A Mutational Analysis of Residues Essential for Ligand Recognition at the Human P2Y₁ Receptor

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SUMMARY

We conducted a mutational analysis of residues potentially involved in the adenine nucleotide binding pocket of the human P2Y₁ 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 P2Y₁ 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 P2Y₁ 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 β- and γ-phosphates of the adenine nucleotides seem to be less important than the α-phosphate in ligand/P2Y₁ 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 γ-phosphate recognition. Taken together, the results suggest that the adenosine and α-phosphate moieties of ATP bind to critical residues in TM3 and TM7 on the exofacial side of the human P2Y₁ 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.¹

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 P2Y₂ receptor (11), which binds both purine and pyrimidine nucleotides, has been studied using mutagenesis. The P2Y₁ receptor subtype is a phospholipase C-activating ATP receptor present in heart, skeletal muscle, and various smooth muscles. Both P2Y₁ (12) and P2Y₂ (11, 13) receptors have been modeled using rhodopsin (14–16)² 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

¹ P2Y₅, P2Y₆, P2Y₇ sequences have been cloned and named in the literature (3), however, several of these may be either receptors of a different family (e.g., P2Y₂ and possibly P2Yₓ) or possibly species homologues (e.g., P2Y₆ and P2Yₓ). The identity and relationship among these clones require further study.

² The coordinates of the P2Y₁ 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 immunoassay; 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\(^3\) R128(3.29), R310(7.39), and S314(7.43) are critical for the binding of nucleotides to human P2Y\(_1\) 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 P2 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\(_1\), but not P2Y\(_2\) 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\(_1\) receptor cDNA (pCDNA3P2Y\(_1\)) 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-MeSATP 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 (Bristol, UK). N-[3H]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 Bioserv Biotechnologies (Laurel, MD). A monoclonal antibody (12CA5) against an HA epitope was purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN), and goat anti-mouse IgG (γ-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\(_1\) was subcloned into the pCD-PS expression vector (22), yielding pCDP2Y\(_1\). All mutations were introduced into pCDP2Y\(_1\) 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 GAT GTG CCA GAC TAC GCG; peptide sequence, YPYDVPDYA) was inserted after the M residue at the extracellular amino terminus of the human P2Y\(_1\) 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\(_1\) 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 × 10\(^5\)) 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 μg of DNA/dish) according to the DEAE-dextran method (26) and grown for an additional 72 hr at 37°C.

**Inositol phosphate determination.** COS-7 cells were subcultured into 12 × 100-mm dishes (Falcon) at a density of −1 × 10\(^6\) cells/dish. After overnight incubation at 37°C and 5% CO\(_2\), the cells were transfected with plasmids containing receptor-coding sequence using the DEAE-Dextran method for 40 min, followed by treatment with 100 μM 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 (~0.75 × 10\(^5\) cells/well; Costar, Cambridge, MA) in DMEM culture medium (plus 100 units/ml of penicillin, 100 μg/ml streptomycin, 2 μmol/ml glutamate, and 10% fetal calf serum) supplemented with 3 μg/ml of nico-[\(^{3}H\)]inositol. After a 24-hr labeling period, cells were preincubated for 30 min at 37°C with 10 μM 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°C and 5% CO\(_2\). The supernatant was removed by aspiration, and 750 μl of cold 20 mM formic acid was added to each well. Cell extracts were collected after a 30-min incubation at 4°C and neutralized with 250 μl of 60 mM NH\(_4\)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. [\(^{3}H\)]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 Wallace 1215 Rackbeta 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 (4–5 × 10\(^5\) 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 μg/ml) for 3 hr at 37°C. 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°C. 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\(_2\)SO\(_4\) solution containing 0.05 M Na\(_2\)SO\(_4\), and the color development was measured spectrophotometrically 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\(_1\) 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\(_1\) receptor (12) to be involved in the binding of the phosphate moiety of nucleotides. Mutated residues also in-
include 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 P2Y1 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 P2Y1 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 P2Y1 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 ± 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).

<table>
<thead>
<tr>
<th>Construct</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>100</td>
</tr>
<tr>
<td>COS-7 (control)</td>
<td>0</td>
</tr>
<tr>
<td>R128A</td>
<td>263 ± 4</td>
</tr>
<tr>
<td>F131A</td>
<td>150 ± 96</td>
</tr>
<tr>
<td>H132A</td>
<td>57.2 ± 4.7</td>
</tr>
<tr>
<td>Y136A</td>
<td>50.6 ± 32.5</td>
</tr>
<tr>
<td>T221A</td>
<td>87.2 ± 16.9</td>
</tr>
<tr>
<td>T222A</td>
<td>123 ± 31</td>
</tr>
<tr>
<td>F226A</td>
<td>144 ± 42</td>
</tr>
<tr>
<td>H277A</td>
<td>129 ± 6</td>
</tr>
<tr>
<td>K280A</td>
<td>148 ± 13</td>
</tr>
<tr>
<td>Q307A</td>
<td>151 ± 9</td>
</tr>
<tr>
<td>R310A</td>
<td>132 ± 9</td>
</tr>
<tr>
<td>R310K</td>
<td>186 ± 40</td>
</tr>
<tr>
<td>R310S/S314R</td>
<td>114 ± 5</td>
</tr>
<tr>
<td>S314A</td>
<td>125 ± 5</td>
</tr>
<tr>
<td>S314T</td>
<td>111 ± 56</td>
</tr>
<tr>
<td>S317A</td>
<td>106 ± 23</td>
</tr>
</tbody>
</table>

Slight increase of inositol phosphates in control COS-7 cells, which was not observed in control cells (Fig. 2). At the high-sensitivity expressing wild-type human P2Y<sub>1</sub> receptors diminished 2-MeSATP response to the potent agonist 2-MeSATP (EC<sub>50</sub> = 1 mM), there was a pronounced effect on agonist activation of phospholipase C by 2-MeSATP (100%). Expression level was determined using a standard curve (5).

TABLE 2
Activation of phospholipase C by mutant human P2Y<sub>1</sub> receptors.

Data are presented as means ± 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).

<table>
<thead>
<tr>
<th>Construct</th>
<th>Residue&lt;sup&gt;a&lt;/sup&gt;</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; 2MeSATP</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; 2MeSADP</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; HT-AMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>3.29</td>
<td>6.75 ± 2.58</td>
<td>1.27 ± 0.01</td>
<td>355 ± 4</td>
</tr>
<tr>
<td>R128A</td>
<td>3.32</td>
<td>44.2 ± 15.9</td>
<td>18.2 ± 2.1</td>
<td>5260 ± 2200</td>
</tr>
<tr>
<td>F131A</td>
<td>3.33</td>
<td>79.9 ± 29.6</td>
<td>15.9 ± 4.4</td>
<td>2390 ± 600</td>
</tr>
<tr>
<td>H132A</td>
<td>3.37</td>
<td>119 ± 103</td>
<td>12.6 ± 1.8</td>
<td>1720 ± 690</td>
</tr>
<tr>
<td>T221A</td>
<td>5.42</td>
<td>604 ± 456</td>
<td>15.9 ± 4.0</td>
<td>1810 ± 280</td>
</tr>
<tr>
<td>T222A</td>
<td>5.43</td>
<td>221 ± 58</td>
<td>11.2 ± 0.6</td>
<td>1210 ± 210</td>
</tr>
<tr>
<td>F226A</td>
<td>5.47</td>
<td>56.8 ± 7.5</td>
<td>20.7 ± 3.8</td>
<td>1850 ± 800</td>
</tr>
<tr>
<td>H277A</td>
<td>6.52</td>
<td>115 ± 28</td>
<td>100 ± 30</td>
<td>4520 ± 640</td>
</tr>
<tr>
<td>K280A</td>
<td>6.55</td>
<td>6400 ± 1380</td>
<td>1790 ± 310</td>
<td>203,000 ± 36,000</td>
</tr>
<tr>
<td>Q307A</td>
<td>7.36</td>
<td>1400 ± 300</td>
<td>455 ± 140</td>
<td>43,000 ± 600</td>
</tr>
<tr>
<td>R310A</td>
<td>7.39</td>
<td>4580 ± 430</td>
<td>418 ± 118</td>
<td>13,400 ± 1700</td>
</tr>
<tr>
<td>R310K</td>
<td>7.39</td>
<td>4580 ± 430</td>
<td>418 ± 118</td>
<td>13,400 ± 1700</td>
</tr>
<tr>
<td>R310S/S314R</td>
<td>7.39/7.43</td>
<td>560 ± 18.0</td>
<td>11.7 ± 1.8</td>
<td>1950 ± 440</td>
</tr>
<tr>
<td>S314A</td>
<td>7.43</td>
<td>600 ± 18.0</td>
<td>11.7 ± 1.8</td>
<td>1950 ± 440</td>
</tr>
<tr>
<td>S314T</td>
<td>7.43</td>
<td>800 ± 1.78</td>
<td>1.58 ± 0.29</td>
<td>611 ± 52</td>
</tr>
</tbody>
</table>

<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.
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 analogues (20). As observed previously with the wild-type receptor (21) and with the P2Y1 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-MeSATP 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 β- and γ-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 was intermediate between those for monophosphates and triphosphates. The consistency of this trend at T221A and T222A mutant receptors suggests a greater contribution of the γ-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 μM, 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 IC50 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 P2Y1 receptors. A mutational analysis of residues potentially involved in the adenine nucleotide binding pocket of the human P2Y1 receptor has been carried out. Single amino acid
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 [35S]α-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 P2Y1 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 [35S]α-thio-2'-deoxy-ATP as radioligand for P2Y1 receptors. Because we already demonstrated the limitations of the binding of another radioligand, [35S]β-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 P2Y1 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 P2Y1 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 A2A 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 P2Y1 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 F226, or H277 resulted in mutant receptors that exhibited a 3–100-fold loss of potency. A2A 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 P2Y1 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.
Fig. 4. Action of antagonists (100 μM) 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 μM (H277A and Q307A), or 10 μM (K280A and R310K).
potency. This result may be indicative of a greater role of these TM5 residues in γ-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 α-phosphate and/or adenosine moieties of ATP.

These findings are largely but not entirely consistent with our P2Y1 receptor molecular model based on a rhodopsin template (12), which predicted that [numbering translated by homology from the chick P2Y1 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 ~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 P2Y1 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 A2A receptors. Although there are numerous parallels between the P2Y1 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 β2 receptor (33), yet it apparently has no role in ligand recognition in the human P2Y1 receptor. Residue F131(3.32) corresponds to the essential D residue in biogenic amine receptors (counterion to ligand). For human P2Y1 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 P2Y2 (formerly called P2U) 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 P2Y1 receptor, the neutral Q307, was proposed in our modeling study (12) to coordinate to the adenine N6-amino group. In the current study, Q307 was found to be strongly modulatory for agonist potency.

In addition, in the mouse P2Y2 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 P2Y1 receptor to be essential for activation by low concentrations of agonist. Residue N253(6.55) in the human A2A 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 P2Y2 receptor, was only modulatory in the P2Y1 receptor, leading to a 17-fold loss of potency of 2-MeSATP. Curiously, this residue is conserved between P2Y and P1 receptors. Mutation of the conserved H250(6.52) in human A2A 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 P2Y2 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 P2Y2 receptor (11). Chemical modification of the cysteinyl mutants of the D2 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 P2Y1 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 β- and γ-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 γ-phosphate binding.

Taken together, the results suggest that the adenosine and α-phosphate moieties of ATP seem to bind to human P2Y1 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 P2Y1 and P2Y2 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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References


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