ACCELERATED COMMUNICATION

Molecular Cloning of the Platelet P2T$_{AC}$ ADP Receptor: Pharmacological Comparison with Another ADP Receptor, the P2Y$_1$ Receptor

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ABSTRACT

Platelet activation plays an essential role in thrombosis. ADP-induced platelet aggregation is mediated by two distinct G protein-coupled ADP receptors, Gq-linked P2Y$_1$, and Gi-linked P2T$_{AC}$, which has not been cloned. The cDNA encoding a novel G protein-coupled receptor, termed HORK3, was isolated. The *HORK3* gene and *P2Y$_1$* gene were mapped to chromosome 3q21-q25. HORK3, when transfected in the rat glioma cell subline (C6–15), responded to 2-methylthio-ADP (2MeSADP) (EC$_{50}$ = 0.08 nM) and ADP (EC$_{50}$ = 42 nM) with inhibition of forskolin-stimulated cAMP accumulation. 2MeSADP (EC$_{50}$ = 1.3 nM) and ADP (EC$_{50}$ = 18 nM) also induced intracellular calcium mobilization in P2Y$_1$-expressing cells. These results show that HORK3 is a Gi/o-coupled receptor and that its natural ligand is ADP. AR-C69931 MX and 2MeSAMP, P2T$_{AC}$ antagonists, selectively inhibited 2MeSADP-induced adenylyl cyclase inhibition in HORK3-expressing cells. On the other hand, A3P5PS, a P2Y$_1$ antagonist, blocked only 2MeSADP-induced calcium mobilization in P2Y$_1$-expressing cells. HORK3 mRNA was detected in human platelets and the expression level of HORK3 was equivalent to that of P2Y$_1$. These observations indicate that HORK3 has the characteristics of the proposed P2T$_{AC}$ receptor. We have also determined that [^{3}H]2MeSADP binds to cloned HORK3 and P2Y$_1$. Competition binding experiments revealed a similarity in the rank orders of potency of agonists and the selectivity of antagonists as obtained in the functional assay. These results support the view that P2Y$_1$ functions as a high-affinity ADP receptor and P2T$_{AC}$ as a low-affinity ADP receptor in platelets.

Extracellular nucleotides, primary ATP, ADP, UTP, and UDP, are important signaling molecules that mediate diverse biological effects through P2 purinergic receptors (Kunapuli and Daniel, 1998; Ralevic and Burnstock, 1998). Based on differences in their molecular structures and signal transduction mechanisms, P2 receptors are subclassified as P2X and P2Y receptors. P2X ionotropic receptors are ATP ligand-gated cation channel receptors, and P2Y metabotropic seven transmembrane domain receptors are coupled to G proteins. To date, seven types of mammalian P2X receptors (P2X$_1$-P2X$_7$) and at least five types of P2Y receptors (P2Y$_1$, P2Y$_2$, P2Y$_4$, P2Y$_6$, and P2Y$_{11}$) have been cloned and characterized. P2Y$_1$ and P2Y$_{11}$ are specifically activated by adenine nucleotides, whereas P2Y$_2$, P2Y$_4$, and P2Y$_6$ respond to uridine nucleotides (King et al., 1998). All the cloned P2Y receptors induce intracellular calcium mobilization through the activation of phospholipase C, and P2Y$_{11}$ also induces cAMP accumulation through the activation of adenylyl cyclase (Communi et al., 1999). Other P2 receptors, including an ADP receptor (P2T$_{AC}$/P2Y$_{ADP}$) negatively coupled to adenylyl cyclase in platelets and a receptor (P2D/P2Y$_{AP4A}$) for diadenosine polyphosphate in synaptosomes, have been proposed based on pharmacological data (Macfarlane et al., 1983; Pintor et al., 1993).

Platelet activation plays an essential role in thrombosis. ADP has been known to induce a platelet shape change and to activate fibrinogen receptors, leading to platelet aggregation (Kunapuli et al., 1998; Kunapuli and Daniel, 1998).

ABBREVIATIONS: GPCR, G protein-coupled receptor; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; ORF, open-reading frame; IBMX, 3-isobutyl 1-methylxanthine; 2MeSADP, 2-methylthio-ADP; RT-PCR, reverse transcription-polymerase chain reaction; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′disulfonic acid; AR-C69931 MX, N 6-[2-methlthio]ethyl]-2-[3,3,3-trifluoropropylthio]-5′-adenylic acid, monohydride with dichloromethylenebiphosphonic acid; 2MeSATP, 2-methylthio-ATP; ADPβS, adenosine 5′-O-(2-thiodiphosphate); 2Me-SAMP, 2-methylthio-AMP; A3P5PS, adenosine 3′-phosphate 5′-phosphosulfate; GTPγS, guanosine 5′-O-(thiotriphosphate).

Specific ADP receptors mediate these responses. Pharmacological studies using selective antagonists showed that ADP-induced platelet aggregation requires intracellular signaling from two distinct G protein-coupled receptors (GPCRs): a Gi-coupled ADP receptor (P2T$_{AC}$/P2Y$_{ADP}$) causing the inhibition of adenylyl cyclase and a Gq-coupled ADP receptor (P2T$_{PLC}$) causing intracellular calcium mobilization (Daniel et al., 1998; Kunapuli, 1998). It has been shown that platelet P2T$_{PLC}$ is the previously cloned P2Y$_1$ receptor, but molecular identification of P2T$_{AC}$/P2Y$_{ADP}$ has remained elusive (Hechler et al., 1998; Jin et al., 1998a; Jin and Kunapuli, 1998; Jantzen et al., 1999).

We have isolated numerous novel sequences that encode structural characteristics common to GPCRs using a computational survey of public dbEST and genomic databases. One of the identified clones, termed HORK3, is predicted to be a novel GPCR encoding a 342-amino-acid protein that is 43% identical with the human UDP-sugar receptor KIAA0001 (Chambers et al., 2000). We report here evidence that HORK3 is a Gi/o-coupled receptor for ADP and that its pharmacological characteristics suggest it is the proposed P2T$_{AC}$.

Experimental Procedures

Cloning of HORK3 and Human P2Y$_1$. Using the TBLASTN algorithm, dbEST and genomic databases (National Center for Biotechnology Information, National Institutes of Health) were queried with the amino acid sequence of CysLT2 (GenBank Accession no. AB038269). One genomic draft-sequence (GenBank Accession no. AC024886) derived from chromosome 3 was predicted to encode a novel GPCR. Primers were generated from the novel GPCR sequence, and the cDNA was amplified by 5′- and 3′-rapid amplification of the cDNA ends (RACE) system from Human Brain Marathon-ready cDNA (CLONTECH, Palo Alto, CA) as described previously (Kamohara et al., 2000). The amplified RACE products were sequenced directly to avoid the influence of PCR error, and a 1029-base-pair open reading frame (ORF) was found. The nucleotide sequence data reported in this article will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB052684. The ORF was amplified from human brain cDNA using the following *Xba*I restriction site-containing primers: 5′-CCTCTA-GAATGCAAGCCGTCGACAACCTCACCTC-3′ and 5′-CCTCTA-GACTATTACATTGGAGTCTCTTCATTTG-3′. For cloning of the human P2Y$_1$, PCR was performed on human placenta cDNA using the following *Spe*I restriction site-containing primers: 5′-AAAAACTAG-TATGACCGAGGTGCTGTGGCC-3′ and 5′-AAAAACTAGTTCA-CAGGCTTGTATCTCCATTCT-3′.

Preparation of HORK3 or P2Y$_1$-Expressing C6–15 Cells. HORK3 cDNA or P2Y$_1$ cDNA was ligated into an expression vector plasmid, pEF-BOS-neo (Mizushima and Nagata, 1990), using the *Xba*I sites. The constructs or pEF-BOS-neo alone were transfected into a rat glioma cell line, C6–15, which was a gift from Prof. Y. Takuwa (Kanazawa University School of Medicine, Kanazawa, Japan). The C6–15 cells expressing HORK3 or P2Y$_1$ were established by selecting 0.6 mg/ml Geneticin-resistant clones.

Assay for Inhibition of Forskolin-Induced Intracellular Accumulation of cAMP. HORK3-expressing cells were plated at a density of 1×10^4 cells/well in 96-well plates and incubated for 24 h. The cells were exposed to ligands with 1 μM forskolin dissolved in Dulbecco's modified Eagle's medium containing 1 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma, St. Louis, MO) and 0.1% bovine serum albumin. After incubation for 30 min, the cells were harvested by 0.2% Triton X-100 in phosphate-buffered saline. Intracellular cAMP was measured using a cAMP homogenous time-resolved fluorescence kit (CIS bio international, Bagnols-sur-Ceze, France). The cell lysates were incubated with cAMP-XL665 and anti-cAMP cryp-

tate for 3 h at 4°C. The fluorescence ratio (665/620 nm $\times$ 10,000) of the samples and standard cAMP were read on Discovery (Packard, Meriden, CT), and the cyclic AMP concentrations were calculated by the displacement curve obtained from standard cAMP.

Calcium Mobilization Experiments. Calcium mobilization studies were conducted using Fluo 3, AM-loaded HORK3, or P2Y$_1$-expressing cells and a microtiter plate-based assay using fluorometric imaging plate reader (Molecular Devices, Sunnyvale, CA). Transient changes in intracellular calcium concentrations caused by agonists and antagonists were monitored as described previously (Takasaki et al., 2000).
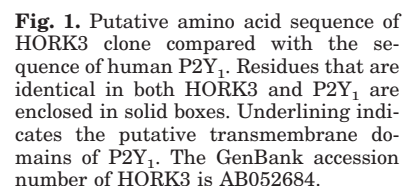
Radioligand Binding Assay. HORK3, P2Y$_1$-expressing C6–15 cells or C6–15 cells were harvested and homogenized in ice-cold 20 mM Tris-HCl, pH 7.4, 1 mM EDTA and a protease inhibitor cocktail set, Complete (Hoffman La Roche, Nutley, NJ), and centrifuged at 200g for 5 min. The supernatant was centrifuged at 100,000g for 20 min at 4°C. The resulting pellets were homogenized again in assay buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1% BSA, 100 mM NaCl, and Complete). The membrane preparations (20 μg of protein) were incubated with concentrations (0.1 to 500 nM) of [^{3}H]2MeSADP (111 GBq/mmol; Moravek Biochemicals Inc., Brea, CA) for 2 h at 25°C. After incubation, the reaction was stopped by the addition of washing buffer (50 mM Tris-HCl, pH 7.4, and 5 mM MgCl$_2$), followed immediately by rapid filtration through Whatman GF/B glass fiber filters. The filters were rinsed three times, and the radioactivity retained on each filter was measured with a liquid scintillation counter. The 2MeSADP-specific binding was calculated by subtracting the nonspecific binding which was defined with 100 μM unlabeled 2MeSADP. Specific binding of [^{3}H]2MeSADP for HORK 3 and P2Y$_1$-expressing cell membrane accounted for 80 to 90% and 75 to 85% of the total binding, respectively. For the competition studies, 1 nM or 10 nM [^{3}H]2MeSADP was added to aliquots of the HORK3 or P2Y$_1$ membrane preparation, which were then incubated with one of several concentrations of compounds. Binding data were analyzed using Prism (Graphpad Software Inc., San Diego, CA).

Quantitative Analysis of HORK3 and P2Y$_1$ mRNA by Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Poly(A)$^+$ RNA from various human tissues were purchased from CLONTECH, and cDNAs were synthesized from these as described previously (Matsumoto et al., 2000). Human mononuclear cells, neutrophils, and eosinophils were isolated from the heparinized blood of informed healthy volunteers using the Ficoll-Paque (Amersham Pharmacia Biotech, Piscataway, NJ) technique and CD16 microbeads (MACS reagents: Milteny Biotech, Gladbach, Germany). Platelet-rich plasma was also prepared from blood, which was collected in a 1/10 volume of 3.8% sodium citrate, by centrifugation at 230g for 15 min at room temperature. The mononuclear cells, neutrophils, eosinophils, and platelets were washed with phosphate-buffered saline. Total RNA preparation and cDNA syntheses were performed as described previously (Kamohara et al., 2000). We quantified HORK3 and P2Y$_1$ mRNA by means of a Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA) with primer sets (5′-GCAAGCCGTCGACAACC-3′ and 5′-TGAT-TTTGTAGTCTCTGGTGCACA-3′ for HORK3; 5′-CCTCCACTGC-CGCCCG-3′ and 5′-AACTGGAAGCCCGTCTTGGT-3′ for P2Y$_1$) and fluorescence-labeled probes (5′-FAM)-CACCTCTGCGCCTGGTAA-CACCAG-(TAMRA)-3′ for HORK3 and 5′-(FAM)-CTCCTCGTCGT-TCAAATGCGCCTT-(TAMRA)-3′ for P2Y$_1$). RT-PCR was carried out in a 25-μl reaction mixture prepared with a TaqMan PCR Core Reagent Kit (PE Biosystems, Warrington, UK) containing an appropriately diluted cDNA solution, 0.2 μM concentrations of each primer, and 0.1 μM probe. To obtain a calibration curve, we amplified a known amount of human genomic DNA in the same manner as the samples. The number of copies of genomic DNA per micro liter was calculated using the equation described previously (Kamohara et al., 2000). Human β-actin mRNA was also measured as an internal control.

Materials. 2MeSADP, PPADS, and reactive blue 2 were purchased from Sigma/RBI (Natick, MA). Other nucleotides, nucleotide derivatives, and pertussis toxin were purchased from Sigma. AR-C69931 MX was synthesized in-house.

Results

Identification and Molecular Characterization of HORK3, a Novel GPCR. The human HORK3 was identified using a combination of genomic database searching and a 5′- and 3′-RACE system. The search of genomic databases suggested a genomic draft-sequence (GenBank Accession no. AC024886), which contains a sequence that encodes a novel GPCR. The ORF encodes a unique putative GPCR protein of 342 amino acids, designated HORK3, which is closely related to the UDP-sugar receptor KIAA0001 with 43% identity and the platelet-activating factor receptor with 30% identity, whereas HORK3 shares a relative low identity (about 25%) to P2Y receptors (Fig. 1). From genomic sequence analysis using the Human Genome Reconstruction Project (HGREP; http://hgrep.ims.u-tokyo.ac.jp/cgi-bin/HTGtool/view.cgi?layer=top), the *HORK3* gene was mapped between D3S1279 and D3S1280 on chromosome 3q21-q25. This region also includes *KIAA0001*, *P2Y₁*, and *H963*, which also shares 31% identity with HORK3.

Pharmacological Characterization of HORK3. To identify possible ligands of HORK3, the changes in intracellular cAMP and calcium after the addition of various bioactive materials were examined using a subline of rat glioma cells (C6–15) transfected with HORK3 cDNA. C6–15 was reported not to express endogenous P2Y receptors at any significant level, as evaluated using the measurements of

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1    M Q A V - - - - - - - - - D N L T S A P G N - - - - - - -    HORK3
1    M T E V L W P A V P N G T D A A F L A G P G S S W G N S T V    P2Y1

14   - - T S L C T R D Y K - - I T Q V L F - - - - - P L L Y T V    HORK3
31   A S T A A V S S S F K C A L T K T G F Q F Y Y L P A V Y I L    P2Y1

35   L F F V G L I T N G L A M R I F - F Q I R S K S N F I I F L    HORK3
61   V F I I G F L G N S V A I W M F V F H M K P W S G I S V Y M    P2Y1
              TM1

64   K N T V I S D L L M I L T F P - - - - - - F K - - - - L S    HORK3
91   F N L A L A D F L Y V L T L P A L I F Y Y F N K T D W I F G    P2Y1
              TM2

84   D A K L G T G P L R T F V C Q V T S V I F Y F T M Y I S I S    HORK3
121  D A - - - - - - - - - - M C K L Q R F I F H V N L Y G S I L    P2Y1
                                              TM3

114  F L G L I T I D R Y Q K T T R P F K T S N P K N L L G A K I    HORK3
141  F L T C I S A H R Y S G V V Y P L K S L G R L K K K N A I C    P2Y1

144  L S V V I W A F M F L L S L P N M I L T N R Q P R D K N V K    HORK3
171  I S V L V W L I V V V A I S P I L F Y S G T G V R K N K T I    P2Y1
              TM4

174  K C - - - - - - S F L K S - - E F G L V W H E I V N Y I C Q    HORK3
201  T C Y D T T S D E Y L R S Y F I Y S M C - - T T V A M F C -    P2Y1

196  V I F W I N F L I V I V C Y T L I T K E L Y R S Y V R T R G    HORK3
228  - - - - V P L V L I L G C Y G L I V R A L - - I Y - - - K D    P2Y1
     TM5

226  V G K V P - R K K V N V K V F I I I A V F I C F V P F H F    HORK3
249  L D N S P L R R K S I Y L V I I V L T V F A V S Y I P F H V    P2Y1
                                              TM6

255  A R I P Y T L S - Q T R D V F D - - - - C T A E N T L F Y V    HORK3
279  M K - - - T M N L R A R L D F Q T P A M C A F N D R V Y A T    P2Y1

280  K E S T L W L T S L N A C L D P F I Y F L C K S F R N S L    HORK3
306  Y Q V T R G L A S L N S C V D P I L Y F L A G D T F R R R L    P2Y1
              TM7

310  I S M L K C P N S A T S L S Q D N R K K E Q D G G - - - - -    HORK3
336  S R A T R K A S R R S E A N L Q S K S E D M T L N I L P E F    P2Y1

335  D P N E E T P M    HORK3
366  K Q N G D T S L    P2Y1
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Fig. 1. Putative amino acid sequence of HORK3 clone compared with the sequence of human P2Y₁. Residues that are identical in both HORK3 and P2Y₁ are enclosed in solid boxes. Underlining indicates the putative transmembrane domains of P2Y₁. The GenBank accession number of HORK3 is AB052684.

intracellular calcium mobilization response and cellular cAMP changes by nucleotides (Chang et al., 1995). Based on the HORK3 sequence homology, we predicted its ligands as UDP-sugars or bioactive lipids. UDP-sugars (UDP-glucose, UDP-galactose etc.), other nucleotide-sugars (CDP-glucose, ADP-glucose etc.) and bioactive lipids (leukotrienes, platelet-activating factor, etc.) showed no effect on HORK3-expressing C6–15 cells, and no clear difference was apparent when these were compared with vector-transfected C6–15 cells.

We also analyzed HORK3 activity using other bioactive materials, such as peptides and nucleotides. Among the nucleotides tested, 2MeSATP, 2MeSADP, ATP, ADP, and ADPβS induced significant inhibition of forskolin-stimulated intracellular cAMP accumulation in HORK3-expressing cells (Fig. 2A). However, other nucleotides, nucleosides and nucleotide derivatives, such as AMP, 2MeSAMP, adenosine, α,β-methylene ATP, 2′ and 3′-O-(4-benzoyl-benzoyl)-ATP, UTP, UDP, and adenosine polyphosphates (up to 10^{-5} M), had no effect on cAMP accumulation in HORK3-expressing cells (data not shown). The rank order of potency of the series of

agonist was 2MeSADP = 2MeSATP > ADP > ADPβS > ATP with respective EC$_{50}$ values of 0.078, 0.098, 42, 404, and 690 nM (Table 1). These nucleotides did not affect mock-transfected cells on cAMP accumulation, and did not significantly induce intracellular calcium mobilization in vector-transfected and HORK3-expressing C6–15 cells (data not shown). The activity of adenylyl cyclase inhibition of HORK3 by 2Me-SADP was completely blocked by 50 ng/ml pertussis toxin (Fig. 2A). These results showed that HORK3 is a Gi/o-coupled receptor and that its most potent natural ligand is ADP.

Pharmacological Comparison with P2Y$_1$. We compared the pharmacological profile of HORK3 with that of another ADP receptor, human P2Y$_1$, transfected in C6–15 cells. Same nucleotides also induced intracellular calcium mobilization in P2Y$_1$-expressing cells with a rank order of potency: 2MeSADP = 2MeSATP > ADP > ADPβS > ATP (Fig. 2B and Table 1).

Next, the antagonistic activity of various compounds on HORK3 activity and P2Y$_1$ activity were analyzed (Fig. 3, A and B; Table 1). Reactive blue 2, a nonselective P2 antagonist, blocked both the adenylyl cyclase inhibition of HORK3 (IC$_{50}$ value of 13 μM) and the intracellular calcium mobilization of P2Y$_1$ (IC$_{50}$ value of 15 μM). Suramin, another nonselective P2 antagonist, was a weak antagonist for both HORK3 and P2Y$_1$ at 100 μM. PPADS and A3P5PS, on the other hand, inhibited P2Y$_1$ activity (IC$_{50}$ values of 3.7 and 8.0 μM, respectively) but did not inhibit HORK3 activity, whereas 2MeSAMP inhibited only HORK3 activity (IC$_{50}$ value of 5.0 μM). In addition, AR-C69931 MX, a potent selective P2T$_{AC}$ antagonist, inhibited HORK3 activity with high potency (IC$_{50}$ value of 2.4 nM) but had no effect on P2Y$_1$-expressing cells (Fig. 3C and Table 1).

Binding of [^{3}H]2MeSADP. No specific binding of [^{3}H]2MeSADP was observed in the membranes from nontransfected C6–15 cells. Saturation binding studies of [^{3}H]2MeSADP were therefore performed using membranes of HORK3 and P2Y$_1$-expressing C6–15 cells. Analysis of binding data revealed that [^{3}H]2MeSADP bound to two sites on the membranes of HORK3-expressing cells with K_d values of 0.23 ± 0.05, and 33 ± 5.2 nM, with respective B_{max} values of 1.4 ± 0.17, and 14 ±
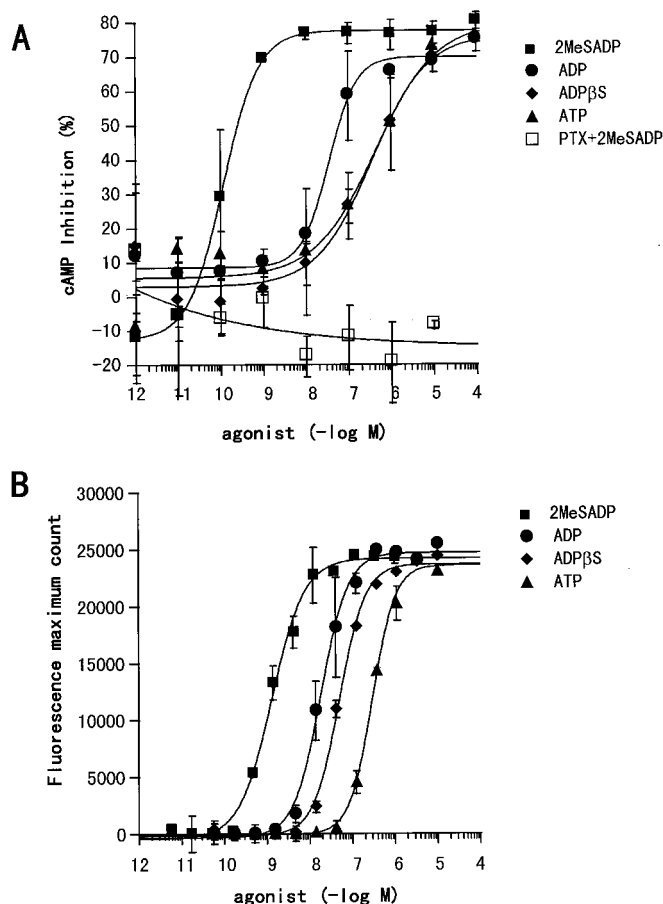
Fig. 2. Nucleotides induced inhibition of cAMP accumulation in HORK3-expressing cells or calcium mobilization in P2Y$_1$-expressing cells. A, the HORK3-expressing C6–15 cells were treated with IBMX (1 mM) and challenged with forskolin (1 μM) and nucleotides. The cAMP concentrations in the presence of forskolin + IBMX and in the presence of IBMX alone were normalized to 0 and 100%, respectively. The effects of pertussis toxin (50 ng/ml, 20 h) on cAMP inhibition by 2MeSADP in HORK3-expressing C6–15 cells were also measured (□). B, the intracellular calcium mobilization of P2Y$_1$-expressing cells was measured after the addition of nucleotides. The data represent the mean ± S.D. of determinations in triplicate from a single experiment performed two to three times.

TABLE 1
Comparison of potencies of agonists and antagonists at HORK3 and P2Y$_1$

IC$_{50}$ values were determined relative to a 1 nM · 2MeSADP stimulation for HORK3 and a 10 nM stimulation for P2Y$_1$.

	HORK3 cAMP inhibition	P2Y$_1$ Calcium mobilization
	nM	
Agonist EC$_{50}$		
2MeSADP	0.078 ± 0.04[a]	1.32[b]
2MeSATP	0.098 ± 0.001[a]	1.36[b]
ADP	41.7 ± 12[a]	18.3[b]
ADPβS	404 ± 133[a]	49.9[b]
ATP	690 ± 344[a]	294[b]
Antagonist IC$_{50}$	*μM*	
2MeAMP	5.01[b]	>100[b]
AR-C69931MX	0.00236[b]	>10[b]
A3P5PS	>100[b]	8.0[b]
Reactive blue 2	13.0[b]	15.3[b]
Suramin	>100[b]	>100[b]
PPADS	>100[b]	3.68[b]

[a] Data are expressed as the mean ± S.D. of three separate experiments performed in triplicate.
[b] Data are expressed as the mean of two separate experiments performed in triplicate.

1.0 pmol/mg protein (the mean $\pm$ SD of three separate experiments; Fig. 4, A and B). The addition of 100 μM guanosine 5'-O-(thiotriphosphate) (GTPγS) during binding assay reduced the high-affinity binding sites of [^{3}H]2MeSADP to an undetectable level. On the other hand, [^{3}H]2MeSADP bound to a single site on the membranes of P2Y$_1$-expressing cells with a K_d value of 49 $\pm$ 9.1 nM, with a respective B_{max} value of 15 $\pm$ 0.2 pmol/mg protein (Fig. 4, C and D). The addition of GTPγS caused a nearly 12-fold reduction in the affinity.

Next, competition-binding experiments were carried out on these membranes (Fig. 5, A and B). 2MeSADP, ADP, and ADPβS competed with the binding of [^{3}H]2MeSADP to the membrane of HORK3 with IC$_{50}$ values of 0.53 nM, 175 nM, and 2.5 μM, respectively ($n = 3$). [^{3}H]2MeSADP binding to P2Y$_1$ was also inhibited by 2MeSADP (IC$_{50}$ = 7.7 nM; $n = 3$), ADP (IC$_{50}$ = 33 nM; $n = 3$) and ADPβS (IC$_{50}$ = 181 nM; $n = 3$). Only 2MeSAMP and AR-C69931 MX inhibited the [^{3}H]2MeSADP binding to HORK3 with IC$_{50}$ values of 4.2 μM and 10 nM, respectively, whereas only A3P5PS inhibited the binding to P2Y$_1$ with an IC$_{50}$ value of 60 μM ($n = 3$).

Tissue Distribution of HORK3. The expression of HORK3 and P2Y$_1$ mRNA in human purified peripheral blood cells (peripheral blood mononuclear cells, neutrophils, eosinophils, and platelets) were examined using real-time quantitative PCR (TaqMan) analysis. As shown in Fig. 6A, high levels of HORK3 and P2Y$_1$ mRNA were detected in the platelets, and the expression level of HORK3 mRNA was equivalent to that of P2Y$_1$ (the mean of three individual mRNA samples). The expression of HORK3 mRNA in human tissues was also evaluated. HORK3 mRNA was detected mainly in the brain and was not expressed in other tissues (Fig. 6B).
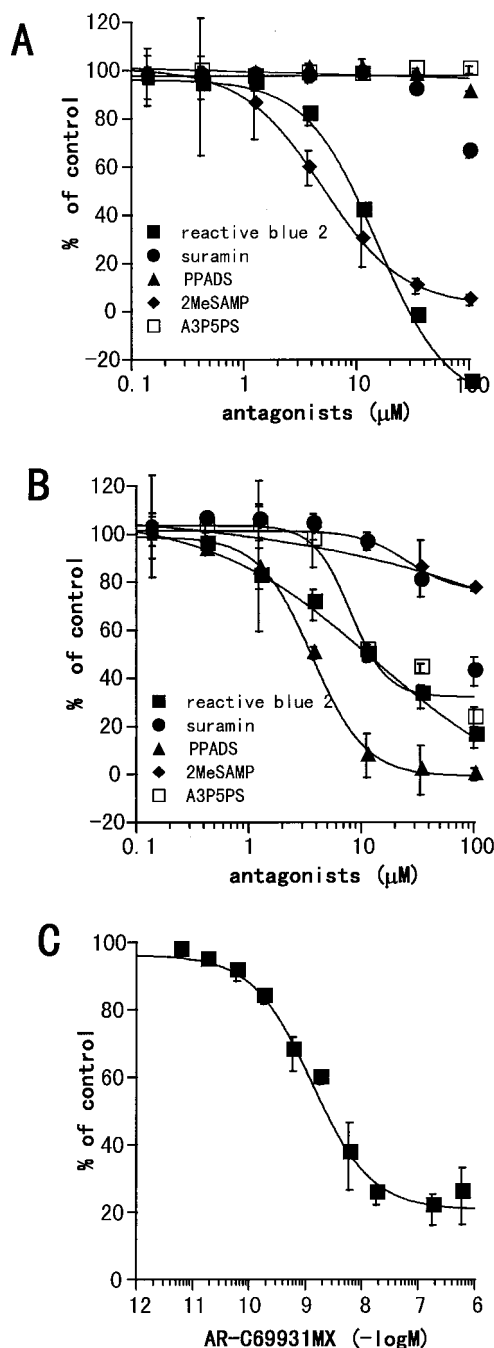
Fig. 3. Effects of P2 antagonists on 2MeSADP-induced adenylyl cyclase inhibition in HORK3-expressing cells and calcium mobilization in P2Y$_1$-expressing cells. A and C, the HORK3-expressing cells were incubated in the absence or the presence of various P2 antagonists for 10 min and then stimulated with forskolin (1 μM) and 2MeSADP (1 nM). The cAMP concentration in the presence of forskolin + 10 nM 2MeSADP was normalized to 100%. B, the P2Y$_1$-expressing cells were incubated in the absence or the presence of antagonists for 5 min and then stimulated with 2MeSADP (10 nM). Calcium mobilization in the presence of 2MeSADP (10 nM) alone was normalized to 100%. The data represent the mean $\pm$ S.D. of determinations in triplicate from a single experiment performed two to three times.
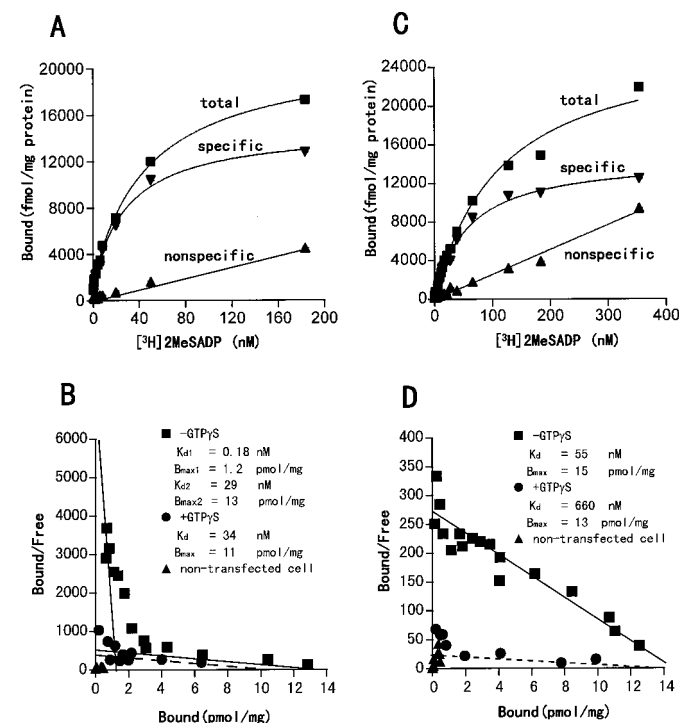
Fig. 4. [^{3}H]2MeSADP-specific binding to HORK3 and P2Y$_1$-expressing C6–15 cell membranes. Membranes from HORK3-expressing cells (A, B), P2Y$_1$-expressing cells (C, D), or nontransfected C6–15 cells were incubated with 0.1–500 nM [^{3}H]2MeSADP in the absence or presence of 100 μM unlabeled 2MeSADP. The data are displayed as saturation binding isotherms (A, C) and Scatchard plots (B, D). The data were best fit by assuming a two-site model for [^{3}H]2MeSADP binding to HORK3 and a single-site model for the binding to P2Y$_1$. These binding reactions were also performed in the presence of 100 μM GTPγS. The data are also displayed as a Scatchard plot. The results from one of three independent experiments are shown.

Discussion

We reported here the identification of a novel GPCR, termed HORK3, which is a Gi/o-coupled ADP receptor. P2T$_{AC}$/P2Y$_{ADP}$, P2D/P2Y$_{AP4A}$, P3, and P4 have been proposed as additional members of the P2Y receptor family (Macfarlane et al., 1983; Forsyth et al., 1991; Pintor et al., 1993; Ralevic and Burnstock, 1998). P2D and P4 are distinct diadenosine polyphosphate receptors in the central nervous system, and both nucleosides and nucleotides activate P3. Neither diadenosine polyphosphate nor adenosine activated HORK3, indicating that HORK3 does not have the characteristics of P2D, P3, and P4. P2T$_{AC}$ has been demonstrated to be a Gi-coupled receptor for ADP in platelets. Our data suggested that the pharmacological characteristics of HORK3 revealed it to be the elusive P2T$_{AC}$.

HORK3, *KIAA0001*, *H963*, and *P2Y$_1$* genes are mapped to 3q21-q25. The three closely related receptors, *HORK3*,

KIAA0001, and *H963*, are localized in tandem within about 1 megabase pair, but the distance from these three receptors to *P2Y$_1$* is about 10 megabase pairs, suggesting that the three closely related receptors have been generated by tandem replication during a recent evolutionary event but that P2Y$_1$ originated from gene duplication during an early evolutionary stage.

In platelets, previous studies comparing the agonist potency indicated that ADP is a more potent agonist in intracellular calcium mobilization via P2Y$_1$ than in adenylyl cyclase inhibition via P2T$_{AC}$. It has also been reported that 2MeSADP is a more potent agonist at P2T$_{AC}$ and P2Y$_1$ than ADP (Hall and Hourani, 1993; Hourani and Hall, 1996). Moreover, 2MeSADP is at least 100 times more potent than ADP at P2T$_{AC}$, but only four to ten times more potent than ADP at P2Y$_1$ (Hourani and Hall, 1996; Daniel et al., 1998; Jin and Kunapuli, 1998). These features are similar to those shown by our data, in which ADP was about two times more potent in calcium mobilization of the P2Y$_1$-expressing cells than in adenylyl cyclase inhibition of HORK3-expressing cells, and the potency of 2MeSADP for HORK3 and for P2Y$_1$ was about 500 times and 13 times higher, respectively, than that of ADP. The rank orders of potency of agonists are
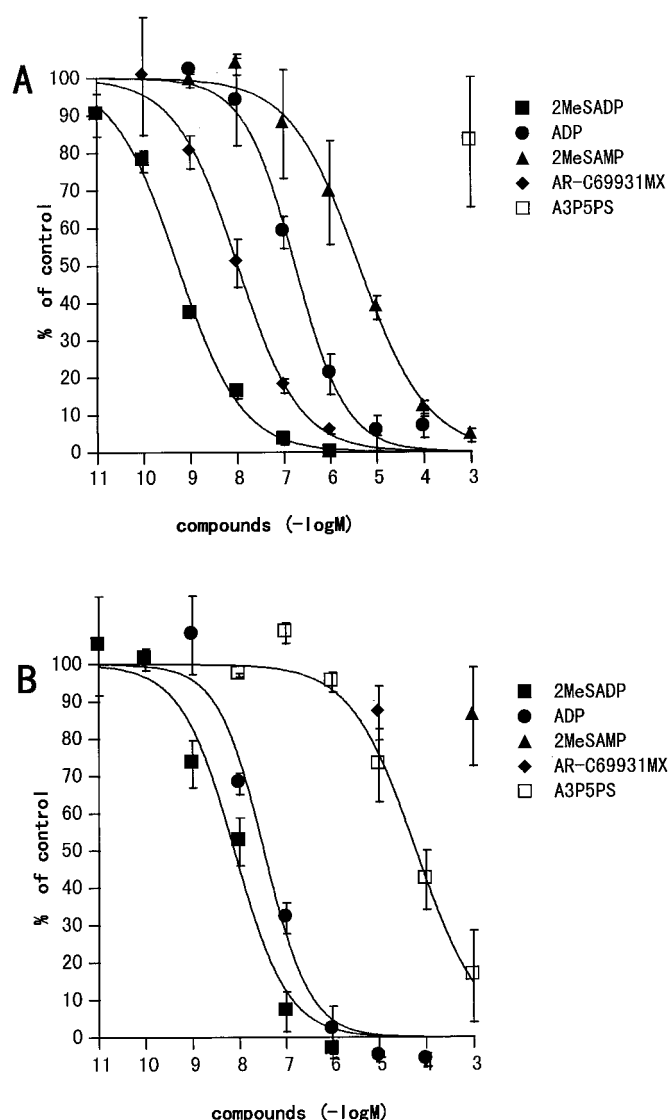
Fig. 5. Competition for [³H]2MeSADP binding to HORK3 and P2Y$_1$-expressing cells. A, [³H]2MeSADP (1 nM) was incubated with membrane of HORK3-expressing cells in the presence of increasing concentrations of the compounds. B, [³H]2MeSADP (10 nM) was incubated with membrane of P2Y$_1$-expressing cells in the presence of the compounds. The data represent the mean ± S.D. of determinations in triplicate.
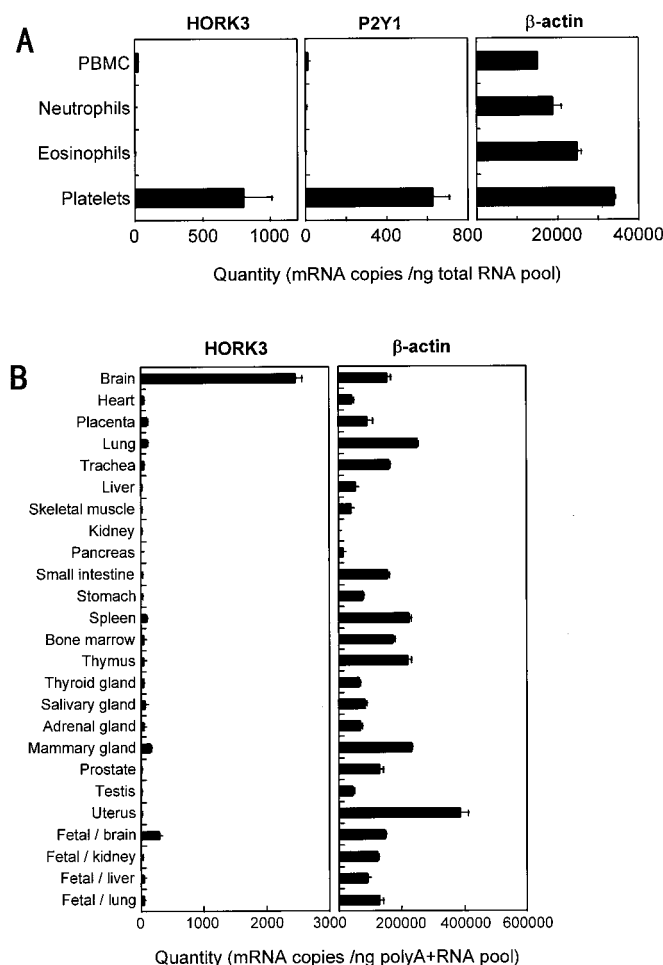
Fig. 6. Expression of HORK3 in human tissues. The cDNA corresponding to 1 ng of total RNA from human blood cells (A) or to 1 ng of poly(A⁺) RNA from human tissues (B) was assessed by TaqMan PCR for HORK3 and P2Y$_1$ mRNA with mRNA for β-actin as a control. The data are presented as the mean ± S.D. of three individual mRNA levels for a subpopulation of human peripheral blood.

consistent with those described for $P2T_{AC}$ and $P2Y_1$ in platelets.

The characteristics of platelet ADP receptor subtypes have also been studied using selective antagonists. AR-C69931 MX is known to be a very potent selective $P2T_{AC}$ antagonist, with an IC_{50} value of 0.4 nM against ADP-induced platelet aggregation (Ingall et al., 1999). 2MeSAMP has also been reported to be a selective antagonist for $P2T_{AC}$, in which 2MeSAMP blocks ADP-induced adenylyl cyclase inhibition ($IC_{50} = 0.57\ \mu M$) in platelets (Jantzen et al., 1999). A3P5PS, a selective $P2Y_1$ antagonist, has been reported to inhibit ADP and 2MeSADP-induced calcium mobilization (IC_{50} values of about 10 μM) in platelets (Boyer et al., 1996; Jin et al., 1998a). In our data, AR-C69931 MX and 2MeSAMP blocked only the adenylyl cyclase inhibition in HORK3-expressing cells, and A3P5PS inhibited only 2MeSADP-induced calcium mobilization in $P2Y_1$-expressing cells with values similar to those previously shown in platelets. PPADS also blocked $P2Y_1$ but not HORK3. PPADS is reported to have the ability to discriminate between $P2Y_1$ and $P2T_{AC}$: it blocks $P2Y_1$ but has no effect on the P2Y receptor coupled to inhibition of adenylyl cyclase (Boyer et al., 1994). The specificity and potency of antagonists also supported the suggestion that HORK3 is a candidate for $P2T_{AC}$.

In our data, ATP and 2MeSATP were agonists at both HORK3 and $P2Y_1$. Leon et al. (1997) reported that ATP and 2MeSATP purified from contaminated nucleotide diphosphates by HPLC did not exhibit agonist activity and acted as a weak antagonist at $P2Y_1$. Palmer et al. (1998) proved that ATP is a partial $P2Y_1$ agonist whose capacity to activate calcium mobilization apparently depends on the degree of $P2Y_1$ reserve. In a preliminary experiment, we purified commercial ATP by Mono Q column and compared the potency at $P2Y_1$. Mono Q-purified ATP still-induced calcium mobilization in $P2Y_1$-expressing cells, but the dose-response curve indicated a right shift, suggesting that the agonist activity of ATP and 2MeSATP may be caused by the contaminant products of ATP and 2MeSATP or an unnatural situation of heterologusly overexpression of $P2Y_1$. Further studies are needed to assess whether ATP or 2MeSATP is a real agonist or antagonist at HORK3.

As is often the case with binding studies using cloned P2Y receptors, it is difficult to discriminate between the response of a heterologously expressed P2Y receptor and that of endogenous nucleotide receptors (Schachter and Harden, 1997). We succeeded in the binding studies with cloned HORK3 and $P2Y_1$ using C6–15 cells. Saturation studies demonstrated the existence of two binding sites of [^{3}H]2MeSADP for HORK3 and the elimination of its high-affinity binding sites with GPTγS. Competition binding experiments revealed similar rank orders of potency of agonists and selectivity of antagonists to those obtained in the functional assay, and all agonists competition curves exhibited Hill coefficients less than unity (0.64–0.71). These results are consistent with the suggestion that 2MeSADP may be associated with two affinity state of HORK3. $P2Y_1$-expressing cells, on the other hand, had a single class of binding sites of [^{3}H]2MeSADP. The specific binding was sensitive to GTPγS and competed with agonists and A3P5PS but not by $P2T_{AC}$ antagonists, suggesting the labeling of a G-protein-coupled $P2Y_1$ subset with [^{3}H]2MeSADP. Commonly, a single population of binding sites is observed with an agonist radioligand because accu-

rate binding data at the high concentrations of radioligand required to define low-affinity binding is unobtainable, and it is not possible to determine whether this is a completely coupled ternary complex form or an uncoupled receptor unless an antagonist radioligand is used to determine the receptor population size (Kenakin, 1996). To obtain an accurate B_{max} value, a better radioligand seems to be necessary. Binding studies have been performed to analyze the platelet ADP receptors using radiolabeled 2MeSADP in combination with selective antagonists, but there is some discrepancy. Savi et al. (1994) reported that $P2Y_1$ represents about 30% of 2MeSADP binding sites ($K_d = 0.9$ nM), which shows a high affinity with ADP ($K_i = 15$ nM), and the remainder are $P2T_{AC}$ ($K_d = 0.7$ nM), which has a low affinity with ADP ($K_i = 486$ nM). Gachet et al. (1995) also demonstrated that $P2Y_1$ and $P2T_{AC}$ represent 30 and 70% of 2MeSADP binding sites, respectively, but the K_d vales are about 10 nM and 5 nM, respectively. Jantzen et al. (1999) reported the inconsistent result that the majority of 2MeSADP binding sites ($K_d = 0.5$ nM) represent $P2T_{AC}$. In our data, ADP inhibited the binding of [^{3}H]2MeSADP for HORK3 with a K_H value of 56 nM and a K_L value of 420 nM, and for $P2Y_1$ with a K_i value of 27 nM. These results suggest that $P2T_{AC}$ and $P2Y_1$ have a high and low affinity with [^{3}H]2MeSADP but a low and high affinity with ADP, respectively, supporting the view that $P2Y_1$ functions as a high-affinity ADP receptor and $P2T_{AC}$ as a low-affinity ADP receptor in platelets. In addition, our distribution study suggests that $P2Y_1$ and $P2T_{AC}$ may be equivalent in human platelets, but this differs from the results of Savi et al. (1994), Gachet et al. (1995), and Jantzen et al. (1999). More studies are needed at the protein level to elucidate these discrepancies.

High expression levels of HORK3 and $P2Y_1$ mRNA were detected in the platelets but not in peripheral blood mononuclear cells. Jin et al. (1998b) reported that human $P2Y_1$ mRNA is detected in lymphocytes and monocytes. One possible explanation for the difference is that $P2Y_1$ mRNA is at quite a low level in leukocyes, but Jin et al. detected $P2Y_1$ mRNA by using a highly sensitive detection system (RT-PCR-Southern analysis). HORK3 was also highly expressed in the brain. A similar receptor to $P2T_{AC}$ has been identified in the rat glioma cell line (C6–2B) and rat brain microvascular endothelial cell line (B10) (Pianet et al., 1989; Boyer et al., 1993; Webb et al., 1996). The rank order of potencies of nucleotides on adenylyl cyclase inhibition in C6–2B and in B10 are reported to be 2MeSATP = 2MeSADP > ADPβS > 2ClATP = ADP > ATPαS > ATP > UTP and 2ClATP > 2MeSATP > ADP > ATP. Moreover, in B10 cells, suramin and reactive blue 2 blocked its activity, but PPADS did not (Webb et al., 1996). These pharmacological profiles are similar to that of HORK3. It is possible that these $P2T_{AC}$-like receptors are identical with HORK3.

Two other groups have cloned and characterized the same ADP receptor during the preparation of this manuscript (Zhang et al., 2000; Hollopeter et al., 2001). The relative potency of agonists and antagonists are similar, but we further confirmed the pharmacological characterization containing a ligand binding study compared with the $P2Y_1$ receptor.

Taken together, the results show that HORK3 has the characteristics of the proposed $P2T_{AC}$ receptor in platelets. Further experiments are required to confirm whether

HORK3 mediates platelet aggregation and whether ADP receptors in C6–2B or B10 are identical with HORK3. The availability of P2T$_{AC}$ cDNA should provide valuable insight into the physiological and pathological roles of ADP in platelets and central nervous system.

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References

Boyer JL, Lazarowski ER, Chen XH and Harden TK (1993) Identification of a P2Y-purinergic receptor that inhibits adenylyl cyclase. *J Pharmacol Exp Ther* **267:**1140–1146.

Boyer JL, Romero-Avila T, Schachter JB and Harden TK (1996) Identification of competitive antagonists of the P2Y1 receptor. *Mol Pharmacol* **50:**1323–1329.

Boyer JL, Zohn IE, Jacobson KA and Harden TK (1994) Differential effects of P2-purinoceptor antagonists on phospholipase C- and adenylyl cyclase-coupled P2Y-purinoceptors. *Br J Pharmacol* **113:**614–620.

Chambers JK, Macdonald LE, Sarau HM, Ames RS, Freeman K, Foley JJ, Zhu Y, McLaughlin MM, Murdock P, McMillan L, et al (2000) A G protein-coupled receptor for UDP-glucose. *J Biol Chem* **275:**10767–10771.

Chang K, Hanaoka K, Kumada M and Takuwa Y (1995) Molecular cloning and functional analysis of a novel P2 nucleotide receptor. *J Biol Chem* **270:**26152–26158.

Communi D, Robaye B and Boeynaems J (1999) Pharmacological characterization of the human P2Y11 receptor. *Br J Pharmacol* **128:**1199–1206.

Daniel JL, Dangelmaier C, Jin J, Ashby B, Smith JB and Kunapuli SP (1998) Molecular basis for ADP-induced platelet activation. I. Evidence for three distinct ADP receptors on human platelets. *J Biol Chem* **273:**2024–2029.

Forsyth KM, Bjur RA and Westfal DP (1991) Nucleotide modulation of norepinephrine release from sympathetic nerves in the rat vas deferens. *J Pharmacol Exp Ther* **256:**821–826.

Gachet C, Cattaneo M, Ohlmann P, Hechler B, Lecchi A, Chevalier J, Cassel D, Mannucci PM and Cazenave JP (1995) Purinoceptors on blood platelets: further pharmacological and clinical evidence to suggest the presence of two ADP receptors. *Br J Haematol* **91:**434–444.

Hall DA and Hourani SM (1993) Effects of analogues of adenine nucleotides on increases in intracellular calcium mediated by P 2T-purinoceptors on human blood platelets. *Br J Pharmacol* **108:**728–733.

Hechler B, Leon C, Vial C, Vigne P, Frelin C, Cazenave JP and Gachet C (1998) The P2Y1 receptor is necessary for adenosine 5′-diphosphate-induced platelet aggregation. *Blood* **92:**152–159.

Hollopeter G, Jantzen HM, Vincent D, Li G, England L, Ramakrishnan V, Yang RB, Nurden A, Julius D and Conley PB (2001) Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature (Lond)* **409:**202–207.

Hourani SM and Hall DA (1996) P2T purinoceptors: ADP receptors on platelets. *Ciba Found Symp* **198:**53–70.

Ingall AH, Dixon J, Bailey A, Coombs ME, Cox D, McInally JI, Hunt SF, Kindon ND, Teobald BJ, Willis PA, et al. (1999) Antagonists of the platelet P2T receptor: a novel approach to antithrombotic therapy. *J Med Chem* **42:**213–220.

Jantzen HM, Gousset L, Bhaskar V, Vincent D, Tai A, Reynolds EE and Conley B (1999) Evidence for two distinct G-protein-coupled ADP receptors mediating platelet activation. *Thromb Haemost* **81:**111–117.

Jin J, Daniel JL and Kunapuli SP (1998a) Molecular basis for ADP-induced platelet activation. II. The P2Y1 receptor mediates ADP-induced intracellular calcium mobilization and shape change in platelets. *J Biol Chem* **273:**2030–2034.

Jin J, Dasari VR, Sistare FD and Kunapuli SP (1998b) Distribution of P2Y receptor subtypes on human haematopoietic cells. *Br J Pharmacol* **123:**789–794

Jin J and Kunapuli SP (1998) Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. *Proc Natl Acad Sci USA* **95:**8070–8074.

Kamohara M, Takasaki J, Matsumoto M, Saito T, Ohishi T, Ishii H and Furuichi K (2000) Molecular cloning and characterization of another leukotriene B4 receptor. *J Biol Chem* **275:**27000–27004.

Kenakin T (1996) The classification of seven transmembrane receptors in recombinant expression systems. *Pharmacol Rev* **48:**413–463

King BF, Townsend-Nicholson A and Burnstock G (1998) Metabotropic receptors for ATP and UTP: exploring the correspondence between native and recombinant nucleotide receptors. *Trends Pharmacol Sci* **19:**506–514.

Kunapuli SP (1998) Multiple P2 receptor subtypes on platelets: a new interpretation of their function. *Trends Pharmacol Sci* **19:**391–394.

Kunapuli SP and Daniel JL (1998) P2 receptor subtypes in the cardiovascular system. *Biochem J* **336:**513–523.

Leon C, Hechler B, Vial C, Leray C, Cazenave JP and Gachet C (1997) The P2Y1 receptor is an ADP receptor antagonized by ATP and expressed in platelets and megakaryoblastic cells. *FEBS Lett* **403:**26–30.

Macfarlane DE, Srivastava PC and Mills DC (1983) 2-Methylthioadenosine[beta-32P]diphosphate. An agonist and radioligand for the receptor that inhibits the accumulation of cyclic AMP in intact blood platelets. *J Clin Invest* **71:**420–428.

Mizushima S and Nagata S (1990) pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res* **18:**5322.

Matsumoto M, Kamohara M, Sugimoto T, Hidaka K, Takasaki J, Saito T, Okada M, Yamaguchi T and Furuichi K (2000) The novel G-protein coupled receptor SALPR shares sequence similarity with somatostatin and angiotensin receptors. *Gene* **248:**183–189.

Palmer RK, Boyer JL, Schachter JB, Nicholas RA and Harden TK (1998) Agonist action of adenosine triphosphates at the Human P2Y1 receptor. *Mol Pharmacol* **54:**1118–1123.

Pianet I, Merle M and Labouesse J (1989) ADP and, indirectly, ATP are potent inhibitors of cAMP production in intact isoproterenol-stimulated C6 glioma cells. *Biochem Biophys Res Commun* **163:**1150–1157.

Pintor J, Diaz-Rey MA and Miras-portugal MT (1993) Ap4A and ADP-beta-S binding to P2 purinoceptors present on rat brain synaptic terminals. *Br J Pharmacol* **108:**1094–1099.

Ralevic V and Burnstock G (1998) Receptors for purines and pyrimidines. *Pharmacol Rev* **50:**413–492.

Savi P, Laplace MC and Herbert JM (1994) Evidence for the existence of two different ADP-binding sites on rat platelets. *Thromb Res* **76:**157–69.

Schachter JB and Harden TK (1997) An examination of deoxyadenosine 5′(alpha-thio)triphosphate as a ligand to define P2Y receptors and its selectivity as a low potency partial agonist of the P2Y$_1$ receptor. *Br J Pharmacol* **121:**338–344.

Takasaki J, Kamohara M, Matsumoto M, Saito T, Sugimoto T, Ohishi T, Ishii H, Ota T, Nishikawa T, Kawai Y, Masuho Y, et al (2000) The molecular characterization and tissue distribution of the human cysteinyl leukotriene CysLT2 receptor. *Biochem Biophys Res Commun* **274:**316–322.

Webb TE, Feolde E, Vigne P, Neary JT, Runberg A, Frelin C and Barnard EA (1996) The P2Y purinoceptor in rat brain microvascular endothelial cells couple to inhibition of adenylate cyclase. *Br J Pharmacol* **119:**1385–1392.

Zhang FL, Luo L, Gustafson E, Lachowicz J, Smith M, Qiao X, Liu YH, Chen G, Pramanik B, Laz TM, et al. (2000) ADP is the cognate ligand for the orphan G protein-coupled receptor SP1999. *J Biol Chem* **276:**8608–8615.

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