British Journal of Pharmacology (1998) 123, 789–794

Distribution of P2Y receptor subtypes on haematopoietic cells

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1 RT–PCR-southern hybridization analyses with radiolabelled P2Y receptor cDNAs as probes indicated that the peripheral blood leukocytes and the human umbilical vein endothelial cells express $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors.

2 Of the haematopoietic cell lines tested, promonocytic U937 cells express $P2Y_2$ and $P2Y_6$, but not $P2Y_1$ or $P2Y_4$; promyelocytic HL-60 cells express the $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptors but not the $P2Y_4$ receptor; K562 cells express $P2Y_1$ but not $P2Y_2$, $P2Y_4$ or $P2Y_6$; and Dami cells express $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors.

3 Of the peripheral blood leukocytes tested, polymorphonuclear cells express $P2Y_4$ and $P2Y_6$ but not $P2Y_1$ or $P2Y_2$ receptors; monocytes express $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors and lymphocytes express $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors.

4 These results suggest a physiological role for different P2Y receptor subtypes in the extracellular nucleotide-mediated stimulation of monocytes, neutrophils, lymphocytes and endothelial cells.

Keywords: P2Y receptors; $P2Y_1$ receptors; $P2Y_2$ receptors; $P2Y_4$ receptors; $P2Y_6$ receptors; haematopoietic cell lines; endothelial cells; peripheral blood leukocytes

Introduction

Extracellular adenine nucleotides activate cell membrane receptors, referred to as P2 receptors (Burnstock, 1978). P2 receptors have been divided into two groups, P2X and P2Y (Abbracchio & Burnstock, 1994). The P2X family of ligand-gated channel receptors includes adenosine 5′-triphosphate (ATP)-activated ion channels, which have long been known to be involved in neurotransmission and smooth muscle contraction (Abbracchio & Burnstock, 1994), as well as a subtype originally referred to as P2Z, first observed to cause a generalized increase in membrane permeability in mast cells but now classified as a P2X subtype (Surprenant *et al*., 1996). The second group comprises receptors coupled to G proteins. These include P2Y, P2U, P2T and P2D subtypes, according to old nomenclature (Abbracchio & Burnstock, 1994). All of these G protein-coupled receptors belong to the seven transmembrane domain family and cause mobilization of intracellular calcium ions and/or activation or inhibition of adenylyl cyclase. Under the new nomenclature proposed by the IUPHAR committee, all the G protein-coupled P2 receptors should be called P2Y, distinct from P2X ligand-gated channel receptors, and the various subtypes are numbered in the order of cloning (Fredholm *et al*., 1994).

Several G protein-coupled P2 receptor subtypes have been cloned and characterized. A receptor designated $P2Y_1$ has been cloned by expressing chick brain cRNA in *Xenopus* oocytes and following the electrophysiological responses induced by ATP, which is the P2Y subtype under the old nomenclature (Webb *et al*., 1993). The human orthologue of the $P2Y_1$ receptor has been cloned in our laboratory (Ayyanathan *et al*., 1996). A cDNA encoding the $P2Y_2$ receptor, equally responsive to ATP and UTP, has been cloned from the NG108-15 murine neuroblastoma-glioma cell line, which is the P2U subtype under the old nomenclature (Lustig *et al*., 1993). The human orthologue of the mouse $P2Y_2$ receptor has been cloned from a human airway epithelial cell line (Parr *et al*., 1994) and from human erythro leukaemia (HEL) cells (Akbar *et al*., 1996a). Another receptor with higher affinity for UDP and ADP than for ATP has also been cloned from the chick brain library and has been designated $P2Y_3$ (Webb *et al*., 1996a). A uridine nucleotide receptor, which responds to UTP and UDP, has been cloned from a human genomic library and designated $P2Y_4$ (Communi *et al*., 1995; Nguyen *et al*., 1995). An orphan receptor cloned from activated chicken T cells has now been identified as a P2 receptor and is designated $P2Y_5$ (Webb *et al*., 1996b). A P2 nucleotide receptor from rat aortic smooth muscle, designated $P2Y_6$, is more responsive to UTP and ADP than to ATP, but it is not clear if it is the rat homologue of $P2Y_3$ (Chang *et al*., 1995). The human orthologue of the $P2Y_6$ receptor has recently been cloned (Communi *et al*., 1996). We have cloned a novel G protein-coupled receptor from a HEL cell cDNA library, originally designated $P2Y_7$ (Akbar *et al*., 1996b), but recent studies have shown that this is indeed a leukotriene B_4 receptor (Yokomizo *et al*., 1997).

The ADP receptor, P2T, which has been pharmacologically characterized has not yet been cloned (Gachet *et al*., 1996; Mills, 1996). The P2T receptor is coupled to inhibition of adenylyl cyclase and plays a role in platelet aggregation (Gachet *et al*., 1996; Mills, 1996). Recent studies have suggested that the $P2Y_1$ receptor is a platelet ADP receptor coupled to inhibition of adenylyl cyclase at which ATP is an antagonist (Leon *et al*., 1997), although it has been regarded primarily as an ATP receptor, due to either contamination of ATP with ADP or breakdown of ATP over the timecourse of the experiment.

Upon vascular injury, ATP and ADP are released into the blood stream from damaged cells and from activated platelets and act on other platelets and leukocytes (Gordon, 1986; Cowen *et al*., 1989). The molecular subtypes of P2 receptors involved in these functional responses have not been identified. The G protein-coupled P2 receptors cloned to date are coupled to intracellular calcium mobilization. It is very difficult to dissect out the pharmacology of a single receptor in cells that express multiple P2 receptor subtypes. The agonist potency

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series, and agonist or antagonist selectivity, found on a given source may not match any actual molecular subtype. The rationale for these studies is to determine the distribution of the cloned G protein-coupled P2 receptors ($P2Y_1-P2Y_6$) in blood cells. These results will then form the basis for understanding the role of the cloned G protein-coupled P2 receptor subtypes in eliciting nucleotide-mediated physiological responses in these cells.

Here we demonstrate the presence of the cloned G protein-coupled P2 receptor subtypes on peripheral blood leukocytes and on their precursor cell lines.

Methods

Isolation of blood cells

Whole blood in citric acid-sodium citrate-dextrose (ACD) was drawn from volunteers and mixed with 0.1 volume of 7.5% polyanhydroglucose and red blood cells were sedimented in polypropylene tubes for 45 min on ice. All subsequent steps were performed at 4°C. Leukocyte-rich plasma was centrifuged at $400\times g$ for 30 min and the remaining red blood cells were lyzed in 155 mM ammonium chloride and 10 mM potassium bicarbonate, pH 7.4. Leukocytes were washed twice in Hank's balanced salt solution containing 20 mM HEPES (HBSS) without Ca^{2+} or Mg^{2+} and the cell count was adjusted for each sample to 5×10^6 ml^{-1}.

Polymorphonuclear cells, monocytes and lymphocytes were isolated from blood from healthy volunteers by Ficoll-histopaque gradient centrifugation (Altman *et al*., 1992; Kappelmayer *et al*., 1993; Wachtfogel *et al*., 1994). Monocytes were purified after adherence of peripheral blood mononuclear cells to plastic petri dishes for 1 h at 37°C, followed by gentle washing with phosphate buffered saline three times to remove non adherent cells (Loudon *et al*., 1996).

Cell culture

Human promonocytic U937 cells and K562 cells were obtained from American Type Culture Collection (ATCC) (Rockville, MD) and were grown in suspension culture in RPMI 1640 medium supplemented with penicillin/streptomycin/amphotericin B solution and 10% foetal calf serum at 37°C with 5% CO_2. These cells were harvested every 4–5 days ($1-1.5\times10^6$ cells ml^{-1}). Megakaryocytic Dami cells were also obtained from ATCC under a transfer agreement with the Brigham and Women's Hospital and the kind co-operation of Dr Sheryl M. Greenberg (Boston, MA). Dami cells were maintained in suspension culture in Iscove's modified Dulbecco's medium supplemented with 10% heat-inactivated horse serum and 4 mM glutamine.

Reverse transcription-coupled polymerase chain reaction (RT–PCR)

The total RNA was isolated from total blood leukocytes or cells by the RNAzol procedure (Tel-Test Inc., Friendswood, TX) and the cDNA was prepared with the first stand synthesis kit (Gibco-BRL, Gaithersberg, MD). The PCR was carried out by use of a set of forward and reverse primers specific for each P2Y receptor (Table 1). After initial denaturation for 5 min at 94°C the amplifications were carried out for 35 cycles with 5.0 units of *pfu* DNA polymerase as follows: denaturation at 94°C for 1 min, annealing at receptor specific temperature for 1 min and extension at 72°C for 1 min, followed by 7 min at 72°C. The annealing temperatures of 54°C, 55°C, 60°C and 58°C were used for the $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors, respectively.

Southern blot analysis

The RT–PCR products were separated on a 1% agarose gel and transferred to a nylon membrane. The P2Y receptor cDNA or genomic DNA insert was radiolabelled to a specific activity of $1-3\times10^9$ c.p.m. μg^{-1} by random priming kit (Stratagene) and was hybridized with the RT–PCR Southern blot for 3 h at 65°C in the Rapid Hybridization buffer (Amersham, Arlington Heights, IL). The blot was washed with $2\times$SSC and 0.1% SDS at room temperature for 1 h followed by several high stringency washes with $0.1\times$SSC and 0.1% SDS at 65°C for 20 min each. The blot was then autoradiographed at −80°C for 15–30 min. The cDNAs for $P2Y_1$ and $P2Y_2$ have been cloned in our laboratory (Akbar *et al*., 1996a; Ayyanathan *et al*., 1996). The genomic clone for $P2Y_4$ (Nguyen *et al*., 1995) was obtained from Dr O'Dowd (University of Toronto, Canada). The $P2Y_6$ cDNA insert was amplified from human genomic DNA by use of primers (Table 1) specific for the $P2Y_6$ receptor cDNA (Communi *et al*., 1996) subcloned into pcDNA3, and the nucleotide sequence was confirmed (Pidlaoan *et al*., 1997).

Materials

α-[^{32}P]-dCTP was from NEN (Boston, MA); other materials were as previously described (Akbar *et al*., 1996a,b; Ayyanathan *et al*., 1996).

Results

Identification of P2Y receptors in cell lines of haematopoietic origin

RNA from various cell lines of haematopoietic origin was analysed by RT–PCR with $P2Y_1-P2Y_6$ receptor specific

Table 1 P2Y receptor primers used for PCR on blood cells

Subtype	*Strand*	*Sequence*	*Corresponds to nt*	*Reference*
$P2Y_1$	+	5′-CGGTCCGGGTTCGTCC-3′	194–209	
	−	5′-CGGACCCCGGTACCT-3′	707–721	(Ayyanathan *et al*., 1996)
$P2Y_2$	+	5′-CTAAAGCCAGCCTACGGGAC-3′	919–938	
	−	5′-TCCTATCCTCTGCATGTC-3′	1281–1297	(Parr *et al*., 1994)
$P2Y_4$	+	5′-CCACCTGGCATTGTCAGACACC-3′	615–636	
	−	5′-GAGTGACCAGGCAGGGCACGC-3′	1019–1039	(Nguyen *et al*., 1995)
$P2Y_6$	+	5′-CGCTTCCTCTTCTATGCCAACC-3′	583–604	
	−	5′-CCATCCTGGCGGCACAGGCGGC-3′	926–947	(Communi *et al*., 1996)

The $P2Y_6$ receptor specific primers also contained additional sequences at the 5′ end for subcloning purposes.

primers and Southern hybridization of the RT–PCR products with the corresponding P2Y receptor subtype cDNA. Since only four molecular subtypes, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ are of human origin, we used specific primers for each of these four molecular subtypes (Table 1) and hybridized the RT–PCR product with the corresponding radiolabelled cDNA probe. As shown in Figure 1, the $P2Y_1$ receptor mRNA was identified in peripheral blood leukocytes, endothelial cells, HL-60 cells, K562 cells and Dami cells, but not in U937 cells. $P2Y_2$ receptor mRNA was detected in peripheral blood leukocytes, endothelial cells, U937 cells, HL-60 cells and Dami cells, but not in K562 cells. The $P2Y_4$ receptor was expressed in peripheral blood leukocytes endothelial cells and Dami cells, but not in U937 cells, HL-60 cells or K562 cells. $P2Y_6$ receptor mRNA was detected in peripheral blood leukocytes, endothelial cells, U937 cells, HL-60 cells and Dami cells, but not in K562 cells. These data are summarized in Table 2. The genomic DNA contamination in the RNA preparation could serve as a template for the PCR primers and result in a signal. In order to rule out such contamination, we used RNA without reverse transcription reaction as template in a PCR reaction. No signal was detected in RNA samples without cDNA synthesis indicating that the samples were free from genomic DNA (data not shown).

Identification of the P2Y receptor subtype mRNA in peripheral blood leukocytes

Since several of the cloned G protein-coupled P2 receptor subtypes were expressed on peripheral blood leukocytes (Figure 1), we investigated the molecular subtypes of these receptors expressed in different leukocyte cell types. The RNA from peripheral blood monocytes, polymorphonuclear cells, and lymphocytes was then analysed for the $P2Y_1$–$P2Y_6$ receptor subtypes by RT–PCR-southern analysis. As shown

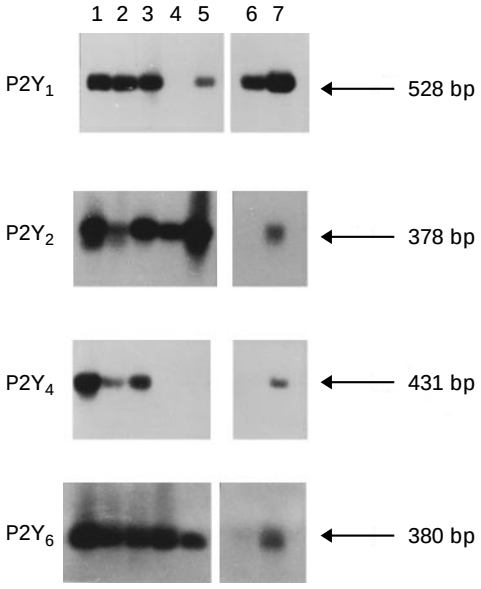

Figure 1 RT–PCR analysis of RNA from haematopoietic cell lines. An autoradiograph is shown of PCR products electrophoresed on a 1.0% agarose gel, Southern blotted to a nylon membrane and probed with the radiolabelled P2Y receptor cDNA. PCR was carried out on RNA from Lane 1, human genomic DNA (positive control); or RT–PCR on RNA from Lane 2, human blood leukocytes; Lane 3, human umbilical vein endothelial cells; Lane 4, promonocytic U937 cells; Lane 5, promyelocytic HL-60 cells; Lane 6, K562 cells; Lane 7, megakaryocytic Dami cells; by use of $P2Y_1$, $P2Y_2$, $P2Y_4$ or $P2Y_6$ receptor specific primers. Four identical gels were separately transferred to nylon membranes and the blots were probed with corresponding radiolabelled P2Y receptor cDNA (as indicated). Autoradiographs of the four blots were carefully aligned for presentation purposes.

Table 2 Distribution of P2Y receptors in haematopoietic cell lines

Cells	*$P2Y_1$*	*$P2Y_2$*	*$P2Y_4$*	*$P2Y_6$*
Endothelial cells	+	+	+	+
U937 cells	–	+	–	+
Hl60 cells	+	+	–	+
K562 cells	+	–	–	–
Dami cells	+	+	+	+
Polymorphonuclear cells	–	–	+	+
Monocytes	+	+	+	+
Lymphocytes	+	+	+	+

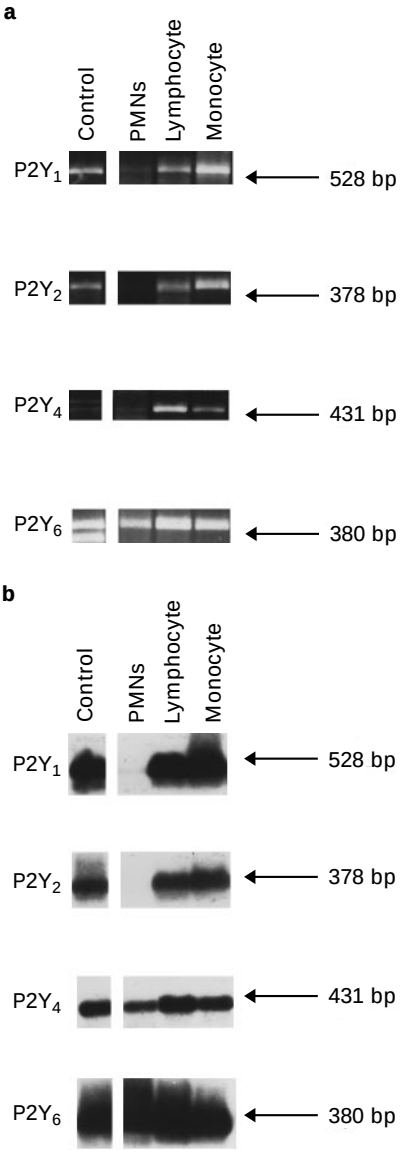

Figure 2 RT–PCR-southern analysis of RNA from peripheral blood leukocytes. An autoradiograph is shown of PCR products electrophoresed on a 1.0% agarose gel and stained with ethidium bromide (a), southern blotted to nylon membrane, and probed with the radiolabelled P2Y receptor cDNA (b). PCR was carried out with human genomic DNA (Control) or cDNA from polymorphonuclear cells (PMNs), monocytes or lymphocytes as indicated.

in Figure 2, polymorphonuclear cells express the $P2Y_4$ and $P2Y_6$ receptors, but not $P2Y_1$ or $P2Y_2$ receptors, while monocytes and lymphocytes express $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors. Control PCR reactions on RNA samples without cDNA synthesis resulted in no signal, indicating that the samples were free from genomic DNA (data not shown).

Discussion and conclusion

Two major sources of extracellular ATP in the blood stream are vascular injury, when broken cells release cytosolic ATP and the degranulation of platelets, releasing stored ATP and ADP. These extracellular nucleotides can act on a number of blood cells to trigger physiological responses (Gordon, 1986; Cowen *et al*., 1989). ADP stimulates other platelets leading to thrombus formation (Hourani & Hall, 1994). In human neutrophils and their precursor cell line HL-60 (Dubyak *et al*., 1988), and in macrophages (Pfeilschifter *et al*., 1989), ATP causes activation of phospholipase C (PLC). Both ADP and ATP stimulate phagocytic activity of neutrophils and monocytes (Sakamoto & Firkin, 1984) and increase intracellular calcium in monocytes and promonocytic U937 cells (Cowen *et al*., 1989). ADP also causes increased binding of fibrinogen to monocytes in a calcium-dependent manner (Altieri *et al*., 1986). The adherence of neutrophils to endothelial cells (Dawicki *et al*., 1995) and of monocytes to surfaces (Ventura & Thomopoulos, 1991) is increased upon stimulation with extracellular nucleotides. Endothelial cells produce prostacyclin and nitric oxide in response to nucleotides (Boarder *et al*., 1995). We have demonstrated that extracellular nucleotides cause increased surface expression of Mac-1 on peripheral blood leukocytes and this was inhibited by R0-31-8220, a protein kinase C specific inhibitor (Akbar *et al*., 1997). Intracellular calcium mobilization by adenine nucleotides was demonstrated in T-leukaemic cells and this response is also mediated by P2Y receptor subtypes (Biffen & Alexander, 1994). P2Y receptor subtypes with a possible role in differentiation have been identified in murine myelomonocytic leukaemic cells (Yamguchi *et al*., 1994).

ATP-induced calcium transients (Cowen *et al*., 1989; Pleass *et al*., 1990; Ventura & Thomopoulos, 1995) and increase in inositol triphosphate formation (Ventura & Thomopoulos, 1995) have been observed in monocytes and U937 cells. The ATP-induced increase in intracellular calcium in U937 cells is mediated by a P2Y receptor subtype which responds to both ADP and ATP (Pleass *et al*., 1990). Extracellular ADP causes increased surface expression of Mac-1 ($\alpha M\beta 2$ integrin, CD11b/CD18) on monocytes (Altieri & Edgington, 1988). RT–PCR analysis of the U937 cell RNA (Figure 1) indicates that these cells express $P2Y_2$ receptors, at which UTP and ATP are at least 100 fold more active than ADP (Erb *et al*., 1995), and $P2Y_6$ receptors, at which UDP and ADP are at least 50 fold more active than ATP, but not $P2Y_4$ receptors, which respond to UTP and UDP and not to ADP (Communi *et al*., 1995; Webb *et al*., 1996b), or the $P2Y_1$ receptor, which responds to ADP but not to ATP (Leon *et al*., 1997). The presence of $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors on monocytes (Figure 2) suggests that different P2Y receptor subtypes may be responsible for the actions of ATP and ADP on these cells.

Neutrophils respond to ATP by increased intracellular calcium by a pertussis toxin sensitive pathway (Boarder *et al*., 1995). This response has been attributed to a $P2Y_2$ receptor, at which both UTP and ATP are equally potent. Our failure to detect the $P2Y_2$ receptor on neutrophils suggests that this receptor subtype may be expressed at low levels. The identification of other P2Y receptor subtypes on neutrophils (Figure 2) and promyelocytic HL-60 cells (Figure 1) indicates that these receptors may also play a role in the stimulation of neutrophils by extracellular nucleotides leading to enhanced adherence to endothelial cells (Dawicki *et al*., 1995).

Vascular endothelial cells are regulated by nucleotides released from platelets, endothelial cells, neurones and damaged cells. Nucleotides stimulate phospholipase A_2 (PLA_2) and nitric oxide synthase in endothelial cells and thereby cause enhanced synthesis and release of prostacyclin and nitric oxide, respectively (Boarder *et al*., 1995). The nucleotides have also been shown to stimulate endothelial cells resulting in enhanced binding of neutrophils (Dawicki *et al*., 1995). Endothelial cells are known to express classical $P2Y_2$ and $P2Y_1$ receptors and respond to nucleotides via pertussis toxin sensitive and insensitive pathways (Boarder *et al*., 1995). The demonstration of the expression of $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors on endothelial cells (Figure 1) indicates that the UTP-induced intracellular responses could be mediated by either the $P2Y_2$ receptor or the $P2Y_4$ on these cells. On the other hand, ADP-induced responses could be mediated by either $P2Y_1$ or $P2Y_6$ receptors.

The $P2Y_4$ receptor, present in placenta, is not expressed in K562 cells or HL-60 cells (Communi *et al*., 1995). Our results confirmed this. The expression of $P2Y_4$ in neutrophils, monocytes, lymphocytes, megakaryocytic Dami cells and in endothelial cells (Figures 1 and 2) suggests a new physiological function for this receptor.

ADP-induced calcium transients have also been observed in various lines of cultured hematopoietic stem cells, including HEL, DAMI, K562, and Meg01 (Murgo *et al*., 1992; 1994; Vittet *et al*., 1992; Kalambakas *et al*., 1993; Akbar *et al*., 1996a). In K562 cells, ADP and 2MeSADP cause a transient increase in $[Ca^{2+}]_i$ and the response is antagonized by ATP (Murgo *et al*., 1992). The identification of $P2Y_1$ receptors on K562 cells (Figure 1) is consistent with the findings of Leon *et al*. (1997) wherein ATP has been shown to be an antagonist of the $P2Y_1$ receptor. On the other hand, the responses seen for ATP, ADP and UTP in Dami cells (Murgo *et al*., 1994) could be mediated by some or all of the four molecular subtypes identified on these cells.

This study is a qualitative study done to find out if a particular P2Y receptor subtype is expressed in a haematopoietic cell or cell line. The quantification of these results in terms of the receptor abundance may not be accurate, since abundant mRNA does not necessarily correlate to abundant protein. Translational regulation of mRNA and protein stability in the cell will determine the ultimate receptor number. Unfortunately, the necessary tools, such as the receptor subtype specific radioligands to determine the exact number of a receptor subtype are not available at present. Additional studies with specific agonists and antagonists, when they become available, and with gene disruption techniques, such as the antisense and knockout approaches, are required to delineate the role of a specific receptor subtype in a functional response.

Note added in proof

Subsequent to the submission of this work, Handin (1997) reported that the Dami cells distributed through ATCC are contaminated with and, appear in fact to be, human erythroleukaemia (HEL) cells. Hence, the present results as well as the previous results with 'Dami cells' cited in the present manuscript should be interpreted in that light.

This work was supported in part by grants from the American Heart Association, Southeastern Pennsylvania Affiliate and the W.W.

Smith Charitable Trust Foundation, H9405. This work was performed during the tenure of an Established Investigator award in Thrombosis from American Heart Association and Genentech to S.P.K. We are grateful to Dr Alison Gagnon, Department of Molecular Pharmacology, Thomas Jefferson University, Philadelphia, for RNA from HL-60 cells. We also thank Dr Keith McCrae, The Sol Sherry Thrombosis Research Center, Temple University School of Medicine, for providing us with human umbilical vein endothelial cells, and Dr Brian O'Dowd, University of Toronto, Toronto, Canada, for his generous gift of the $P2Y_4$ genomic clone. We also would like to acknowledge the help of Dr Robert Loudon, Department of Molecular Pharmacology, Thomas Jefferson University, Philadelphia in the separation of monocytes and lymphocytes from peripheral blood leukocytes. We thank Dr David C.B. Mills, Sol Sherry Thrombosis Research Center, Temple University Medical School, for critically reviewing this manuscript.

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(Received July 15, 1997
Revised October 21, 1997
Accepted November 11, 1997)