ADP Is the Cognate Ligand for the Orphan G Protein-coupled Receptor SP1999*

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P2Y receptors are a class of G protein-coupled receptors activated primarily by ATP, UTP, and UDP. Five mammalian P2Y receptors have been cloned so far including P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11. P2Y1, P2Y2, and P2Y6 couple to the activation of phospholipase C, whereas P2Y4 and P2Y11 couple to the activation of both phospholipase C and the adenyl cyclase pathways. Additional ADP receptors linked to Ga_i have been described but have not yet been cloned. SP1999 is an orphan G protein-coupled receptor, which is highly expressed in brain, spinal cord, and blood platelets. In the present study, we demonstrate that SP1999 is a Ga_i-coupled receptor that is potently activated by ADP. In an effort to identify ligands for SP1999, fractionated rat spinal cord extracts were assayed for Ca^{2+} mobilization activity against Chinese hamster ovary cells transiently transfected with SP1999 and chimeric Ga subunits (Ga_{q/i}). A substance that selectively activated SP1999-transfected cells was identified and purified through a series of chromatographic steps. Mass spectral analysis of the purified material definitively identified it as ADP. ADP was subsequently shown to inhibit forskolin-stimulated adenyl cyclase activity through selective activation of SP1999 with an EC_{50} of 60 nM. Other nucleotides were able to activate SP1999 with a rank order of potency 2-Cl-ATP = 2-MeS-ATP > ADP = adenosine 5’-O-2-(thiotriphosphate) > ATP-s = adenosine 5’-O-(thiotriphosphate). Thus, SP1999 is a novel, Ga_i-linked receptor for ADP.

Purine and pyrimidine nucleotides are known to modulate a variety of physiological functions by interaction with two types of cell surface receptors: P2X and P2Y receptors (1, 2). P2X receptors are ligand-gated ion-channels, whereas P2Y receptors are G protein-coupled receptors (GPCRs). Five mammalian P2Y receptors have been cloned so far including P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11 (2, 3). All of these receptors share high degree of sequence homology with each other. P2Y1, P2Y2, and P2Y6 couple to the activation of phospholipase C (PLC), whereas P2Y4 and P2Y11 couple to the activation of both PLC and the adenyl cyclase pathways. P2Y1, P2Y2, and P2Y11 are selectively activated by ATP, whereas P2Y6 is selectively activated by UDP, and P2Y4 can be selectively activated by both ATP and UTP (1-3). Additionally, receptors for ADP have been identified in rat C6 gliona cells and human blood platelets, but these have not been cloned yet (4-6). In C6 cells ADP causes inhibition of adenyl cyclase, whereas in platelets ADP appears to cause both inhibition of cAMP and activation of PLC (4-6).

SP1999 is an orphan G protein-coupled receptor cloned from a human hypothalamus cDNA library (7). Phylogenetic analysis shows that SP1999 shares homology with a group of G protein-coupled receptors, most of which are orphans as well. Its closest known receptors are the recently identified UDP-glucose receptor and the platelet-activating factor receptor (8, 9). In contrast, SP1999 shares little homology with the known P2Y receptors. Nevertheless, the present study demonstrates that SP1999 is a Ga_i-linked receptor that is potently activated by ADP. Using a Ca^{2+} mobilization assay, a substance was identified in fractionated rat spinal cord extracts which specifically activated SP1999 when cotransfected with the chimeric G protein (Ga_{q/i}) (10, 11). The substance was purified to homogeneity and identified as ADP by mass spectrometry. ADP was subsequently shown to inhibit forskolin-stimulated adenyl cyclase activity through selective activation of SP1999 with an EC_{50} of 60 nM. Other nucleotides were able to activate SP1999 with a rank order of potency 2-Cl-ATP = 2-MeS-ATP > ADP > ATP-s > ATP-s > 2-Cl-ATP. Thus, SP1999 is a novel, Ga_i-linked receptor for ADP.

EXPERIMENTAL PROCEDURES

Reagents and Materials—All nucleotides were obtained from either Sigma or RBI. [^{3}H]Adenine (20–40 Ci/mmol, 1 mCi/ml) was from PerkinElmer Life Sciences. Fluo-3-AM and pluronic acid were from Molecular Probes. Cell culture media and reagents were from Life Technologies, Inc. All cloning work was performed according to standard procedures. Scintillation mixture (Ready Safe™) for aqueous sample was obtained from Beckman Coulter. Human chimeric Ga proteins (Ga_{q/xz}, Ga_{q/y}, Ga_{q/x2}, Ga_{q/y2}, Ga_{q/et}, Ga_{q/x12}, Ga_{q/truncated}) were constructed by replacing the five C-terminal residues of human Ga with the five amino acid residues of the corresponding human G protein except that, for Ga_{q/truncated}, the C-terminal five residues of Ga was deleted (11). All chimeric G proteins were cloned into the mammalian expression vector pCR3.1 (Invitrogen).

Cloning and Expression of SP1999—Full-length cDNA of SP1999 was first cloned by Human Genome Sciences Inc. from a human hypothalamus cDNA library. The sequence was disclosed as EBI-2 receptor

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The abbreviations used are: GPCR, G protein-coupled receptor; PLC, phospholipase C; FLIPR, fluorometric image plate reader; HPLC, high performance liquid chromatography; ADP/S, adenosine 5’-O-2-(thiophosphate); ATP-s/S, adenosine 5’-O-(thiotriphosphate); AMP-PCP, adenosine 5’-β,γ-methylene triphosphate; CHO, Chinese hamster ovary; DHFR, dihydrofolate reductase; RT, reverse transcription; PCR, polymerase chain reaction; CV, column volume; GHPDH, glyceraldehyde-3-phosphate dehydrogenase.
in patent WO 98/50549. To avoid confusion with EBI-2 cloned previously (12), we have designated the clone as SP1999. The open reading frame of SP1999 was subcloned into the pcDNA3.1 expression vector (Invitrogen). SP1999 was then transfected into CHO-DHFR or NIH3T3 cells using LipofectAMINE (Life Technologies, Inc.). Stable cell lines were selected by selection under 1 mg/ml G418 (Life Technologies, Inc.) 24 h after transfection.

**Dot Blot and In Situ Hybridization**—To determine the distribution of SP1999 in human tissues, vector primers (T3/T7) were used to amplify a 1.3-kilobase insert from SP1999 plasmid DNA, which was then gel-purified. The purified amplicon was random-prime labeled (Prime-It II, Stratagene) with [32P]dCTP and hybridized overnight at 42 °C with either multiple tissue Northern blots or RNA Master blots (both from CLONTECH). For the RNA Master blots, the hybridization buffer (ExpressHyb, CLONTECH) contained 0.1 mg/ml sheared salmon sperm DNA (Life Technologies, Inc.), 6 µg/ml human C-t-1 DNA, and 2 x 10^5 cpm of probe. Only the probe was added to the Express-Hyb for hybridization with the Northern blots. The following day, the blots were washed with increasing stringency according to the manufacturer’s protocol, wrapped in Saran Wrap, and exposed to Kodak Biomax MS film for 24–72 h at -70 °C. The films were analyzed for semi-quantitative autoradiography using the M4/MCID image analysis package (Imaging Research).

**RT-PCR in Human Blood Platelets**—Total RNA was isolated from washed human platelets using Qiagen RNeasy Mini Kit. First-strand complementary DNA (cDNA) was synthesized utilizing a gene-specific primer (CD2 is agggagggtgacaacattgactggg, and 3'-primer of CD2 is tactc, and 3'-primer of SP1999 is tgccagactagaccgaactct. 5'-Primer of CD2 is gtcggtgacactagacagcttacte, and 3'-primer of SP1999 is tgcagaactgacacgaactct. 5'-Primer of CD2 is gtcggtgacactagacagcttacte, and 3'-primer of CD2 is agggagtgggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggtggt
as shown in Fig. 2A. The RNA Master blot contains a variety of human tissues including different regions of brain, heart, spleen, lung, etc. As shown in Fig. 2A, hybridization of the SP1999 probe showed very strong signals in all brain regions and spinal cord as well as in fetal brain. The most intense signals were from substantia nigra, putamen, thalamus, and temporal cortex. In addition, weak signals can be observed in the lung, appendix, pituitary, and adrenal gland. The brain distribution of SP1999 was further demonstrated by Northern blot of mRNA from multiple regions of human brain (Fig. 2B).

The Northern blot showed a predominant 3.2-kilobase band across all brain regions with a minor 2.4-kilobase band in some brain regions such as cortex and medulla. To detect the expression of SP1999 in human blood platelets, RT-PCR experiments were performed (Fig. 2C). A specific 140-base pair product for SP1999 was amplified in cDNA from human platelets but not in the cDNA from the human platelets without reverse transcriptase and from human leukocyte, indicating that SP1999 is expressed in human blood platelets. β2-Integrin was used as a negative control here to exclude the possibility of contaminating of human platelets by leukocytes, whereas CD2 and GH-PDH were used as positive control. Therefore, SP1999 is expressed in human brain, spinal cord, and human blood platelets.

Identification of the Ligand for SP1999—To understand the function of SP1999, we set out to identify its endogenous ligand; SP1999 was cotransfected with a mixture of chimeric G protein plasmids encoding Gaq12, Gap16, Goq11, Gaq6, and Goq13.
Gaq/Gai/Gao to CHO-DHFR, whereas empty pCDNA-3.1 was cotransfected with chimeric G protein mixture as negative control. Each G protein chimera used here is a Gaq subunit with its five C-terminal residues substituted by the corresponding residues of other Ga subunits such as Gas, except that, for GaqD5, its five C-terminal residues was deleted (10, 11).

As SP1999 was predominantly expressed in brain tissue and spinal cord, spinal cord was used as raw material for purification. Rat spinal cords (100 g) were extracted according to “Experimental Procedures,” loaded onto a C18 column (Vydac C18, 218TP510), and gradient elution was performed. The chromatogram is shown in Fig. 3A. Each fraction was screened for Ca^{2+} mobilization using CHO-DHFR cells transfected with SP1999 and the chimeric G proteins using the FLIPR assay. Using different dilutions of the fractions, it was possible to identify regions exhibiting activity only in the SP1999-transfected cells (Fig. 3A). Furthermore, this SP1999 specific activity required the coexpression of chimeric G proteins. Two peaks (fractions 19–23 and fractions 30–35) were identified that activated SP1999 specifically. These two peaks were later shown to be identical (data not shown). Subsequent experiments using chi-
meric G proteins transfected individually with SP1999 indicated that the $G_{aq/i3}$ chimera provided the most robust response (data not shown). The pool of fractions 30–35 was subsequently purified further by cation-exchange (SP8HR), anion exchange (Mono Q), anion exchange (DEAE), reverse phase (C18 column), and size exclusion (Superdex HR10/30). Fig. 3B

![Diagram](image)

**Fig. 3. Purification of endogenous ligand for SP1999.** A, reverse-phase chromatography of crude extracts from rat spinal cord. Acetonitrile gradient and absorbance of eluted materials are indicated by dotted and solid lines. Fractions were assayed using CHO-DHFR cells transfected with SP1999 and Go$_{aq/i3}$ by FLIPR (see “Experimental Procedures”); regions of specific activity are indicated by bar. B, size-exclusion chromatography. Active fractions from C18 chromatography (see “Experimental Procedures”) were finally purified by size-exclusion chromatography. Fractions were assayed as described. All activity was contained in peak A. C, mass spectrum of the purified material. The pooled fractions of peak A from size-exclusion chromatography were analyzed by electrospray ionization mass spectrometry in negative mode. The individual molecular ion was further analyzed by fragmentation using electrospray ionization mass spectrometry/mass spectrometry and the suggested structures are shown next to the ions.
A SP1999 or the vector pCDNA3.1 was cotransfected with chimeric G protein coupling specificity of SP1999, single chimeric G protein G\textsubscript{q/o} into CHO-DHFR\textsuperscript{a}, and Ca\textsuperscript{2+} mobilization of transfected cells in response to increasing doses of ADP was measured by FLIPR. B, ADP dose response in NIH3T3 cells. SP1999 or the vector pCDNA3.1 was cotransfected with or without chimeric G\textsubscript{q/i}, into NIH3T3 cells, and ADP dose response was measured as described.

Fig. 4. ADP dose response. Dose-response curves are from a representative experiment. A, ADP dose response in CHO-DHFR\textsuperscript{a} cells. SP1999 or the vector pCDNA3.1 was cotransfected with chimeric G\textsubscript{q/i} into CHO-DHFR\textsuperscript{a}, and Ca\textsuperscript{2+} mobilization of transfected cells in response to ADP dose response in NIH3T3 cells. SP1999 or the vector pCDNA3.1 was cotransfected with or without chimeric G\textsubscript{q/i} into NIH3T3 cells, and ADP dose response was measured as described.

Consistent with the structure of 5'-ADP, fragmentation of the ions suggest that the ion at m/z 426.1 is a dimer of trifluoroacetic acid (trifluoroacetic acid, formula weight = 114), and the m/z 249.0 peak is an adduct of the trifluoroacetic acid dimer and a sodium ion. Since the sample was purified by reverse phase HPLC in the presence of trifluoroacetic acid, it is not surprising to observe the adduct peaks at m/z 270.0 and 249.0 peaks. Trifluoroacetic acid has been shown to be inactive at SP1999 (data not shown); thus, 5'-ADP appears to be the active component in the sample. When commercially available 5'-ADP was compared with the purified sample, the activities were shown to be identical. Using the CHO cells cotransfected with SP1999 and chimeric G\textsubscript{q/o}, the dose response to ADP was examined, and an EC\textsubscript{50} of 330 nM was observed (Fig. 4A). However, ADP also caused significant

Fig. 5. Signal pathway of SP1999. Dose-response curves are from a representative experiment. A, G protein coupling of SP1999. SP1999 (5 \muM) was transiently cotransfected to NIH3T3 cells with 0.5 \muM each of human chimeric G protein G\textsubscript{q/o}, G\textsubscript{q/s}, G\textsubscript{q/z}, G\textsubscript{q/12}, or G\textsubscript{q/o}, and G\textsubscript{o}. The response to ADP (300 nM) was then measured by FLIPR. B, inhibited without chimeric G protein. B, inhibition of cAMP by SP1999. SP1999 stably transfected CHO-DHFR\textsuperscript{a} cells were first labeled with 5 \muCi/ml \[^{3}H\]adenine, then incubated with 50 \muM forskolin and the indicated amount of ADP. The \[^{3}H\]cAMP generated was purified using Dowex and alumina chromatography and quantitated by scintillation counter.

Ca\textsuperscript{2+} mobilization in pCDNA and G\textsubscript{q/i3} transfected CHO cells, with an EC\textsubscript{50} of 14 \muM (Fig. 4A). The same Ca\textsuperscript{2+} mobilization response was also observed in untransfected, wild type CHO cells (data not shown). To avoid the Ca\textsuperscript{2+} response to ADP inherent in the CHO cells, the activity of ADP was tested on several cell lines. It was found that NIH3T3 cells were devoid of endogenous ADP responses in the FLIPR assay. However, when transiently transfected with SP1999 and chimeric G\textsubscript{q/i3}, ADP potently stimulated Ca\textsuperscript{2+} mobilization with an EC\textsubscript{50} of 74 nM (Fig. 4B). The discrepancy in the EC\textsubscript{50} values for ADP in the two cell lines is likely due to the addition of the endogenous ADP response observed in the CHO cells.

Intracellular Signaling Pathway of SP1999—To confirm the G protein coupling specificity of SP1999, single chimeric G proteins were cotransfected with SP1999 into NIH3T3 cells, and the response to 300 nM ADP was then measured by FLIPR. As shown in Fig. 5A, strong Ca\textsuperscript{2+} flux signals were observed for cells transfected with SP1999 and G\textsubscript{q/i3}, G\textsubscript{q/s}, or G\textsubscript{q/o} while much weaker signals were observed for cells transfected with SP1999 and all other chimeric G proteins. These results suggest that SP1999 should normally couple to G\textsubscript{o} proteins of the G\textsubscript{o} class (10, 11).

The coupling between SP1999 and the G\textsubscript{o} proteins also suggests that ADP should act to inhibit the activity of adenyl cyclase in SP1999-transfected cells (11, 15). To confirm this prediction, a cAMP assay was performed using CHO-DHFR\textsuperscript{a} cells stably transfected with SP1999. To measure cAMP, the cells were first labeled with \[^{3}H\]adenine, then the \[^{3}H\]cAMP generated after ADP stimulation was purified by column chromatography and quantitated by scintillation spectrometry (13).
ADP is only observed when the cells are transfected with both P2Y6, and P2Y11 receptors in that ADP is the most potent of the naturally occurring nucleotides at the P2Y1 receptor. Although ADP is also the most potent of the naturally occurring nucleotides for P2Y2, P2Y4, P2Y6, and P2Y11 receptors, ATP, UTP, or UDP (2). Although ADP is also the most potent of the naturally occurring nucleotides at the P2Y1 receptor, P2Y1 couples to Gα, while SP1999 couples to Goi. Although we currently cannot rule out an interaction of ATP, UTP, ITP, or Ap4A with SP1999 at UDP (UDP was found to be inactive), these interactions must be of low affinity (EC50 > 10 μM) as their background EC50 values in NIH3T3 cells are larger than 10 μM.

Despite the overall low sequence homology with the known nucleotide receptors, SP1999 does contain several amino acid residues that are conserved in the P2Y family, and which have previously been implicated in nucleotide binding. Amino acids Phe-105, Tyr-106, Tyr-110, Phe-198, His-253, Arg-256, and Ser-288 are identical to residues highly conserved among the P2Y1, P2Y2, P2Y4, and P2Y6 receptors. Through the use of molecular modeling and site-directed mutagenesis, these residues have been shown to be involved in the binding of purinergic ligands to the P2Y1 receptor. Although several residues found to be absolutely required for ligand interaction at the P2Y1 receptor (Arg-128 and Arg-310 in P2Y1) are not present in SP1999, the conservation of the residues mentioned above supports the finding that ADP can act as a ligand at this receptor.

ADP receptors have been identified in both C6 glioma cell lines and blood platelets but have not been cloned (5, 6). In rat C6 glioma cells, an ADP receptor has been described to couple to Goi. The order of its agonist potency was 2-MeSATP > 2-MeS-ADP > ADP > ATP, which is very similar to that of SP1999. There are also two different G protein-coupled ADP receptors identified

**DISCUSSION**

The results of the present study clearly indicate that SP1999 is a high affinity receptor for ADP, despite the fact that SP1999 does not possess a particularly high degree of homology with the known purinergic receptors. It is unlikely that the results presented here are due to the unsuspected expression of a known purinergic receptor, since the profile of activity observed exhibits several properties that distinguish it from the previously cloned P2 purinergic receptors. Notably, the responses presented exhibit a unique pharmacological and second messenger profile and are only observed in conjunction with expression of transfected SP1999. Although the previously cloned P2 receptors are all capable of coupling through Goi to activation of phospholipase C and Ca2+ mobilization, SP1999 couples only to the Goi class of G proteins to inhibit adenylate cyclase activity. In NIH3T3 cells, Ca2+ mobilization in response to ADP is only observed when the cells are transfected with both SP1999 and a chimeric G protein of the Goi class (Gαi, Goi, or Gi). Pharmacologically, SP1999 differs from the P2Y2, P2Y4, P2Y6, and P2Y11 receptors in that ADP is the most potent of the compounds tested at SP1999. The rank order of potency is 2-MeS-ATP > 2-MeS-ADP > ADP > ATP-S. Table II lists the nucleotides that were not active at SP1999 at concentrations up to 8 μM. The nucleotides ATP, UTP, ITP, and Ap4A exhibited activity in wild-type NIH3T3 cells, and with EC50 values of about 15 μM. The ability of the compounds active at SP1999 to inhibit cAMP in SP1999-transfected CHO cells was also examined. With the exception of ATP-S, the rank order of EC50 values is comparable to those determined using the FLIPR assay. Three compounds were also identified that were able to antagonize the activity of ADP at SP1999: reactive blue-2, suramin, and 2-MeS-ATP. The Kᵢ for reactive blue-2, suramin, and 2-MeS-ADP in the presence of 200 nM ADP were 1.3, 3.6, and 10 μM, respectively. Reactive blue-2 and suramin have been shown to be nonselective antagonists for P2Y receptors (2).
in blood platelets. One ADP receptor, P2TAC, couples to the inhibition of adenylyl cyclase; another ADP receptor, P2Y1, couples to mobilization of intracellular calcium stores through inositol phosphate production (5). Since SP1999 couples to the inhibition of adenylyl cyclase and the pharmacological profiles between SP1999 and P2TAC are also very similar and, moreover, SP1999 mRNA was found in platelets, SP1999 may also be a candidate for the uncloned P2TAC receptors. However, to confirm whether SP1999 is the ADP receptor identified in C6 cells or blood platelets, further experiments must be done.

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Note added in Proof—While this paper was in press, G. Hollopeter et al. published a paper (Hollopeter, G., Jantzen, H.-M., Vincent, D., Li, G., England, L., Ramakrishnan, V., Yang, R.-B., Nurden, P., Julius, D., and Conley, P. B. (2001) Nature 409, 202–207). The receptor P2Y12 in the paper is identical to SP1999.

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