

# A Mutational Analysis of Residues Essential for Ligand Recognition at the Human P2Y<sub>1</sub> Receptor

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## SUMMARY

We conducted a mutational analysis of residues potentially involved in the adenine nucleotide binding pocket of the human P2Y<sub>1</sub> receptor. Mutated receptors were expressed in COS-7 cells with an epitope tag that permitted confirmation of expression in the plasma membrane, and agonist-promoted inositol phosphate accumulation was assessed as a measure of receptor activity. Residues in transmembrane helical domains (TMs) 3, 5, 6, and 7 predicted by molecular modeling to be involved in ligand recognition were replaced with alanine and, in some cases, by other amino acids. The potent P2Y<sub>1</sub> receptor agonist 2-methylthio-ATP (2-MeSATP) had no activity in cells expressing the R128A, R310A, and S314A mutant receptors, and a markedly reduced potency of 2-MeSATP was observed with the K280A and Q307A mutants. These results suggest that residues on the exofacial side of TM3 and TM7 are critical determinants of the ATP binding pocket. In contrast, there was no change in the potency or maximal effect of 2-MeSATP with the S317A mutant receptor. Alanine replacement of F131,

H132, Y136, F226, or H277 resulted in mutant receptors that exhibited a 7–18-fold reduction in potency compared with that observed with the wild-type receptor. These residues thus seem to subserve a less important modulatory role in ligand binding to the P2Y<sub>1</sub> receptor. Because changes in the potency of 2-methylthio-ADP and 2-(hexylthio)-AMP paralleled the changes in potency of 2-MeSATP at these mutant receptors, the  $\beta$ - and  $\gamma$ -phosphates of the adenine nucleotides seem to be less important than the  $\alpha$ -phosphate in ligand/P2Y<sub>1</sub> receptor interactions. However, T221A and T222A mutant receptors exhibited much larger reductions in triphosphate (89- and 33-fold versus wild-type receptors, respectively) than in diphosphate or monophosphate potency. This result may be indicative of a greater role of these TM5 residues in  $\gamma$ -phosphate recognition. Taken together, the results suggest that the adenosine and  $\alpha$ -phosphate moieties of ATP bind to critical residues in TM3 and TM7 on the exofacial side of the human P2Y<sub>1</sub> receptor.

Extracellular ATP acts via membrane-bound receptors as a neurotransmitter in the central and peripheral nervous systems and as a regulator of vascular and smooth muscle tone (1). Two pharmacologically distinct families of ATP receptors, the P2X receptor class of ligand-gated ion channels and the P2Y receptor class of GPCRs, have been described (2, 3). As many as seven subtypes have been cloned within each family.<sup>1</sup>

P2Y receptors are members of the superfamily of rhodopsin-like GPCRs. Many other members of this superfamily, including adenosine receptors, have been probed using site-directed mutagenesis (4–10). Among P2Y receptors, only the

P2Y<sub>2</sub> receptor (11), which binds both purine and pyrimidine nucleotides, has been studied using mutagenesis. The P2Y<sub>1</sub> receptor subtype is a phospholipase C-activating ATP receptor present in heart, skeletal muscle, and various smooth muscles. Both P2Y<sub>1</sub> (12) and P2Y<sub>2</sub> (11, 13) receptors have been modeled using rhodopsin (14–16)<sup>2</sup> or bacteriorhodopsin (17), respectively, as a template. There are substantial pharmacological differences between these two subtypes, and the overall percent identity of amino acids in the human sequences is only 38%.

In this study, we attempted to characterize the determinants of ligand recognition in the family of P2Y receptors by

<sup>1</sup> P2Y<sub>1</sub>-P2Y<sub>7</sub> sequences have been cloned and named in the literature (3), however, several of these may be either receptors of a different family (e.g., P2Y<sub>5</sub> and possibly P2Y<sub>7</sub>) or possibly species homologues (e.g., P2Y<sub>3</sub> and P2Y<sub>6</sub>). The identity and relationship among these clones require further study.

<sup>2</sup> The coordinates of the P2Y<sub>1</sub> receptor model are available from the protein database (Brookhaven National Laboratory) using the URL <http://www.pdb.bnl.gov/cgi-bin/browse> under the ID code 1ddd.

**ABBREVIATIONS:** 2-MeSATP, 2-methylthio-ATP; 2-MeSADP, 2-methylthio-ADP; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; HA, hemagglutinin; HT-AMP, 2-(hexylthio)adenosine-5'-monophosphate; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; TM, (helical) transmembrane domain; PPADS, pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid.

site-directed mutagenesis. Activation of phospholipase C by three nucleotide ligands was used as an indicator of receptor function. We explored the role of positively charged and conserved residues in TM3, TM6, and TM7 and found that residues<sup>3</sup> R128(3.29), R310(7.39), and S314(7.43) are critical for the binding of nucleotides to human P2Y<sub>1</sub> receptors.

A long-range goal of this investigation is the design of more selective pharmacological agents based on structural differences in receptors. A limitation in the study of P<sub>2</sub> receptors is the lack of high affinity antagonist radioligands. Although no specific, high affinity agonists or antagonists are available for the cloned subtypes, we previously introduced agonist probes that reveal pharmacological heterogeneity within the two superfamilies (18–20). One such agonist used in this study is HT-AMP (20), which activates P2Y<sub>1</sub> but not P2Y<sub>2</sub> receptors with a potency greater than that of the endogenous agonist ATP. The availability of a potent AMP derivative allowed us to compare various agonists to probe the phosphate binding region of the receptor.

## Experimental Procedures

**Materials.** The human P2Y<sub>1</sub> receptor cDNA (pcDNA3P2Y<sub>1</sub>) was prepared as previously described (21). KlenTaq polymerase for the PCR was purchased from Clontech (Palo Alto, CA). All endonuclease restriction enzymes used in this study were obtained from New England Biolabs (Beverly, MA). The agonists 2-MeSATP and 2-MeSADP were from RBI (Natick, MA). The agonist HT-AMP was synthesized as previously described (18, 20) as the ammonium salt, which was more soluble in aqueous medium than the triethylammonium salt. The antagonists suramin and PPADS were from Tocris Cookson (St. Louis, MO). Reactive Blue 2 was from Aldrich (Milwaukee, WI). *myo*-[<sup>3</sup>H]inositol (15 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). FBS and *o*-phenylenediamine dihydrochloride were purchased from Sigma Chemical (St. Louis, MO). The Sequenase Kit, ATP, and cAMP were from United States Biochemical (Cleveland, OH). All oligonucleotides were synthesized by Bioserve Biotechnologies (Laurel, MD). A monoclonal antibody (12CA5) against an HA epitope was purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN), and goat anti-mouse IgG ( $\gamma$ -chain specific) antibody conjugated with horseradish peroxidase was purchased from Sigma. DEAE-dextran was obtained from Pharmacia-LKB (Piscataway, NJ).

**Plasmid construction and site-directed mutagenesis.** The coding region of pcDNA3P2Y<sub>1</sub> was subcloned into the pCD-PS expression vector (22), yielding pCDP2Y<sub>1</sub>. All mutations were introduced into pCDP2Y<sub>1</sub> using standard PCR mutagenesis techniques (23). The accuracy of all PCR-derived sequences was confirmed by dideoxy sequencing of the mutant plasmids (24).

**Epitope tagging.** A 9-amino acid sequence derived from the influenza virus HA protein (TAC CCA TAC GAC GTG CCA GAC TAC GCG; peptide sequence, YPYDVPDYA) was inserted after the M residue at the extracellular amino terminus of the human P2Y<sub>1</sub> ATP receptor gene (21). Oligonucleotides containing the HA-tag sequence were designed and used to generate a PCR fragment, which was then used to replace the corresponding wild-type P2Y<sub>1</sub> sequence. A HexaHis tag (25) was also included at the carboxyl terminus immediately after the L residue, resulting in a construct suitable for potential affinity chromatography using a nickel column.

**Transient expression of mutant receptors in COS-7 cells.** COS-7 cells ( $2 \times 10^6$ ) were seeded onto 100-mm culture dishes

containing 10 ml of DMEM supplemented with 10% FBS. Cells were transfected ~24 hr later with plasmid DNA (4  $\mu$ g of DNA/dish) according to the DEAE-dextran method (26) and grown for an additional 72 hr at 37°.

**Inositol phosphate determination.** COS-7 cells were subcultured into 12  $\times$  100-mm dishes (Falcon) at a density of  $\sim 1 \times 10^6$  cells/dish. After overnight incubation at 37° and 5% CO<sub>2</sub>, the cells were transfected with plasmids containing receptor-coding sequence using the DEAE-Dextran method for 40 min, followed by treatment with 100  $\mu$ M chloroquine for 2.5 hr. Typically, two dishes of COS-7 cells were transfected for each mutant tested.

The assay was carried out according to the general approach of Harden *et al.* (27). At ~24 hr after transfection, the cells were split into six-well plates ( $\sim 0.75 \times 10^6$  cells/well; Costar, Cambridge, MA) in DMEM culture medium (plus 100 units/ml of penicillin, 100  $\mu$ g/ml streptomycin, 2  $\mu$ mol/ml glutamate, and 10% fetal calf serum) supplemented with 3  $\mu$ Ci/ml of *myo*-[<sup>3</sup>H]inositol. After a 24-hr labeling period, cells were preincubated for 30 min at 37° with 10 mM LiCl. The mixtures were swirled to ensure uniformity before they were allowed to stand for 20 min at room temperature. 2-MeSATP was added, and the cells were incubated for 30 min at 37° and 5% CO<sub>2</sub>. The supernatant was removed by aspiration, and 750  $\mu$ l of cold 20 mM formic acid was added to each well. Cell extracts were collected after a 30-min incubation at 4° and neutralized with 250  $\mu$ l of 60 mM NH<sub>4</sub>OH. The inositol monophosphate fraction was then isolated by anion exchange chromatography (28). The contents of each well was applied to a small anion exchange column (AG-1-X8; BioRad, Hercules, CA) that had been pretreated with 15 ml of 0.1 M formic acid/3 M ammonium formate, followed by 15 ml of water. The columns were then washed with 15 ml of a solution containing 5 mM sodium borate and 60 mM sodium formate. [<sup>3</sup>H]Inositol phosphates were eluted with 4.5 ml of 0.1 M formic acid/0.2 M ammonium formate and quantified by liquid scintillation counting (LKB Wallac 1215 Rack-beta scintillation counter).

Pharmacological parameters were analyzed using KaleidaGraph (version 3.01; Abelbeck/Synergy Software, Reading, PA). Statistical analysis was performed using the Alternate *t* test (InStat version 2.04; GraphPAD, San Diego, CA).

**ELISA.** For indirect cellular ELISA measurements, cells were transferred to 96-well dishes ( $4\text{--}5 \times 10^4$  cells/well) at 1 day after transfection. At ~72 hr after transfection, cells were fixed in 4% formaldehyde in PBS for 30 min at room temperature. After washing three times with PBS and blocking with DMEM (containing 10% FBS), cells were incubated with HA specific monoclonal antibody (12CA5; 20  $\mu$ g/ml) for 3 hr at 37°. Plates were washed and incubated with a 1:2000 dilution of a peroxidase-conjugated goat anti-mouse IgG antibody (Sigma) for 1 hr at 37°. Hydrogen peroxide and *o*-phenylenediamine (each 2.5 mM in 0.1 M phosphate-citrate buffer, pH 5.0) served as substrate and chromogen, respectively. The enzymatic reaction was stopped after 30 min at room temperature with 1 M H<sub>2</sub>SO<sub>4</sub> solution containing 0.05 M Na<sub>2</sub>SO<sub>3</sub>, and the color development was measured bichromatically with a BioKinetics reader (EL 312; Bio Tek Instruments, Winooski, VT) at 490 and 630 nm (baseline).

## Results

Sequence alignments for selected transmembrane regions of P2Y receptors and other G protein-coupled receptors are shown in Fig. 1. The residues of the human P2Y<sub>1</sub> receptor selected as targets for site-directed mutagenesis are shown in bold; they include the positively charged residues R128, K280, and R310 in TM3, TM6, and TM7, respectively, which also appear in some but not all of the other P2Y sequences. K280 and R310 were suggested in our modeling study of the chick P2Y<sub>1</sub> receptor (12)<sup>2</sup> to be involved in the binding of the phosphate moiety of nucleotides. Mutated residues also in-

<sup>3</sup> Residue identifiers as described in van Rhee and Jacobson (4) specify the helix (X) and the position relative to a key conserved residue in that helix, which is designated (X0.50). For example, for the helices mutated in the present study, the key conserved residues are R149(3.50), P229(5.50), P275(6.50), and P321(7.50).

TM3					
		128	131	132	136
P2Y <sub>1</sub>	125	KLQ <b>R</b> F <b>I</b> F <b>H</b> VNLYGSILFLTCISAHRY	150		
P2Y <sub>2</sub>	107	KLVRFLFYTNLYCSILFLTCISVHRC	132		
P2Y <sub>3</sub>		KFVRFQFYTNLHGSILFLTCISVQRY			
P2Y <sub>4</sub>		KFVRFLFYWNLYCSVLFLTCISVHRY			
P2Y <sub>6</sub>		RLVRFLFYANLHGSILFLTCISFQRY			
AT1a	102	KIASASVSFNLYASVLLTCLSIDRY	127		
β <sub>2</sub>	107	EFWTSIDVLCVTASIELTLCVIAVDY	132		
m3ACh	142	EFWTSIDVLCVTASIELTLCVIAVDY	167		
A2a	78	LFIACFVLVL <b>T</b> QSSIFSLLAIAIDRY	103		
TM5					
		221	222	226	
P2Y <sub>1</sub>	215	FIYSMC <b>T</b> <b>T</b> VAM <b>F</b> CVPLVLILGCYGLIVRA	243		
P2Y <sub>2</sub>	195	VAYSSVMLGLLFAVPFAVILVCYVLMARR	223		
P2Y <sub>3</sub>		FPYGITLTITGFLLPFAAILACYCSMARI			
P2Y <sub>4</sub>		VHFSSAVMGLLFGVPCLVTLVCYGLMARR			
P2Y <sub>6</sub>		MPYGMALTIVIGFLLPFAALLACYCLLACR			
AT1a	193	IGLGLTKNILGFLFPFLIILTSYTLIWKA	221		
β <sub>2</sub>	197	QAYAIASSIVSFYVPLVIMVFVYSRVFQE	225		
m3ACh	229	PTITFGTAIAAFYMPVTIMTILYWRIYKE	257		
A2a	175	NYMVYFN <b>F</b> FACVLVPLLLMLGVYLRIFLA	203		
TM6					
		277	280		
P2Y <sub>1</sub>	260	YLVIIVLTVFAVSYP <b>F</b> HVM <b>K</b> TMNLRAR	287		
P2Y <sub>2</sub>	244	RTIAVVLAVFALCFLP <b>F</b> HV <b>R</b> TLYYSF	271		
P2Y <sub>3</sub>		RMIIIVVIVFSISFFPFHLTKTIYLIVR			
P2Y <sub>4</sub>		RTIAVVLTVFAVCFVPFHITRTIYYLAR			
P2Y <sub>6</sub>		RMAVVVAAFAISFLPFHITKTAYLAVR			
AT1a	240	KIIMAIVLFFFSWIPHQIFTFDLVLIQ	267		
β <sub>2</sub>	273	KTLGIIMGTFTLCWLPPFIVNIVHVIQD	300		
m3ACh	491	QTLSAILLAFIITWTPYNIMVLVNTFCD	518		
A2a	233	KSLAIIIVGLFALCWLPL <b>H</b> II <b>N</b> CFT <b>F</b> FCP	260		
TM7					
		307	310	314	317
P2Y <sub>1</sub>	300	DRVYATY <b>Q</b> V <b>T</b> RGLAS <b>L</b> NSCVDPILYFLAGDTFR	332		
P2Y <sub>2</sub>	281	NAINMAY <b>K</b> V <b>T</b> RPLASANSCLDPVLYFLAGQRLV	313		
P2Y <sub>3</sub>		QAFAIAYKCTRPFASMNSVLDPILFYFTQKKFR			
P2Y <sub>4</sub>		NIVNVVYKVTRPLASANSCLDPVLYLLTGDKYR			
P2Y <sub>6</sub>		AAVRTMYPITLCIAVANSVLDPILFYFTQKKFR			
AT1a	278	DIVDTAMPITICIAFYFNNCLNPLFYGFLGKKFK	310		
β <sub>2</sub>	302	LIRKEVYILLNWIGYVNSGFNPLIYCRSPDFRI	334		
m3ACh	520	CIPKTFWNLGWLCYINSTVNPVCYALCNKTFR	552		
A2a	264	HAPLWLW <b>M</b> YLA <b>I</b> VLS <b>H</b> TNSVVNPFYIAYRIREFR	296		

**Fig. 1.** Location of mutations of the human P2Y<sub>1</sub> receptor (*bold*) and alignment of the transmembrane domains of selected GPCRs for purines/pyrimidines (P2Y and adenosine), biogenic amines (β-adrenergic, muscarinic cholinergic), and peptides (angiotensin). Sites mutated in the P2Y<sub>2</sub> receptor by Erb *et al.* (11) and sites in the human A<sub>2A</sub> adenosine receptor found to be involved in ligand recognition by Kim *et al.* (5) are also *bold*. All are human sequences except P2Y<sub>3</sub> (chicken). Accession numbers are P2Y<sub>1</sub>, U42030; P2Y<sub>2</sub>, U07225; P2Y<sub>3</sub>, X98283; P2Y<sub>4</sub>, X91852; P2Y<sub>6</sub>, X97058; AT1A, M93394; A2A, U40771; m3ACh, U29589; β<sub>2</sub>, J02960.

clude F226 and S317, which are highly conserved among GPCRs. Another mutated residue, H277, is conserved among six of the seven known P2Y receptor sequences and among three of the four adenosine receptor sequences. Other mutated residues include H132, T221, and Q307, which are unique to P2Y<sub>1</sub> receptors within the P2Y class of GPCRs. In addition, other uncharged residues targeted for mutagenesis that have been predicted through molecular modeling to be in proximity to the ligand binding site are: F131 and Y136 in TM3, T222 in TM5, and Q307 and S314 in TM7. Each of these amino acid residues was individually replaced with alanine and/or other amino acids (see below). In addition, each mutant contained an epitope-tag sequence included at the amino terminus for immunological detection (see below) and a hexa-His tag at the carboxyl terminus, potentially for future purification using Ni-affinity chromatography (25). The pharmacological properties were compared with those of the wild-type receptor that was similarly modified.

**ELISA assay for receptors located at the cell surface.** Mutant P2Y<sub>1</sub> receptors were detected immunologically at the

plasma membrane surface by virtue of the HA-tag and the 12CA5 monoclonal antibody. This assay is specific for receptor proteins in which the amino-terminal sequence is accessible to the extracellular medium (i.e., of the proper orientation in the plasma membrane). This ELISA procedure does not interfere with the intactness of the plasma membrane barrier (5). To estimate approximate levels of receptor protein present in the plasma membrane, a standard curve was constructed from different batches of transfected COS-7 cells expressing different levels of HA-tagged P2Y<sub>1</sub> wild-type receptors (see Experimental Procedures; Ref. 5).

Table 1 shows that the mutant receptors were expressed on the cell surface using ELISA (5). Expression levels for the various mutants (HA-tagged wild-type receptor = 100%) determined by this method (eight experiments) ranged from 50% to 260% (Table 1). The combination of ELISA and the functional assays discussed below indicates which residues are important, either directly for activation of the receptor or indirectly for the high affinity binding of agonists.

TABLE 1

**ELISA detection of human P2Y<sub>1</sub> receptor mutants on the surface of COS-7 cells.**

Data are presented as means  $\pm$  standard deviation of eight independent determinations. Table shows expression level as percentage of Ha-tagged P2Y<sub>1</sub> wild-type (100%). Expression level was determined using a standard curve (5).

Construct	Expression
	%
Wild-type	100
COS-7 (control)	0
R128A	263 $\pm$ 4
F131A	150 $\pm$ 96
H132A	57.2 $\pm$ 4.7
Y136A	50.6 $\pm$ 32.5
T221A	87.2 $\pm$ 16.9
T222A	123 $\pm$ 31
F226A	144 $\pm$ 42
H277A	129 $\pm$ 6
K280A	148 $\pm$ 13
Q307A	151 $\pm$ 9
R310A	132 $\pm$ 9
R310K	188 $\pm$ 40
R310S/S314R	114 $\pm$ 5
S314A	125 $\pm$ 5
S314T	111 $\pm$ 56
S317A	106 $\pm$ 23

**Functional assay.** Agonist-promoted production of inositol phosphates was measured to determine whether mutant receptors were functionally active (Table 2). COS-7 cells transiently expressing wild-type human P2Y<sub>1</sub> receptors displayed a substantial increase of inositol phosphates in response to the potent agonist 2-MeSATP ( $EC_{50}$  = 6.75 nM), which was not observed in control cells (Fig. 2). At the highest concentration of the agonist tested (1 mM), there was a slight increase of inositol phosphates in control COS-7 cells, presumably from an endogenous nucleotide receptor. The difference in basal inositol phosphate levels of the various expressed receptors occurs due to release of endogenous ATP and concomitant differential activation of receptors that are

sensitive to activation by low concentrations of nucleotide versus mutant receptors that are only activated by higher concentrations of nucleotide. The broad concentration-effect curves reflect both the noise introduced by a limited number of data points and the fact that the assays were for a length of time (30 min) during which agonist-induced desensitization of the inositol lipid signaling response occurred.

The most critical residues for activation of phospholipase C by 2-MeSATP were found in TM3, TM6, and TM7 (Fig. 3). The ability of the agonist 2-MeSATP to stimulate production of inositol phosphates at the R128A, R310A, and S314A mutant receptors was virtually abolished because at 1 mM 2-MeSATP, <20% of the stimulation found in mutant receptors was observed. The ligand recognition properties of the S314A mutant could be rescued substantially by substitution with T instead of A (Fig. 2B). Thus, the potency of 2-MeSATP at S314T mutant receptors was only 8.9-fold reduced compared with wild-type receptors. The effect of substituting R310 with another positively charged residue, K, was tested. The resulting R310K mutant receptor remained poorly responsive to agonist, with 2-MeSATP 680-fold less potent than at wild-type receptors. An attempt to rescue the activity in an R310 mutant with the double revertant mutant R310S/S314R resulted in a nonfunctional receptor (Fig. 2B).

The K280A and Q307A mutant receptors displayed greatly diminished potency with 2-MeSATP (950- and 210-fold less, respectively) compared with wild-type receptors, although the receptors were well expressed (Table 1). In contrast, replacement of other amino acids, located in TMs 3, 5, 6, and 7 (but more distal from the exofacial side than the above critical residues as shown in Fig. 3) did not have as pronounced an effect on agonist activation of phospholipase C (Table 2). The maximal effect and potency of 2-MeSATP at the S317A mutant receptor were identical to those observed with the native receptor. Moreover, basal activity was unchanged from wild-type receptors, whereas other mutant receptors showed lower basal activity levels. Alanine substitu-

TABLE 2

**Activation of phospholipase C by mutant human P2Y<sub>1</sub> receptors.**

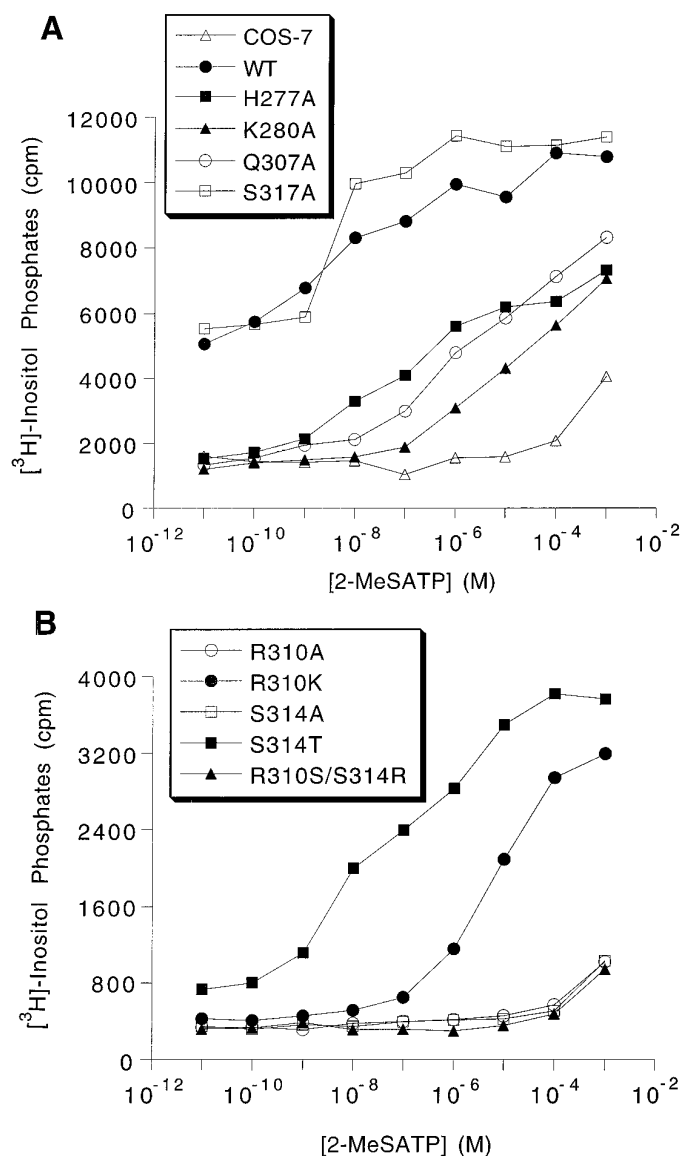
Data are presented as means  $\pm$  standard deviation of two to four independent experiments, each performed in duplicate. All constructs contain the Ha-epitope tag sequence at the amino terminus (5) and a hexa-His tag at the carboxyl terminus (25).

Construct	Residue <sup>a</sup>	EC <sub>50</sub>		
		2MeSATP	2MeSADP	HT-AMP
			<i>nM</i>	
Wild-type		6.75 ± 2.58	1.27 ± 0.01	355 ± 4
R128A	3.29	<i>b</i>	<i>b</i>	<i>b</i>
F131A	3.32	44.2 ± 15.9	18.2 ± 2.1	5260 ± 2200
H132A	3.33	79.9 ± 29.6	15.9 ± 4.4	2390 ± 600
Y136A	3.37	119 ± 103	12.6 ± 1.8	1720 ± 690
T221A	5.42	604 ± 456	15.9 ± 4.0	1810 ± 280
T222A	5.43	221 ± 58	11.2 ± 0.6	1210 ± 210
F226A	5.47	56.8 ± 7.5	20.7 ± 3.8	1850 ± 800
H277A	6.52	115 ± 28	100 ± 30	4520 ± 640
K280A	6.55	6400 ± 1380	1790 ± 310	203,000 ± 36,000
Q307A	7.36	1400 ± 300	455 ± 140	43,000 ± 600
R310A	7.39	<i>b</i>	<i>b</i>	<i>b</i>
R310K	7.39	4580 ± 430	418 ± 118	13,400 ± 1700
R310S/S314R	7.39/7.43	<i>b</i>	<i>b</i>	<i>b</i>
S314A	7.43	<i>b</i>	<i>b</i>	<i>b</i>
S314T	7.43	60.0 ± 18.0	11.7 ± 1.8	1950 ± 440
S317A	7.46	8.00 ± 1.78	1.58 ± 0.29	611 ± 52

<sup>a</sup> Using the sequence identifier as defined in footnote 3 (4).

<sup>b</sup> All agonists at the highest concentrations caused only a small increase or no change in inositol phosphates.





**Fig. 2.** Stimulation of phospholipase C in COS-7 cells transiently expressing wild-type HA-tagged-hP2Y<sub>1</sub> or mutant hP2Y<sub>1</sub> receptors (A, alanine substitutions; B, nonalanine substitutions). Transfected COS-7 cells were incubated for 30 min at 37° (see Experimental Procedures for details). Data are presented as absolute accumulations of tritiated inositol phosphates above basal levels in the absence of 2-MeSATP for a representative experiment. EC<sub>50</sub> values (average of two to four independent experiments, each carried out in duplicate) are given in Table 2.

tion at residues F131, H132, Y136, T221, T222, F226, and H277 resulted in intermediate potency of the agonist, suggesting a modulatory and less critical role of those residues in ligand binding. The potency of 2-MeSATP was reduced compared with wild-type receptors by 15–100-fold in Y136, T221, T222, and H277 mutant receptors and by 3–15-fold in F131A, H132A, and F226A mutant receptors.

The potencies of the agonists 2-MeSADP and HT-AMP were also measured in wild-type and mutant receptors. HT-AMP was selected as the monophosphate to be tested in this comparison rather than 2-MeSAMP to allow precise determination of potency in the functional assay. The extended chain at the 2-position enhanced potency over simple AMP ana-

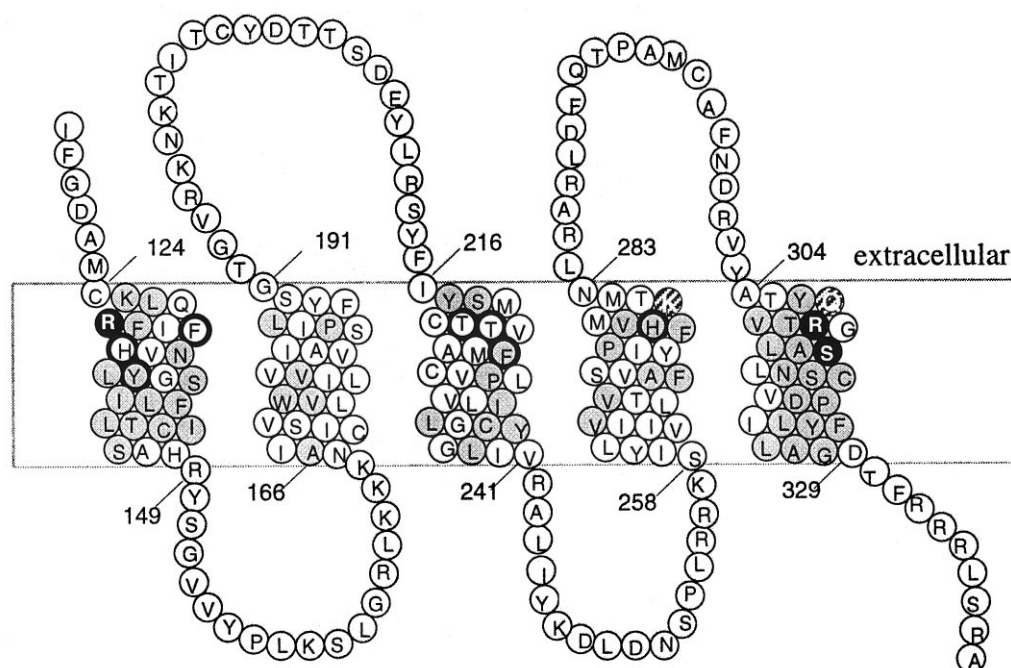
logues (20). As observed previously with the wild-type receptor (21) and with the P2Y<sub>1</sub> receptor in turkey erythrocytes (18), the diphosphate analogue was more potent than the triphosphate. Potency changes for the agonists 2-MeSADP and HT-AMP in general paralleled changes in potency for 2-MeSATP (Table 2). The ratio for potency of diphosphate versus triphosphate was generally 5–10-fold. An exception was the H277A mutant receptor, where diphosphates and triphosphates were equipotent. The potency of 2-MeSADP at most mutant receptors was generally 100–200-fold greater than for HT-AMP. In the case of the S317A mutant receptor, the potency ratio for 2-MeSADP versus HT-AMP was ~400.

These results suggest that receptor structure-function relationships are independent of specific interactions of the  $\beta$ - and  $\gamma$ -phosphates with the receptor. The residues that were found most critical for activation by 2-MeSATP (R128, R310, and S314) were also absolutely critical for activation of the receptor by monophosphates and diphosphates. In addition, the K280A mutant receptor had greatly diminished potency of monophosphates and diphosphates as well as the triphosphates. The one instance of an alanine mutation with no effect on triphosphate potency (S317A) yielded identical results for monophosphates and diphosphates. However, subtle differences were noted in the relative effects of a given mutation on potency of monophosphates versus triphosphates. For example, the F131A mutation was relatively more important in reducing the potency of HT-AMP (15-fold) than of 2-MeSATP (6.5-fold). The potency shifts for agonists at other mutant receptors versus wild-type receptors were less pronounced for HT-AMP versus 2-MeSATP: H132A (6.7- versus 12-fold), Y136A (4.8- versus 18-fold), T221A (5.1- versus 89-fold), and T222A (3.4- versus 33-fold). Among these cases of divergence between HT-AMP and 2-MeSATP, the differences between the two agonists were the greatest at T221A and T222A mutant receptors. The change in potencies of 2-MeSADP at T221A and T222A mutant receptors (13- and 8.8-fold, respectively) versus wild-type receptors were intermediate between those for monophosphates and triphosphates. The consistency of this trend at T221A and T222A mutant receptors suggests a greater contribution of the  $\gamma$ -phosphate moiety of ATP in ligand recognition at this region of TM5.

The effects of ATP antagonists (29) at wild-type receptors and at six mutant receptors at which 2-MeSATP was fully active (S317A, S314T, H277A, Q307A, K280A, and R310K) were probed using the same functional assay (Fig. 4). The weak, nonselective antagonists suramin, PPADS, and Reactive Blue 2, at concentrations of 100  $\mu$ M, were tested in the presence of a concentration of 2-MeSATP that caused ~50% stimulation of phospholipase C in each case (Fig. 4A). Suramin and PPADS each partially (~20%) blocked the agonist effects at this concentration; however, Reactive Blue 2 had no significant effect. Although IC<sub>50</sub> values were not determined, it seemed that both suramin and PPADS retained antagonist properties at all of the mutant receptors examined (Fig. 4B).

## Discussion

**Stimulation of phospholipase C by mutant human P2Y<sub>1</sub> receptors.** A mutational analysis of residues potentially involved in the adenine nucleotide binding pocket of the human P2Y<sub>1</sub> receptor has been carried out. Single amino acid



**Fig. 3.** Partial topology of hP2Y<sub>1</sub> receptors (TM3-TM7) showing mutation sites in which replacement by alanine significantly affected the ability of either 2-MeSATP or HT-AMP to activate the receptor. *Solid circles*, >1000-fold loss of potency; *striped circles*, 100-1000-fold loss of potency; *circles with thick outline*, 3-100-fold loss of potency. S317A mutation (not indicated in figure) had no effect on the agonist potency. The categorization of the results using HT-AMP as agonist were identical for the cases in which  $\geq 100$ -fold loss of potency was observed with 2-MeSATP. *Gray circles*, residues conserved between P2Y<sub>1</sub> and P2Y<sub>2</sub> receptors (R128, R310, and S314 are also conserved).

residues in TMs 3, 5, 6, and 7 predicted by molecular modeling to be involved in ligand recognition were targeted for replacement with alanine and, in some cases, by other amino acids. Mutated receptors were expressed in COS-7 cells with an epitope tag that permitted confirmation of expression in the plasma membrane. A functional assay (i.e., agonist-promoted inositol phosphate accumulation) was used as a measure of receptor activity. We have attempted to supplement these results by using the radioligand [<sup>35</sup>S] $\alpha$ -thio-2'-deoxy-ATP, as reported by Barnard *et al.* (30). However, untransfected cells displayed high levels of binding that was comparable to COS-7 cells transfected with wild-type P2Y<sub>1</sub> receptors but no comparable activation of phospholipase C by 2-MeSATP (data not shown). Similar conclusions were reached by Schachter *et al.* (31) concerning the unsuitability of [<sup>35</sup>S] $\alpha$ -thio-2'-deoxy-ATP as radioligand for P2Y<sub>1</sub> receptors. Because we already demonstrated the limitations of the binding of another radioligand, [<sup>35</sup>S] $\beta$ -thio-ADP binding (32), for characterization of P2Y receptors, no suitable radioligand was available.

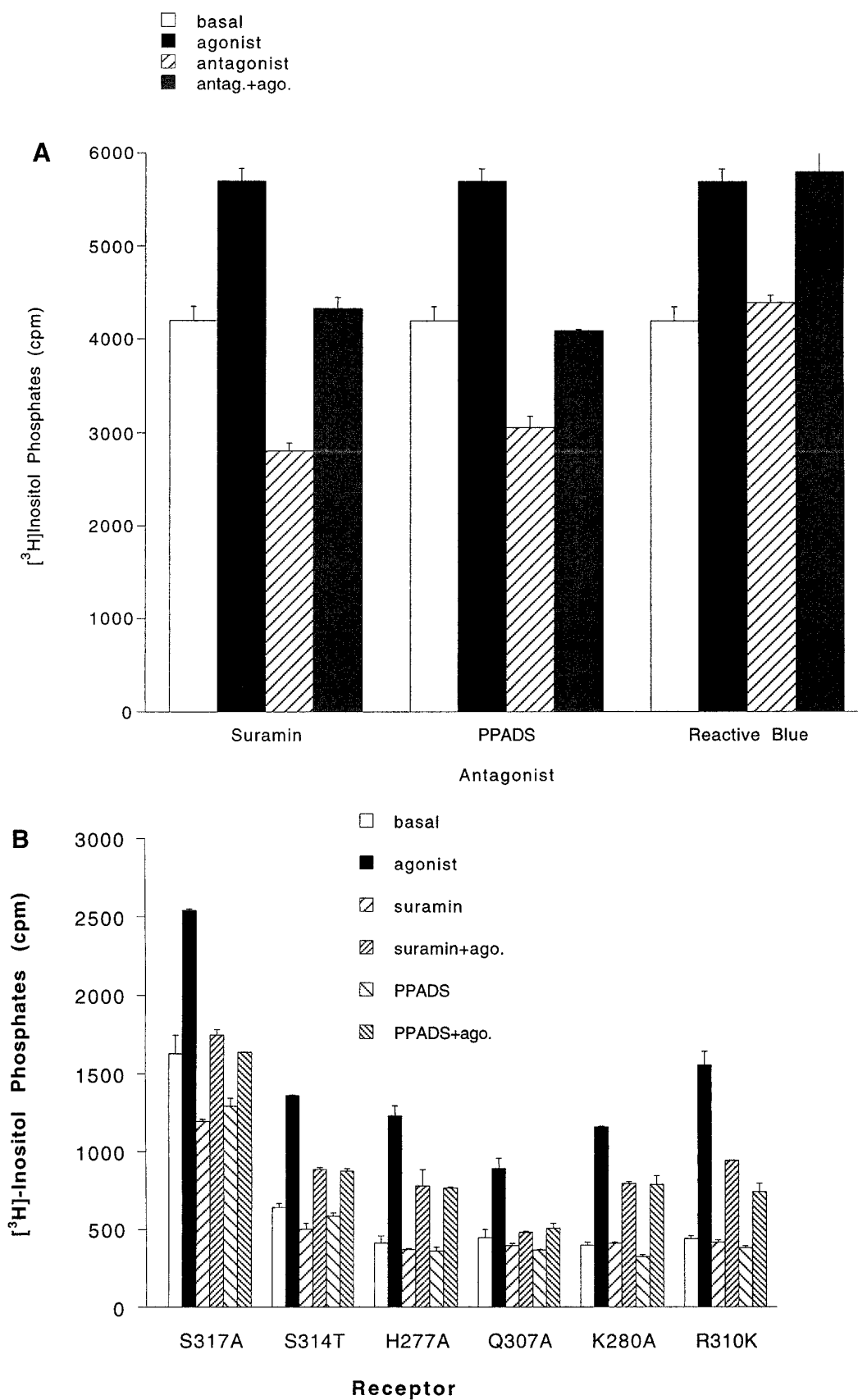
The current study clearly demonstrates that particular residues of the human P2Y<sub>1</sub> receptor are involved in ligand binding/receptor activation. The functional assay provides direct information concerning receptor activation and only indirect information concerning ligand recognition. However, in consideration of the location of the mutated residues proximal to the exofacial side, the most likely source of major shifts in agonist potency observed in mutant receptors is interference with ligand recognition. This region was also predicted by molecular modeling of P2Y<sub>1</sub> receptors to be in proximity to the bound ligand (11-13). Many of the mutated sites are homologous to ligand recognition sites in the human A<sub>2A</sub> adenosine receptor (5) (Fig. 1). In contrast, the predominant regions for effector coupling in GPCRs are known to be located near the cytosolic side of the receptor (4).

Alanine scanning mutagenesis showed that residues R128, K280, Q307, R310, and S314 are critical for high potency

agonist-induced activation of phospholipase C. For K280A and Q307A mutant receptors, nearly complete concentration-response curves were measured, and 2-MeSATP was shown to be a full agonist, although at markedly reduced potency compared with wild-type receptors. Thus, the ability of these mutant receptors to effect signal transduction was not eliminated. Furthermore, the rescue of function in the S314T mutant receptor emphasizes the necessity at this position of a hydroxyl group, which may be available to hydrogen bond to the ligand. The modeling study places the homologous residue of the chick P2Y<sub>1</sub> receptor in proximity to the 2'-hydroxyl group of the ribose moiety of bound ATP. Overall, these results suggest that residues on the exofacial side of TM3, TM6, and TM7 are critical determinants of the ATP binding pocket.

In contrast, there was no change in the potency or maximal effect of 2-MeSATP with the S317A mutant receptor. Thus, this residue is insensitive in adenine nucleotide binding. Furthermore, alanine replacement of F131, H132, Y136, F226, or H277 resulted in mutant receptors that exhibited a 7-18-fold reduction in potency of 2-MeSATP compared with that observed with the wild-type receptor. These five residues in TM3, TM5, and TM6 thus seem to subserve a less important modulatory role in ligand binding to the P2Y<sub>1</sub> receptor. The three residues in TM3 in this modulatory category would be aligned along the same face of the helix as the critical residue R128 but are located further from the exofacial side by approximately one (F131 and H132) or two (Y136) helical turns.

Because changes in the potency of 2-MeSATP and HT-AMP paralleled the changes in potency of 2-MeSATP at these mutant receptors, the  $\beta$ - and  $\gamma$ -phosphates of the adenine nucleotides seem to be less important than the  $\alpha$ -phosphate in ligand/P2Y<sub>1</sub> receptor interactions. However, T221A and T222A mutant receptors exhibited much larger reductions in the potency of 2-MeSATP (89- and 33-fold versus wild-type receptors, respectively) rather than HT-AMP and 2-MeSADP



**Fig. 4.** Action of antagonists (100  $\mu$ M) at wild-type receptors (A) and at mutant receptors using 2-MeSATP (10 nM) as agonist. B, 2-MeSATP was used as agonist at a concentration of 10 nM (S317A), 100 nM (S314T), 1  $\mu$ M (H277A and Q307A), or 10  $\mu$ M (K280A and R310K).



potency. This result may be indicative of a greater role of these TM5 residues in  $\gamma$ -phosphate recognition. The remaining residues in TMs 3, 5, 6, and 7 found to be either essential or modulatory for agonist potency did not show any striking differences among monophosphate, diphosphate, and triphosphate agonists. Thus, they would be expected to be associated preferentially with the  $\alpha$ -phosphate and/or adenosine moieties of ATP.

These findings are largely but not entirely consistent with our P2Y<sub>1</sub> receptor molecular model based on a rhodopsin template (12), which predicted that [numbering translated by homology from the chick P2Y<sub>1</sub> receptor sequence] H132(3.33), Y136(3.37), F226(5.47), H277(6.52), K280(6.55), and R310(7.39), but not R128(3.29), are in proximity to the triphosphate moiety of bound ATP. The model further predicted that S314 and S317 are in proximity to the ribose moiety. However, only mutation of the former residue, which is located closer to the exofacial side, affects ligand binding. Residues F131 and Q307 were predicted to be in proximity to the adenine moiety (the latter in potentially hydrogen-bonding proximity from the exocyclic amine), and indeed mutation of these residues caused a substantial loss of agonist potency (particularly at Q307).

PPADS and suramin retained potency as antagonists (1, 29) in the H277, K280, Q307A, R310K, S314T, and S317A mutant receptors. Thus, the noncritical (and possibly modulatory) residues H277, K280, and Q307A seem to be involved especially in agonist recognition. The S317A mutation seems to be inconsequential for agonist binding. The rescued mutants R310K and S314T are functional for both agonists and antagonists, although the potencies determined precisely for agonist are  $\sim 1$  (S314T) or several orders of magnitude (R310K) less than those for wild-type receptors. It is not feasible with the present experimental paradigms to test the ability of the nonactivatable R310K and S314T mutants to recognize the antagonists PPADS and suramin.

**Structural homology of human P2Y<sub>1</sub> receptors to other GPCRs.** The essential S314(7.43) corresponds to important hydrogen-bonding residues in other GPCRs, such as tyrosine in adrenergic and muscarinic acetylcholine receptors, and the essential H278 in human A<sub>2A</sub> receptors. Although there are numerous parallels between the P2Y<sub>1</sub> receptors and homologous sites in other GPCRs (4), there are some striking differences. For example, S317(7.46) corresponds to a critical serine residue in the hamster  $\beta_2$  receptor (33), yet it apparently has no role in ligand recognition in the human P2Y<sub>1</sub> receptor. Residue F131(3.32) corresponds to the essential D residue in biogenic amine receptors (counterion to ligand). For human P2Y<sub>1</sub> receptors, this site is less critical for ligand recognition than in biogenic amine receptors, because its mutation to A resulted in only a 6.5-fold loss of 2-MeSATP potency.

These findings can be compared with those of Erb *et al.* (11) with the human P2Y<sub>2</sub> (formerly called P<sub>2U</sub>) receptor subtype (Fig. 1), which is activated equipotently by UTP and ATP. The latter study found that although K289(7.36) is positively charged at physiological pH, it is not involved in phosphate binding. The homologous residue in the human P2Y<sub>1</sub> receptor, the neutral Q307, was proposed in our modeling study (12) to coordinate to the adenine N<sup>6</sup>-amino group. In the current study, Q307 was found to be strongly modulatory for agonist potency.

In addition, in the mouse P2Y<sub>2</sub> receptor (11), three positively charged residues, H262(6.52), R265(6.55), and R292(7.39), were the most critical. Neutralization of each of these residues individually by replacement with leucine or isoleucine caused a 100–850-fold decrease in the potency of ATP and UTP (11, 13). Of these three sites, we found only the homologous residues K280(6.55) and R310(7.39) in the human P2Y<sub>1</sub> receptor to be essential for activation by low concentrations of agonist. Residue N253(6.55) in the human A<sub>2A</sub> adenosine receptor (5), which is homologous to K280, is essential for both agonist and antagonist binding. The residue H277(6.52), homologous to R265 in the P2Y<sub>2</sub> receptor, was only modulatory in the P2Y<sub>1</sub> receptor, leading to a 17-fold loss of potency of 2-MeSATP. Curiously, this residue is conserved between P2Y and P<sub>1</sub> receptors. Mutation of the conserved H250(6.52) in human A<sub>2A</sub> receptors resulted in a dramatic loss of both agonist and antagonist affinity (5).

There is another charged residue K125(3.26) in the TM region that was not mutated in the current study. In the P2Y<sub>2</sub> receptor, replacement of the homologous residue K107(3.26) by the uncharged L had no effect on agonist potency.

The current study demonstrates the critical importance of R128(3.29), not predicted by molecular modeling. The homologous residue was not mutated in the P2Y<sub>2</sub> receptor (11). Chemical modification of the cysteinyl mutants of the D<sub>2</sub> dopamine receptor (34) suggests that the residue equivalent in position, V111C(3.29), is solvent exposed in the binding cleft.

**Conclusions.** The present site-directed mutagenesis study confirms that many of the residues of TM3, TM5, TM6, and TM7 predicted in a modeling study of P2Y<sub>1</sub> receptors to form the nucleotide binding pocket (12) are indeed involved in ligand recognition. Thus, it seems that positively charged residues near the exofacial side of both TM3 and TM7 and, to a lesser extent TM6, are important for recognition of ATP, presumably through interaction with the negatively charged phosphate moiety. The potency of monophosphate and diphosphate nucleotide analogues generally varied in parallel to changes for the triphosphate analogue. Therefore, most structure-function relationships for the receptor were not dependent on interactions with the  $\beta$ - and  $\gamma$ -phosphates. An exception was the T221T222 sequence in TM5, for which mutation to A was more detrimental to triphosphate than either monophosphate or diphosphate potency, suggesting a greater role of this region in  $\gamma$ -phosphate binding.

Taken together, the results suggest that the adenosine and  $\alpha$ -phosphate moieties of ATP seem to bind to human P2Y<sub>1</sub> receptors preferentially in a TM region proximal to the exofacial side, with the most critical residues located in TM3 and TM7. This study has revealed differences between P2Y<sub>1</sub> and P2Y<sub>2</sub> receptors (11), in which positively charged residues of TM6 and TM7 principally have been proposed to coordinate the phosphate group of uridine nucleotides. The characterization of structure function differences among P2Y receptor subtypes will likely aid in the design of highly potent and selective ligands.

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