascular injury.

PLATELETS

ADP has been known to activate platelets since 1961 [10]. Exposure of platelets to ADP results in an increase in intracellular Ca^{2+}, rapid Ca^{2+} influx, phospholipase C (PLC) activation and inhibition of stimulated adenylate cyclase [11–13]. ADP causes platelets to change shape and activates fibrinogen receptors, leading to platelet aggregation [11,12]. Whether platelets exposed to ADP release their contents of α granules and produce thromboxane A_2 remains controversial [14–21]. Thromboxane A_2 produced by the action of ADP on platelets converts reversible aggregation into irreversible aggregation, which is also referred to as the second wave of aggregation [11,12]. Recently, Bennett et al. [22] reported that ADP causes activation of integrin $\alpha_v\beta_3$ on platelets, which might play an important role in anchoring platelets to disrupted atherosclerotic plaques and the walls of the injured arteries.

All the physiological and intracellular signalling events triggered by ADP in platelets were attributed initially to a single cell-surface receptor. Since the molecular nature of this receptor was unknown, it was designated P_{2T} (P_2 receptors on thrombocytes) [9]. MacKenzie et al. [23] demonstrated an ADP-gated ion channel on platelets, proposed to be a P_{2X1} receptor, causing rapid influx of Ca^{2+}. The mRNA for the P_{2X1} receptor was detected in platelets and several megakaryocytic cell lines by

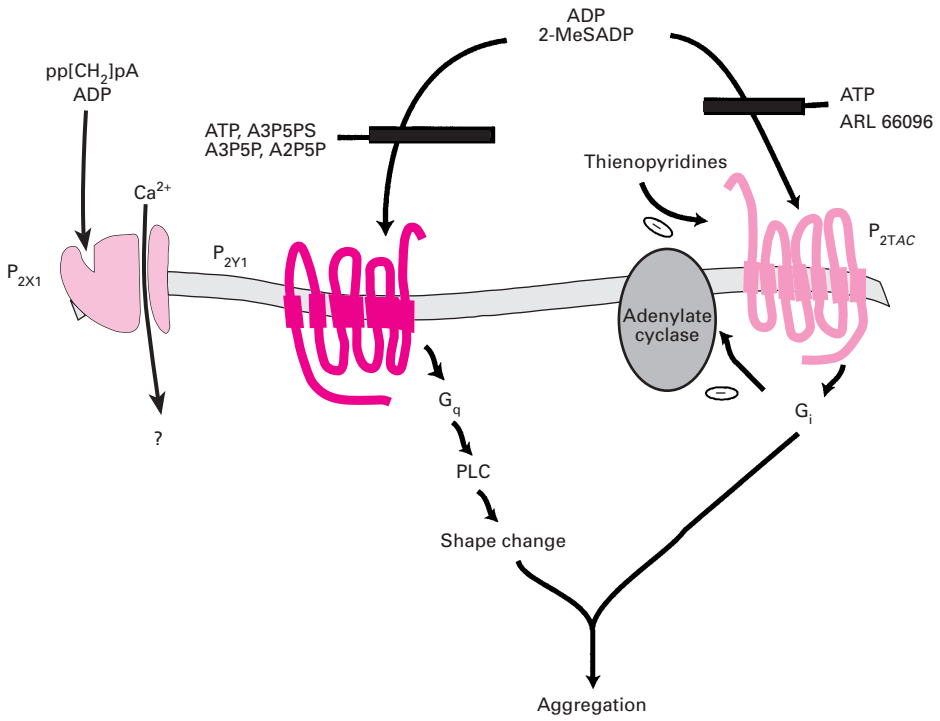

Figure 1 Functional role of three P_2 receptor subtypes in ADP-induced platelet activation

See the text for details. Modified from [143] Trends Pharmacol. Sci. **19**, Kunapuli, S. P. Multiple P_2 receptor subtypes on platelets: a new interpretation of their function, pp. 391–394, copyright 1998, with permission from Elsevier Science.

reverse transcription–PCR (RT-PCR) [24], and the P_{2X1} receptor cDNA was recently cloned from human platelets [25].

Model for activation of platelets by ADP

The availability of selective P_2 receptor ligands has led to a modified view of the effects of ADP on platelets. ARL 66096 (2-propylthio-D-β,γ-difluoromethylene-ATP), a potent inhibitor of ADP-induced platelet aggregation, blocks the ADP-induced inhibition of adenylate cyclase, but does not inhibit ADP-mediated increases in the intracellular Ca^{2+} concentration [7]. Both ADP and 2-methylthio-ADP (2-MeSADP) induce increases in Ins(1,4,5)P_3 and Ins(1,3,4)P_3 levels which peak with a similar time constant to that of the intracellular Ca^{2+} increases. Adenosine 5′-[α,β-methylene]triphosphate (pp[CH_2]pA), a selective agonist for P_{2X1} receptors, has no effect on InsP_3 formation. ARL 66096 does not inhibit intracellular Ca^{2+} mobilization, InsP_3 formation or shape change.

Based on these observations, we proposed a three-receptor model [7], with one receptor coupled to the inhibition of stimulated adenylate cyclase (designated P_{2TAC}), a second coupled to the mobilization of Ca^{2+} from intracellular stores through the activation of PLC and InsP_3 formation (designated P_{2TPLC}), and the third an ionotropic P_{2X1} receptor mediating the rapid influx of Ca^{2+} (Figure 1). Thus the concept of the 'P_{2T}' receptor [9] is resolved into three distinct functional P_2 receptor subtypes. Several other recent studies [26–29] also support the three-receptor model. A cDNA clone encoding the P_{2Y1} receptor has been isolated from a human platelet cDNA library by homology screening and was shown to be the P_{2TPLC} receptor using P_{2Y1}-receptor-selective antagonists [30]. The P_{2Y1} mRNA was demonstrated in both platelets and megakaryocytic cell lines by RT-PCR analysis [31,32].

Platelet shape change

In the presence of extracellular Ca^{2+}, pp[CH_2]pA, a P_{2X1}-selective agonist, causes rapid Ca^{2+} influx, but fails to elicit platelet shape change [30]. pp[CH_2]pA neither causes nor inhibits shape change induced by ADP [30]. ARL 66096, a selective antagonist of the P_{2TAC} receptor, abrogates the ADP-induced inhibition of stimulated adenylate cyclase, but does not inhibit ADP-induced shape change [7].

A group of P_{2Y1}-receptor-selective antagonists [33], adenosine 3′-phosphate 5′-phosphosulphate (A3P5PS), adenosine 3′-phosphate 5′-phosphate (A3P5P) and adenosine 2′-phosphate 5′-phosphate (A2P5P), inhibit ADP- or 2-MeSADP-induced intracellular Ca^{2+} mobilization and shape change in platelets in a concentration-dependent manner [30]. The EC_{50} for the effect of ADP on PLC activation at the cloned P_{2Y1} receptor is $\sim 0.3\ \mu M$ [34], and this is also the EC_{50} for platelet shape change [35]. Studies with mice lacking the G-protein G_q revealed that signalling through G_q is essential for ADP-induced shape changes [36]. All the agents that cause platelet shape change, such as thrombin, thromboxane and 5-hydroxtryptamine (serotonin), also activate PLC [37]. These data thus implicate PLC activation as the essential step in platelet shape change. Thus the P_{2Y1} receptor solely mediates ADP-induced platelet shape change.

ADP-induced platelet aggregation

The P_{2TAC} receptor is essential for ADP-induced platelet aggregation. Antagonists of this receptor, i.e. ATP and ARL

66096, block both ADP-induced adenylate cyclase inhibition [7,38] and platelet aggregation [38,39]. Furthermore, a significant correlation was found between antagonist affinity constant values for eight nucleotide analogues as blockers of both ADP-induced aggregation and adenylate cyclase inhibition [40]. The thienopyridines, ticlopidine and clopidogrel, when administered *in vivo*, are presumably converted into an active metabolite which abrogates both ADP-induced inhibition of adenylate cyclase and aggregation [41]. Two patients with defective ADP-induced platelet adenylate cyclase inhibition also had abnormal ADP-induced aggregation, suggesting that the receptor coupled to the inhibition of adenylate cyclase is essential for platelet aggregation. Furthermore, platelet shape change was not impaired in these two patients [42,43]. Hence P_{2TAC} receptor activation is required for ADP-induced platelet aggregation.

The P_{2Y1}-receptor-selective antagonists A3P5PS, A3P5P and A2P5P also inhibit ADP-induced human platelet aggregation in a concentration-dependent manner [44], without affecting the ADP-induced inhibition of adenylate cyclase [30]. Hence intracellular signalling from both the P_{2TAC} and P_{2Y1} receptors is essential for ADP-induced platelet aggregation. Abrogation of signalling through either receptor, by specific antagonists, is sufficient to abolish ADP-induced platelet aggregation. Furthermore, in the presence of ARL 66096, signalling through the P_{2TAC} receptor can be substituted by adrenaline [44] acting on α_{2A}-adrenergic receptors, which are also coupled to G_i. On the other hand, activation of 5-hydroxytryptamine receptors can replace signalling through the P_{2Y1} receptor in both human [44] and rabbit [45] platelets. Moreover, this novel mechanism of ADP-induced platelet aggregation can be mimicked by co-activation of two non-ADP receptors coupled to G_i and G_q (α_{2A}-adrenergic receptors and 5-hydroxytryptamine receptors respectively) [44]. Thus ADP-induced platelet aggregation results from concomitant signalling from both the P_{2TAC} and P_{2Y1} receptors, a novel mechanism by which G-protein-coupled receptors elicit a physiological response [44]. It remains to be seen if a similar mechanism is responsible for ADP-induced integrin $\alpha_v\beta_3$ activation on platelets [22].

In the presence of extracellular Ca^{2+}, pp[CH_2]pA, a P_{2X1}-selective agonist, causes rapid Ca^{2+} influx, but it neither causes platelet aggregation nor inhibits aggregation induced by ADP [44,46]. Furthermore, selective co-activation of P_{2X1} receptors and either P_{2TAC} or P_{2Y1} receptors also does not lead to platelet aggregation [44]. Thus the P_{2X1} receptor does not play any significant role in ADP-induced platelet aggregation.

Although ADP has been known to activate platelets for nearly four decades, the molecular mechanisms involved have become clear only in recent years. The data on ADP receptors in platelets probably can be explained using the three-receptor model. Whether shape change and aggregation are mediated by the same or different ADP receptors may have been resolved. The shape change receptor, i.e. the P_{2Y1} receptor, is also essential for aggregation. On the other hand, the P_{2TAC} receptor, which is also essential for aggregation, has no significant role in platelet shape change. The molecular structures of two of these three receptors, i.e. the P_{2X1} and P_{2Y1} receptors, are known, and future investigations will depend on cloning the P_{2TAC} receptor. Cloning of this receptor subtype has proven difficult. A similar receptor, which couples to G_i, has been identified in C6 glioma cells [47,48]. The effects of ADP at the C6 glial P_{2Y} receptor are also antagonized by ATP [48] and hence this receptor may represent the P_{2TAC} subtype. Thus molecular cloning of the P_{2Y} receptor from C6 glioma cells may facilitate the cloning of the P_{2TAC} receptor from platelets. Future studies may also delineate the functional role, if any, of the P_{2X1} receptor subtype in ADP-induced platelet activation. The intracellular signalling events mediated by these three receptors will provide a better understanding of how ADP causes physiological responses in platelets and, in general, of the molecular mechanisms of agonist-induced platelet activation.

PERIPHERAL BLOOD LEUCOCYTES

During vascular injury, the extracellular nucleotides released from broken cells and degranulation of platelets can act on a number of blood cells to trigger physiological responses (Figure 2) [9,49]. In human neutrophils and their precursor cell line HL-60 [50], and in macrophages [51], ATP causes the activation of PLC. Both ADP and ATP stimulate phagocytic activity of neutrophils and monocytes [52], and increase the intracellular Ca^{2+} concentration in monocytes and promonocytic U937 cells [49]. ATP causes the secretion of granule contents from neutrophils and monocytes [53]. ADP also induces increased binding of fibrinogen to monocytes in a Ca^{2+}-dependent manner [54]. The adherence of neutrophils to endothelial cells [55] and of monocytes to surfaces [56] is increased upon stimulation with extracellular nucleotides. We have demonstrated that extracellular nucleotides cause increased surface expression of Mac-1 ($\alpha_M\beta_2$ integrin; CD11b/CD18) on peripheral blood leucocytes, which was inhibited by Ro 31-8220, a specific inhibitor of protein kinase C (PKC) [57].

ATP-induced Ca^{2+} transients [49,58,59] and increases in $InsP_3$ formation [59] have been observed in monocytes and U937 cells. ATP-induced intracellular Ca^{2+} increases in U937 cells are mediated by a P_{2Y} receptor subtype that responds to both ADP and ATP [58]. Extracellular ADP causes increased surface expression of Mac-1 on monocytes [60]. RT-PCR analysis of U937 cell RNA [61] indicated that these cells express P_{2Y2} receptors [62] and P_{2Y6} receptors, but not the P_{2Y4} receptor [63,64] or the P_{2Y1} receptor [32]. The presence of P_{2Y1}, P_{2Y2}, P_{2Y4} and P_{2Y6} receptors on monocytes [61] suggests that different P_{2Y} receptor subtypes may be responsible for the actions of ATP and ADP on these cells.

Neutrophils respond to ATP by an increase in intracellular Ca^{2+} via a pertussis-toxin-sensitive pathway [65]. This response has been attributed to a P_{2Y2} receptor, at which both UTP and ATP are equally potent. An inability to detect the P_{2Y2} receptor on neutrophils by RT-PCR [61] suggests that this receptor subtype may be expressed at low levels. The identification of P_{2Y4} and P_{2Y6} receptor subtypes on neutrophils and of P_{2Y1}, P_{2Y2} and P_{2Y6} receptor subtypes on promyelocytic HL-60 cells [61] indicates that these receptors may also play a role in the stimulation of neutrophils by extracellular nucleotides, leading to enhanced adherence to endothelial cells [55]. In undifferentiated HL-60 cells, a novel nucleotide receptor subtype, possibly the P_{2Y11} subtype [66], was identified which couples to activation of adenylate cyclase [67].

Mobilization of intracellular Ca^{2+} by adenine nucleotides was demonstrated in T-leukaemic cells, and this response is also mediated by P_{2Y} receptor subtypes [68]. P_{2Y} receptor subtypes with a possible role in differentiation have been identified in murine myelomonocytic leukaemic cells [69]. Extracellular ATP inhibits cytotoxicity mediated by macrophages [70] and natural killer cells [71,72], but is also an effector in cytotoxic T-lymphocyte-mediated cell lysis [73]. Since lymphocytes express mRNAs for the P_{2Y1}, P_{2Y2}, P_{2Y4} and P_{2Y6} receptors [61], the functional effects may be mediated by one or more of these subtypes. Furthermore, the P_{2Y5} receptor subtype was cloned from activated chicken lymphocytes [74], and hence may have a role in ATP-induced lymphocyte activation.

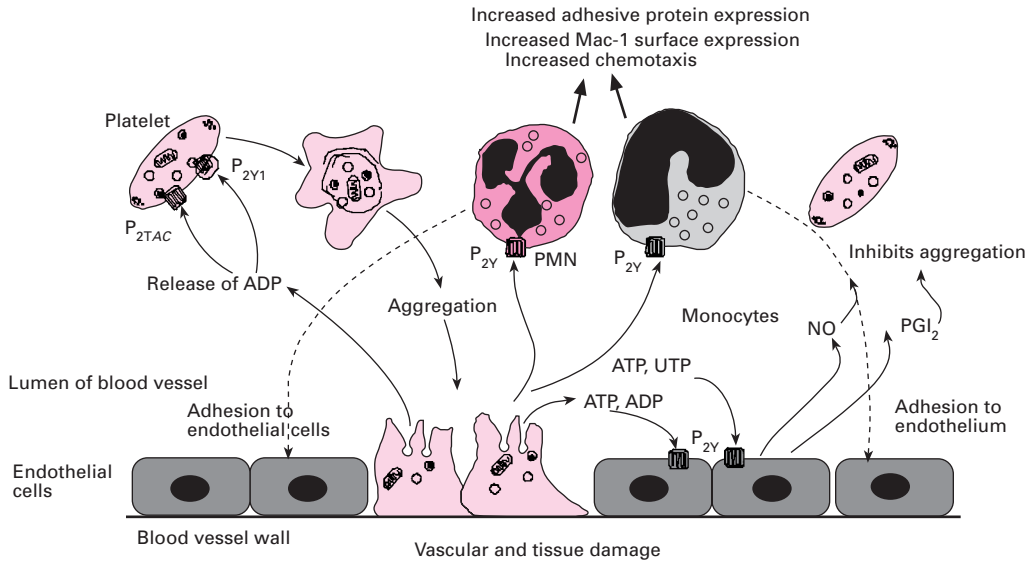

Figure 2 Functional role of nucleotides during vascular injury

The nucleotides released from broken cells and activated platelets activate peripheral blood leucocytes and endothelial cells. See the text for details. Abbreviations: PMN, polymorphonuclear leucocyte; PGI_2, prostaglandin I_2. Modified from [65] Trends Pharmacol. Sci. **16**, Boarder, M. R., Weisman, G. A., Turner, J. T. and Wilkinson, G. F. G-protein-coupled P_2 purinoceptors: from molecular biology to functional responses, pp. 133–139, copyright 1995, with permission from Elsevier Science.

Mast cells express a unique subtype of P_2 receptors, originally designated P_{2Z} receptors [75]. These receptors required ATP^{4-} as an agonist and mediate non-selective pore formation [76]. ATP released from secretory granules of mast cells acts on other mast cells. The physiological effects of P_{2Z} receptor activation include shedding of L-selectin and activation of phospholipase D [77,78]. Molecular cloning has revealed that the P_{2Z} receptor is indeed a P_{2X} subtype, designated P_{2X7} [79].

The subtype distribution of P_{2X} receptors in haematopoietic cells has not been determined as yet, and P_{2X} subtypes might mediate some of the physiological effects. Extracellular nucleotides elicit a variety of responses in peripheral blood leucocytes. The stimulation of monocytes and neutrophils by nucleotides contributes to inflammatory responses. However, chemotaxis, surface expression of adhesive proteins, adhesion to the endothelium and secretion of granule contents are also effects elicited by chemokines such as leukotriene B_4 [80]. Since nucleotides have been known to activate phospholipase A_2 and liberate arachidonic acid in both platelets and endothelial cells [65], it is conceivable that nucleotides can elicit similar responses in leucocytes, wherein arachidonic acid could be converted into leukotrienes. Thus the effects of nucleotides on peripheral blood monocytes and neutrophils could occur indirectly through the generation of a potent agonist such as leukotriene B_4. Hence, in addition to the identification of the P_2 receptor subtypes involved in nucleotide-induced physiological responses, future work needs to address any autocrine regulation of leucocyte function.

ENDOTHELIAL CELLS

Vascular endothelial cells are regulated by nucleotides released from platelets, endothelial cells, neurons and damaged cells (Figure 2). The first indications of the importance of purine nucleotides to the physiological function of endothelial cells came from two studies [81,82] showing that ATP induces endothelial cells to produce prostacyclin. Later it was shown that ATP and ADP cause the release of endothelium-derived relaxing factor, now known to be nitric oxide [83]. Both prostacyclin and NO are potent vasodilators and inhibitors of platelet function. Nucleotides stimulate endothelial cells, resulting in the enhanced binding of neutrophils [55].

Pharmacological profiles of the response of endothelial cells to different nucleotides indicated the presence of two distinct P_2 receptors; one activated by adenine nucleotides and the other by UTP [84,85]. P_{2Y1} receptor cDNA has been cloned and sequenced from a bovine aortic endothelial cell library [86]. Pirotton et al. [87] demonstrated that P_{2Y1} and P_{2Y2} receptors are co-expressed on bovine aortic endothelial cells. The demonstration by RT-PCR of the mRNAs for P_{2Y1}, P_{2Y2}, P_{2Y4} and P_{2Y6} receptors in human umbilical vein endothelial cells [61] indicates that the UTP-induced intracellular responses could be mediated by either the P_{2Y2} or P_{2Y4} receptors on these cells. On the other hand, ADP-induced responses could be mediated by P_{2Y1} or P_{2Y6} receptors.

ADP and ATP produce an increase in Ins(1,4,5)P_3 in bovine aortic endothelial cells [88]. The nucleotide profile of InsP_3 formation indicates that both P_{2Y1} and P_{2Y2} receptors are coupled to PLC activation. UTP-induced InsP_3 production was inhibited by pertussis toxin and was unaffected by PMA [87]. The response induced by 2-methylthio-ATP (2-MeSATP) was inhibited by PMA, but not by pertussis toxin. These results suggested that P_{2Y1} and P_{2Y2} receptors are coupled to G_q/G_{11}- and G_i/G_o-proteins respectively.

In rat brain capillary endothelial cells, in addition to the PLC-activating P_{2Y} receptors, P_2 receptors that cause increases in intracellular Ca^{2+} independent of InsP_3s have been reported [89]. ATP is a partial agonist at these receptors, while ADP, 2-MeSATP and 2-chloro-ATP (2-Cl-ATP) are agonists [90,91]. In the B10 microvascular endothelial cell line, a P_{2Y} receptor couples to the inhibition of stimulated adenylate cyclase; based on RT-PCR analysis this was reported to be the P_{2Y1} receptor [92].

Production of prostacyclin (prostaglandin I_2) requires the activation of phospholipase A_2, which releases arachidonic acid from membrane phospholipids. At first, it was thought that the adenine-nucleotide-induced increase in cytosolic Ca^{2+} was the primary activator of phospholipase A_2 [93,94]. However, possible activation of phospholipase A_2 by PKC was demonstrated by treatment of endothelial cells with PMA in the absence of a change in the cytosolic Ca^{2+} concentration [95]. Pretreatment of the cells with PMA prior to agonist stimulation enhanced phospholipase A_2 activity in a complicated way. These effects have been suggested to be due to the activation of PKC-ϵ [96]. Activation of P_{2Y} receptor subtypes leads to the tyrosine phosphorylation and activation of both the p42 and p44 forms of mitogen-activated protein kinase (MAPK). Adenine-nucleotide-induced prostaglandin I_2 formation was blocked in a dose-dependent manner by the selective MAPK kinase inhibitor PD98059. Based on these results, Patel et al. [97] proposed that both of the endothelial P_{2Y} receptors are linked to the activation of MAPK, and that activation of this pathway is a requirement for ATP/ADP-induced prostaglandin I_2 production.

The primary mechanism of activation of constitutive NO synthase is known to be via Ca^{2+}/calmodulin [98]. In addition, there is conflicting evidence that, during purinergic activation of NO synthase, phosphorylation by PKC either may have an inhibitory role [99] or may be mandatory for activity [100].

Heterogeneity of expression of P_2 receptors on different endothelial cells is an issue that needs more complete evaluation. In adrenal medulla endothelial cells, for example, only P_{2Y2}-like receptor activity was detected by Purkiss et al. [101]; however, others have proposed that both P_{2Y1} and P_{2Y2} receptors are found on these cells [102].

The basic functions of the vascular endothelium, in addition to physically preventing interaction of platelets with the sub-endothelium, are the regulation of vascular tone, through the production of potent vasodilators, and the inhibition of platelet function through the generation of inhibitors, i.e. prostacyclin and NO. Extracellular nucleotides released during vascular injury contribute to endothelial function through the production of prostacyclin and NO. The distribution of P_{2X} receptor subtypes on these cells has not yet been determined. Future work needs to address the distribution of specific P_{2X} and P_{2Y} receptor subtypes on different endothelial cells and the contribution of these receptors to endothelial function.

VSMC

ATP is found in sympathetic neurons and acts on vascular smooth muscle to cause vasoconstriction. P_{2X1} is the primary P_{2X} subtype expressed on vascular smooth muscle [103]. Recently Nori et al. [104] used *in situ* hybridization and RT-PCR to show the presence of P_{2X1}, P_{2X2} and P_{2X4} in rat aortic smooth muscle. Responses to both ATP and UTP suggested the presence of P_{2Y2} receptors on smooth muscle cells [105,106]. The cDNA for the P_{2Y6} receptor was also cloned from rat aortic smooth muscle cells [107]. P_{2Y6} and P_{2Y2} receptor mRNAs were detected in vascular smooth muscle by Northern blot analysis [107].

It has been generally accepted that P_{2X1} receptors are the primary receptors coupled to vasoconstriction [8]. However, P_{2Y} receptors may also induce contractile responses in smooth muscle. Miyagi et al. [106] demonstrated that P_{2Y2} receptors can also contribute to the contractile response of smooth muscle after adenine nucleotide addition. Other experiments are consistent with a role for P_{2Y4} in smooth muscle contractility [108]. Both P_{2X} and P_{2Y} receptors are capable of causing elevation of the intracellular Ca^{2+} concentration [109]. An apparent conflict existed in the case of the portal vein; purinergic agonists had been shown both to contract [110] and to relax [111] portal vein vascular smooth muscle. However, non-endothelial-dependent relaxation of portal vein smooth muscle has been shown to require ectonucleotidase activity, which converts adenine nucleotides into adenosine [112].

Smooth muscle cells in culture lose their P_{2X1} receptors, while retaining their P_{2Y} responses [109,113]. Adenine nucleotides also produce increases in cytosolic Ca^{2+} in cultured smooth muscle cells, in spite of the lack of P_{2X1} receptors. Extracellular ATP is a mitogen for cultured VSMC and stimulates DNA synthesis, protein synthesis, an increase in cell number, immediate-early gene expression, cell-cycle progression and tyrosine phosphorylation [113]. ATP also acts synergistically with other growth factors. The mitogenic effects of ATP have been demonstrated in rat, pig and bovine VSMC and in cells from human coronary arteries, aorta, and subcutaneous arteries and veins [113]. P_{2Y2} and other P_{2Y} receptors mediate these effects [113]. In contrast, in VSMC from spontaneously hypertensive rats, the P_{2Y4} receptor, but not P_{2Y1}, P_{2Y2} or P_{2Y6}, has been proposed to mediate cell proliferation through activation of the MAPK pathway [114]. In VSMC from control Wistar–Kyoto rats, however, nucleotide-induced cell proliferation is mediated by the P_{2Y2} receptor [114]. Multiplex RT-PCR analysis of VSMC from spontaneously hypertensive rats revealed the expression of P_{2Y2}, P_{2Y4} and P_{2Y6}, but not P_{2Y1}, mRNA [114]. Since RT-PCR was not carried out on VSMC from the control Wistar–Kyoto rats [114], it is not known whether P_{2Y} receptor subtype expression levels are different in these two strains.

Adenine nucleotides produce increases in intracellular Ca^{2+} through opening of P_{2X1} channels and P_{2Y}-dependent activation of PLC-β_1 via $G_q/_{11}$-proteins [113,115]. Murthy and Makhlouf [115] also indicate that, in gastric smooth muscle, adenine nucleotides act through $G_{i3}\beta\gamma$ to activate PLC-β_3, and suggest that both P_{2Y} and P_{2X} receptors are expressed on the same cell and that P_{2Y} dominates over P_{2X1} in causing smooth muscle contraction. In addition to activation of PLC, ATP activates phospholipase D, PKC isoforms α and δ, Raf-1, MAPK kinase and MAPK in vascular smooth muscle [113].

Under physiological conditions, ATP, released from sympathetic neurons, regulates vascular tone by acting on VSMC. ATP is an agonist at P_{2X1}, P_{2Y2} and rat P_{2Y4} receptors, and an antagonist at the P_{2Y1} receptor (Table 2). Thus the absence of P_{2Y1} receptor mRNA from VSMC [114] is not unexpected. Under pathophysiological conditions, wherein the vascular endothelium is damaged, ATP, ADP and UTP released from activated platelets can act directly on VSMC, causing contraction. Such nucleotide-induced contraction reduces haemorrhage during vascular injury. Thus, although ATP released from platelet secretory granules is an antagonist of ADP-induced platelet activation, it may have physiological role during haemorrhage. UTP, also released from secretory granules, albeit in smaller quantities [116], may also cause VSMC contraction during vascular injury. The role of two different P_{2Y} receptor subtypes in VSMC from normal and hypertensive rats [114] suggests that more work needs to be done on the regulation of P_2 receptor subtype gene expression in disease states.

CARDIAC MYOCYTES

As early as 1936, ATP was shown to influence cardiac contraction [117,118]. Extracellular ATP influences cardiac function both indirectly, via blood vessels and nerves, and directly, via myocytes. ATP regulates coronary vascular tone via P_{2X} receptors that are coupled to vasoconstriction synergistically with α-

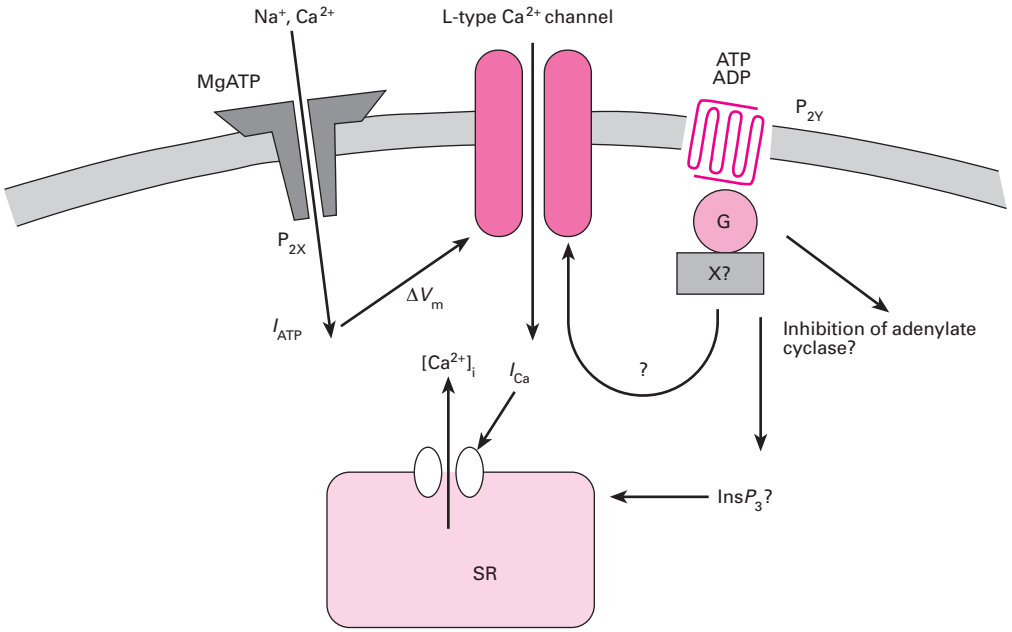

Figure 3 ATP-mediated signal transduction pathways in cardiac myocytes

See the text for details. Abbreviations: I_{ATP} and I_{Ca}, ATP and Ca^{2+} currents respectively; ΔV_m, change in membrane potential, m; G, G-protein; SR, sarcoplasmic reticulum. Modified from Zheng et al. [128], with permission of the American Physiological Society.

adrenoceptors [119], while in some coronary vessels it acts via P_{2Y} receptors to produce vasodilatation in concert with β-adrenoceptor activation. ATP has also been shown to modulate autonomic control of the heart [120,121].

In rat ventricular myocytes, ATP consistently increases intracellular Ca^{2+} levels [122–125] and the degree of cell shortening [124,126]. Extracellular ATP is known to cause cardiac muscle contraction through modulation of L-type Ca^{2+} currents in rat cardiac myocytes [127,128]. ATP has been demonstrated to have positive inotropic effects and to increase the Ca^{2+} transients of electrically stimulated rat cardiac preparations [124,129]. Furthermore, ATP transiently increases the intracellular Ca^{2+} concentration of quiescent cells [129]. Electrophysiological studies have shown that ATP activates a non-specific cationic current in rat cardiac myocytes, possibly through P_{2X} receptors [130], and increases the L-type Ca^{2+} current amplitude in the rat [127,128]. The transient increase in intracellular Ca^{2+} and the positive inotropism induced by ATP have also been attributed to the G-protein-coupled P_{2Y} receptors [122,131]. The EC_{50} value for the ATP-mediated increase in Ca^{2+} current is 0.36 μM, with a rank order of potency of 2-MeSATP = ATP = adenosine 5′-[γ-thio] triphosphate [132]. Under photoaffinity labelling conditions, radioactive 8-azido-ATP specifically labelled a species of 45–48 kDa on an SDS/polyacrylamide gel, and UV-photolysed 8-azido-ATP irreversibly and specifically inhibited the ATP-induced cytosolic Ca^{2+} currents [133].

On the other hand, in isolated ferret ventricular myocytes, extracellular ATP can directly inhibit L-type Ca^{2+} currents, the action potential plateau, the myoplasmic Ca^{2+} transient and the degree of cell shortening in response to stimulation [134]. These results are consistent with the uncertainty regarding the effect of ATP on Ca^{2+} transients: both an increase [124] and a decrease [122] have been observed. Furthermore, in rat ventricular myocytes, ATP causes an initial transient inhibition of the Ca^{2+} current, followed by stimulation [130].

In order to characterize the ATP receptor subtypes involved in the regulation of cardiac function, degenerate oligonucleotides were used to clone receptors of both the P_{2X} and P_{2Y} families from human fetal heart. Using this approach, P_{2X1}, P_{2X3} and P_{2X4} and P_{2Y6}, P_{2Y2} and P_{2Y4} receptors have been identified [135]. It is unclear whether, as a general rule, receptor expression changes with development, i.e. whether all these receptors continue to be expressed in adult hearts and if new receptor subtypes become expressed. In one study, Webb et al. [136] used RT-PCR to show expression of P_{2Y1}, P_{2Y2}, P_{2Y4} and P_{2Y6} receptor transcripts in the whole heart, neonatal cardiac fibroblasts, neonatal cardiac myocytes and adult cardiac myocytes. The amount of each receptor subtype changed during development. All receptor sequences could be amplified from neonatal rat whole heart, with P_{2Y6} being the most abundant. In adult myocytes, P_{2Y1}, P_{2Y2} and P_{2Y6} could be amplified, but P_{2Y4} could not be detected. However RT-PCR may not be the best technique to use to make quantitative conclusions. The P_{2X5} receptor was cloned from a rat heart cDNA library, its mRNA is abundantly expressed in the heart, and this receptor is insensitive to pp[CH_2]pA [137]. Thus the P_{2X5} receptor subtype might mediate the non-selective cation inward current (Figure 3) in myocytes [128].

In patch–clamp experiments, the effect of ATP on the Ca^{2+} current was additive with maximal stimulation induced by a β-adrenergic agonist or after internal perfusion with cAMP, indicating a separate mechanism of action [125,126]. Activation of PKC attenuated the response [138]. Other studies using whole-cell patch–clamp showed that, while guanosine 5′-[β-thio]-diphosphate inhibited the ATP-induced increases in Ca^{2+} current, guanosine 5′-[γ-thio]triphosphate increased the Ca^{2+} current on its own. This suggested a role for P_{2Y} subtypes in mediating the ATP-induced increase in Ca^{2+} current. There have been conflicting reports on the role of second messengers in increases in Ca^{2+} current in myocytes (Figure 3). In myocytes, ATP causes both an increase in inositol phosphates and a decrease in cAMP

[139]. However, the ATP-induced inhibition of adenylate cyclase, but not PLC activation, is abolished by pertussis toxin, suggesting a role for different G-proteins or different P_2 receptor subtypes [139]. On the other hand, both inositol phosphate formation and attenuation of cAMP formation are inhibited by PKC activation, possibly due to heterologous desensitization of the receptors [139]. ATP-mediated InsP_3 production in cardiac myocytes was also reported by Vassort and co-workers [140]. The same group also reported, based on electrophoretic mobility and susceptibility to cholera toxin and anti-$G_s\alpha$ serum, that P_2 receptors couple to a 'G_s'-protein in myocytes, leading to stimulation of cardiac Ca^{2+} channels, without a change in cAMP accumulation [127]. pp[CH_2]pA was reported to stimulate InsP_3 formation [140], while it was reported to have no effect on the Ca^{2+} current at 100 μM [128].

Although ATP has been known to cause cardiac contraction since 1934 [118], the molecular mechanisms have not been elucidated. In recent years, not much has been reported on the physiology of the P_2 receptor subtypes in cardiac myocytes. In addition, future work also needs to address the discrepancies regarding the effects of ATP on Ca^{2+} transients (increase compared with decrease) and in different species, e.g. rat and ferret ventricular myocytes.

CONCLUDING REMARKS

Ever since Burnstock [141] proposed the concept of cell-surface receptors for nucleotides, this field has expanded enormously. Several nucleotide receptor subtypes were identified by pharmacological and physiological studies, and subsequently several molecular subtypes have been cloned [142]. The progress in the molecular pharmacology of P_2 receptors has clarified the subtypes responsible for classical pharmacological P_2 receptor responses in some cases, but has further complicated the picture in others. For example, the platelet P_{2T} receptor has now been resolved into three P_2 receptor subtypes with distinct functions [143]. On the other hand, in endothelial cells, for example, it remains to be clarified whether the classical P_{2U} receptor responses are mediated by P_{2Y2} or P_{2Y4} [61]. ATP is a very weak agonist at the human P_{2Y4} receptor, but is equally as potent as UTP at the rat P_{2Y4} receptor [144]. Again, it remains to be clarified if 2-MeSATP, a selective agonist for classical P_{2Y} receptors, is truly an agonist, or whether its hydrolysis product 2-MeSADP is the real agonist [145]. Although some of the initial studies using impure nucleotides are currently under scrutiny and are being re-examined using purified nucleotides, in order to resolve the function of each of the subtypes, agonists and antagonists specific for these subtypes need to be developed. Progress in the development of selective P_{2X} receptor antagonists was recently reported [146,147]. Such reagents may prove to have therapeutic potential in several of the pathophysiological conditions of the cardiovascular system.

Two molecular biological techniques that have advanced our knowledge of P_2 receptors, i.e. RT-PCR and heterologous expression, appear to have created some confusion in this field (and in other fields too). First, RT-PCR is a very sensitive technique that detects even the rare mRNAs in a cell; however, a positive RT-PCR signal should not be taken as sole evidence for receptor subtype expression. For example, multiplex RT-PCR analysis on VSMC indicates that the P_{2Y6} mRNA is more abundantly expressed than the P_{2Y4} mRNA, but responses selective for P_{2Y6} agonists were not detected in these cells [114]. Again, RT-PCR analysis of pure platelet mRNA indicated the expression of P_{2Y1}, P_{2Y2} and P_{2Y6}, but not P_{2Y4}, receptor mRNAs, with the P_{2Y6} receptor mRNA being the most abundant (J. Jin and S. P. Kunapuli, unpublished work). However, platelets do not respond to UDP, a P_{2Y6} agonist. Hence the identification of various P_2 receptor subtypes in leucocytes and endothelial cells [61], neonatal [135] and adult [136] rat heart and VSMC [114] should be more critically evaluated. RT-PCR analysis together with pharmacological evaluation and/or protein expression studies might provide better insight into the function of the receptor subtypes.

Heterologous expression is essential to demonstrate that the cloned cDNA is a functional P_2 receptor. While this technique has established the pharmacological profiles of several cloned P_2 receptors, identical receptor subtypes revealed different agonist/antagonist profiles in some cases. For example, UTP is severalfold more potent than ATP at the human P_{2Y4} receptor, while the two are equally potent at the rat P_{2Y4} receptor [144,148]. Similar differences in agonist and antagonist profiles were observed with the human and rat P_{2X7} receptors [147]. These differences could result from levels of receptor expression, constitutively active compared with inactive states, and desensitization. When G-protein-coupled receptors are overexpressed, they could promiscuously couple to multiple G-proteins. Hence coupling of the heterologously expressed P_{2Y11} receptor to both cyclase activation and Ca^{2+} mobilization [66] should be confirmed in cells that natively express this receptor subtype, using specific agonists. Thus the interpretation of the physiological responses based on heterologous expression could become extremely complex and unreliable.

The problems associated with RT-PCR and heterologous expression in demonstrating the expression of a particular receptor subtype and its physiological function might, at least in some cases, be overcome by gene-knockout approaches. Knockout animals for the P_2 receptors are still 'in the works', and when available might shed light on the role of each subtype in various cardiovascular systems. It is possible that multiple receptor subtypes are expressed in each cell and take over the function of a missing subtype. In such a case no physiological abnormality may be noticed, and this may lead to the conclusion that the particular receptor subtype might not have any function in the system. On the other hand, the receptor subtype might be essential for the growth and development of the mice, and hence the subtype knockout may prove to be lethal.

In conclusion, although we have made tremendous progress in the molecular cloning and identification of various P_2 receptor subtypes in the cardiovascular system, their function remains to be established. Future progress in the development of subtype-specific reagents and transgenic approaches should elucidate the function of each of the P_2 receptor subtypes in the cardiovascular system.

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