



Published in final edited form as:

*J Biol Chem.* 1999 May 21; 274(21): 14639–14647.

## The Role of Amino Acids in Extracellular Loops of the Human P2Y<sub>1</sub> Receptor in Surface Expression and Activation Processes\*

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### Abstract

The P2Y<sub>1</sub> receptor is a membrane-bound G protein-coupled receptor stimulated by adenine nucleotides. Using alanine scanning mutagenesis, the role in receptor activation of charged amino acids (Asp, Glu, Lys, and Arg) and cysteines in the extracellular loops (EL) of the human P2Y<sub>1</sub> receptor has been investigated. The mutant receptors were expressed in COS-7 cells and measured for stimulation of phospholipase C induced by the potent agonist 2-methylthioadenosine-5'-diphosphate (2-MeSADP). In addition to single point mutations, all receptors carried the hemagglutinin epitope at the N-terminus for detection of cell-surface expression. The C124A and C202A mutations, located near the exofacial end of transmembrane helix 3 and in EL2, respectively, ablated phospholipase C stimulation by 100 μM 2-MeSADP. Surface enzyme-linked immunosorbent assay detection of both mutant receptors showed <10% expression, suggesting that a critical disulfide bridge between EL2 and the upper part of transmembrane 3, as found in many other G protein-coupled receptors, is required for proper trafficking of the P2Y<sub>1</sub> receptor to the cell surface. In contrast, the C42A and C296A mutant receptors (located in the N-terminal domain and EL3) were activated by 2-MeSADP, but the EC<sub>50</sub> values were >1000-fold greater than for the wild-type receptor. The double mutant receptor C42A/C296A exhibited no additive shift in the concentration-response curve for 2-MeSADP. These data suggest that Cys<sup>42</sup> and Cys<sup>296</sup> form another disulfide bridge in the extracellular region, which is critical for activation. Replacement of charged amino acids produced only minor changes in receptor activation, with two remarkable exceptions. The E209A mutant receptor (EL2) exhibited a >1000-fold shift in EC<sub>50</sub>. However, if Glu<sup>209</sup> were substituted with amino acids capable of hydrogen bonding (Asp, Gln, or Arg), the mutant receptors responded like the wild-type receptor. Arg<sup>287</sup> in EL3 was impaired similarly to Glu<sup>209</sup> when substituted by alanine. Substitution of Arg<sup>287</sup> by lysine, another positively charged residue, failed to fully restore wild-type activity.

P2 receptors have been divided into two structurally distinct families as follows: the P2X receptor class of ligand-gated ion channels, which are primarily activated by ATP, and the P2Y receptor class of G protein-coupled receptors (GPCRs),<sup>1</sup> which are activated by both extracellular adenine and uridine nucleotides (1–3). As many as 11 subtypes of P2Y

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<sup>1</sup>The abbreviations used are: GPCR, G protein-coupled receptor; 2-MeSADP, 2-methylthioadenosine-5'-diphosphate; 2-MeSATP, 2-methylthioadenosine-5'-triphosphate; DMEM, Dulbecco's modified Eagle's medium; EL, extracellular loops; 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; PLC, phospholipase C; TM, (helical) transmembrane domain; AT, angiotensin.

receptors have been cloned, but only 5 of these are from mammalian species and have been shown to be functionally activated by extracellular nucleotides. Of these 11, several may be either non-nucleotide receptors such as P2Y<sub>5</sub>, P2Y<sub>7</sub>, P2Y<sub>9</sub>, and P2Y<sub>10</sub> or species homologues like p2y3 and P2Y<sub>6</sub>. The P2Y<sub>1</sub> subtype specifically recognizes adenine nucleotides, which act as either potent agonists (e.g. 2-methylthio-ADP) or selective antagonists (e.g. MRS 2179). The P2Y<sub>1</sub> receptor, which is coupled to activation of phospholipase C (PLC), is expressed in heart muscle, skeletal muscle, and various smooth muscle cells and is associated with the vasodilatory action of ATP (3). In platelets, it serves as one of the receptors through which ADP induces aggregation, an action attenuated by ATP and other triphosphate derivatives (4).

Previously we have investigated the determinants of ligand recognition in P2Y<sub>1</sub> receptors using site-directed mutagenesis of specific amino acid residues in the transmembrane helical domains (TMs) (5), molecular modeling based on similarity to rhodopsin in sequence and overall geometry (6), and the chemical synthesis of novel agonists (7) and antagonists (8). Positively charged and other conserved residues in TM3, TM6, and TM7, such as Arg<sup>128</sup>, Arg<sup>310</sup>, and Ser<sup>314</sup>, were found to be critical for the activation by nucleotides of human P2Y<sub>1</sub> receptors. A molecular model of the TM domains based on these findings has been proposed (6).

Most of the interest in GPCRs to date has focused on the TM domains for locating amino acids that are specifically involved in recognition of small, non-peptide ligands, including nucleotides (5, 9, 10). However, for peptide receptors residues within the extracellular loops (ELs) are essential for ligand recognition (11). Currently, there is accumulating evidence that residues within the ELs are also important in GPCRs that recognize small molecules as ligands. In adenosine receptors, amino acids in EL2 were shown to be involved in agonist and antagonist binding (12) and high affinity binding of xanthine antagonists (13). In the thyrotropin-releasing hormone receptor, amino acids in EL2 and EL3 were found to be important for ligand binding (14). In the  $\alpha_1$ -adrenergic receptor, three amino acids at the C-terminal end of EL2 were shown to be responsible for subtype-specific antagonist binding (15). In angiotensin II type 2 receptors, amino acid residues in EL2 and EL3 contribute to angiotensin II binding (16), whereas in angiotensin II type 1 receptors, an additional residue in EL1 is involved in this process (17).

A conserved disulfide bridge between Cys residues in the second extracellular loop (EL2) and the exofacial end of TM3 has been shown to be essential for various biogenic amine and other GPCRs. It is generally thought that this disulfide bond is required to maintain overall receptor geometry, although its role in transport to the cell surface is contradictory (18, 19). An additional disulfide bridge between a cysteine in the EL3 and the N-terminal domain was found in the angiotensin receptor (20). The extracellular portions of the human P2Y<sub>1</sub> receptor carry four cysteines, and sequence comparison has revealed that all of these cysteines are conserved among P2Y receptors (21). Hence P2Y receptors might form two disulfide bridges within the extracellular receptor portions.

Interestingly, the P2Y receptors are GPCRs that recognize small molecules as ligands, but they exhibit the highest sequence identity with peptide receptors such as somatostatin, platelet-activating factor, angiotensin II, and neuropeptide Y receptors (22). Because of this paradox (binding of a small ligand and sequence homology with peptide receptors), we have investigated the role of amino acids in the ELs in human P2Y<sub>1</sub> receptor activation.

The aim of this study was to identify amino acids that potentially may be involved in either direct ligand contact or in conformational restriction of the ELs. We hypothesized that disruption of either of the two potential disulfide bonds in the ELs or substitution of amino

acids potentially involved in ionic interactions (Asp, Glu, Lys, or Arg) might have a marked effect on ligand recognition and thus receptor-mediated activation of PLC.

## EXPERIMENTAL PROCEDURES

### Materials

The expression construct coding for the human P2Y<sub>1</sub> receptor (pCDP2Y<sub>1</sub>) was prepared as described previously (5). Vent DNA polymerase and 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 Research Biochemicals (Natick, MA). The agonist 2-hexylthioadenosine-5'-monophosphate (HT-AMP) was synthesized as described (7, 23) as the ammonium salt, which was more soluble in aqueous medium than the triethylammonium salt. *myo*-[<sup>3</sup>H]inositol (15 Ci/mmol) was obtained from American Radiolabeled Chemicals. Fetal bovine serum (FBS) was from Life Technologies, Inc. *o*-Phenylenediamine dihydrochloride was purchased from Sigma. The Sequenase Kit version 2.0 was from Amersham Pharmacia Biotech. All oligonucleotides were synthesized by Bioserve Biotechnologies (Laurel, MD). A monoclonal antibody (12CA5) against a hemagglutinin epitope (HA) was purchased from Roche Molecular Biochemicals, and goat anti-mouse IgG ( $\gamma$ -chain-specific) antibody conjugated with horseradish peroxidase was purchased from Sigma. DEAE-dextran was obtained from Amersham Pharmacia Biotech.

### Plasmid Construction and Site-directed Mutagenesis

All mutations were introduced into pCDP2Y<sub>1</sub> (5) using standard polymerase chain reaction mutagenesis techniques (24). The accuracy of all polymerase chain reaction-derived sequences was confirmed by dideoxy sequencing of the mutant plasmids (25).

### Epitope Tagging

As described previously (5) a 9-amino acid sequence derived from the influenza virus hemagglutinin (HA) protein (TAC CCA TAC GAC GTG CCA GAC TAC GCG; peptide sequence: YPYDVPDYA) was inserted after the initiating methionine residue in the extracellular N terminus of the human P2Y<sub>1</sub> receptor gene. A hexahistidine tag (26) was also included at the C terminus immediately after the ultimate leucine residue resulting in a construct suitable for potential affinity chromatography using a nickel column.

### Transient Expression of Mutant Receptors in COS-7 Cells

$4 \times 10^6$  COS-7 cells were seeded into 150-mm culture dishes containing 25 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, and 2  $\mu$ mol/ml glutamine. Cells were transfected approximately 24 h later with plasmid DNA (10  $\mu$ g DNA/dish) using the DEAE-dextran method (27) for 40 min, followed by treatment with 100  $\mu$ M chloroquine for 2.5 h, and grown for an additional 24 h at 37 °C and 5% CO<sub>2</sub>.

### Inositol Phosphate Determination

Assays were carried out according to the general approach of Harden *et al.* (28). About 24 h after transfection, the cells were split into 6-well plates (Costar,  $\sim 0.75 \times 10^6$  cells/well) in DMEM culture medium supplemented with 3  $\mu$ Ci/ml *myo*-[<sup>3</sup>H]inositol. After a 24-h labeling period, cells were preincubated with 10 mM LiCl for 20 min at room temperature. The mixtures were slightly swirled to ensure uniformity. Following the addition of agonists, the cells were incubated for 30 min at 37 °C, 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. After a 30-min incubation at 4 °C, cell extracts were neutralized with 250  $\mu$ l of 60 mM NH<sub>4</sub>OH. The

inositol monophosphate fraction was then isolated by anion exchange chromatography (29). The contents of each well were applied to a small anion exchange column (Bio-Rad AG-1-X8) 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 10 ml of water followed by 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 quantitated by liquid scintillation spectrometry (LKB Wallace 1215 Rackbeta scintillation counter).

Pharmacological parameters were analyzed using the KaleidaGraph program (Abelbeck Software, version 3.01).

## ELISA

For cell surface ELISA measurements, cells were transferred to 96-well dishes ( $4-5 \times 10^4$  cells per well) 1 day after transfection. About 72 h after transfection, cells were fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature. After washing three times with PBS and blocking with DMEM (containing 10% FBS), cells were incubated with the HA-specific monoclonal antibody (12CA5), 20  $\mu\text{g}/\text{ml}$ , for 3 h 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 h 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<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 in the BioKinetics reader (EL312, BioTek Instruments, Inc., Winooski, VT) at 490 and 630 nm (base line). The reading for cells transfected with expression constructs coding for the human P2Y<sub>1</sub> wild-type receptor was approximately 0.5 OD units, and approximately 0.2 OD units for cells transfected with vector DNA alone. The difference was normalized as 100% surface expression.

## Western Blotting

Wild-type and all cysteine mutant receptors were detected with the polyhistidine monoclonal antibody (0.5  $\mu\text{g}/\text{ml}$ , clone BMG-His-1, Roche Molecular Biochemicals) directed against the hexa-His tag present at the C-terminal domain of all receptors. Samples containing 50  $\mu\text{g}$  of solubilized membrane protein prepared from transfected COS-7 cells as described previously (30) were resolved by SDS-polyacrylamide gel electrophoresis (4–20% miniplus sepragel, Owl Separation Systems, Woburn, MA), electroblotted onto nitrocellulose, and probed with the polyhistidine monoclonal antibody as described (31). Immunoreactive proteins were detected by incubation with horseradish peroxidase-conjugated sheep anti-mouse antibody (1:2500, Amersham Pharmacia Biotech) and visualized using an enhanced chemiluminescence system (Amersham Pharmacia Biotech).

## RESULTS

### Cysteine Residues

Among P2Y receptors, four cysteines in the extracellular domains are conserved (21). For the human P2Y<sub>1</sub> receptor these cysteines are Cys<sup>42</sup> in the N-terminal domain, Cys<sup>124</sup> near the exofacial end of TM3, Cys<sup>202</sup> in EL2, and Cys<sup>296</sup> in EL3. Each of these cysteine residues was individually mutated to alanine, and the corresponding mutants were tested for activation of PLC by three different agonists (2-MeSATP, 2-MeSADP, and HT-AMP). The results are summarized in Table I. The C202A mutant receptor showed no activation up to 100  $\mu\text{M}$  concentration of all agonists used in this study. Analogously, the C124A mutant

receptor exhibited the same properties, with no increased stimulation of PLC detectable at 100  $\mu$ M agonist.

All mutant receptors contained an HA epitope at the N terminus, allowing cell-surface expression levels to be measured by ELISA (Table I). Both C124A and C202A mutant receptors showed strongly diminished surface expression. The C124A mutant receptor was detectable at approximately 10% of the level of the wild-type receptor, whereas the level of the C202A mutant receptor was not significantly different from vector-transfected control. Western blot analyses of membrane fractions prepared from transfected cells showed that these cysteine mutant proteins were expressed in almost equal amounts compared with the wild-type receptor (Fig. 1). These results, together with the lack of agonist-promoted functional activity, suggest that a disulfide bridge between Cys<sup>124</sup> and Cys<sup>202</sup> is critical for proper receptor trafficking of the human P2Y<sub>1</sub> receptor to the cell surface. These results are in concordance with previous studies indicating that this conserved disulfide bridge found in the vast majority of GPCRs is important for proper receptor function (11, 32).

In contrast to the above cysteine mutant receptors, the C42A and C296A mutant receptors were activated by 2-MeSADP; however, the 2-MeSADP concentration-response curves were shifted by more than 1000-fold to the right compared with the wild-type receptor. The same relative shift in receptor activation was observed for the two other agonist ligands (Table I), indicating a similar influence on the general recognition of the agonist ligands. The double point mutant C42A/C296A exhibited a similarly impaired response as the single alanine mutants, indicating that the effect of this cysteine substitution is not additive (Fig. 2).

Although the expression levels of C42A, C296A, and C42A/C296A mutant receptors were low, activation of PLC was clearly detectable. Control experiments (inositol phosphate assay and ELISA) in which the amount of DNA used for transfection was varied showed that the wild-type P2Y<sub>1</sub> receptor was significantly stimulated, even at expression rates as low as 10% of the normal expression level attained using 10  $\mu$ g of DNA. No significant shift in EC<sub>50</sub> values was observed with a 10% expression rate of wild-type receptor (Fig. 3, A and B); thus, the EC<sub>50</sub> values observed for these three cysteine mutant receptors likely reflect the intrinsic activity of these constructs.

### Extracellular Loop 1

Only two positions in EL1 were selected for investigation of their influence on agonist activation of P2Y<sub>1</sub> receptors as follows: Cys<sup>124</sup> (see above) and Lys<sup>125</sup>, which is functionally conserved within the P2Y family. The K125A mutant showed no difference compared with the wild-type receptor in agonist-promoted PLC activation. ELISA data for this mutant receptor showed approximately 45% of wild-type receptor expression, indicating that Lys<sup>125</sup> does not influence receptor activation. No further mutations were made in EL1, since this loop is presumed to be located distal to the ATP-binding site (6).

### Extracellular Loop 2

Seven positively or negatively charged amino acids and one cysteine residue (see above) are located in EL2. All of these amino acids were mutated individually to alanine. Although R195A, K196A, K198A, D208A, and R212A mutant receptors showed no detectable changes in activation of PLC and were well expressed on the cell surface (Table I), the D204A and E209A mutant receptors behaved much differently. The D204A mutant receptor was activated at 20–25-fold higher agonist concentrations compared with the wild-type receptor. Furthermore, this shift was observed for all agonists, independent of the length of the 5'-phosphate chain. To investigate further the role of Asp<sup>204</sup> in receptor activation, we constructed D204E and D204N mutant receptors. Extension of the alkyl carboxylate side

chain at position 204 (D204E) further impaired agonist-promoted activation of the receptor, resulting in a 55–65-fold shift of the concentration-response curves. Surprisingly, the relatively small change of a carboxylate to carboxamide in the D204N mutant receptor caused an even greater change (200–270-fold shift in  $EC_{50}$  values) in receptor activation (Fig. 4 and Table I).

In contrast, the E209A mutant receptor exhibited a dramatically different activation pattern. This mutant receptor was activated by >1000-fold higher concentrations of each agonist than the wild-type receptor (Fig. 5 and Table I). The nature of the interaction of this carboxylate side chain with agonists was further tested by additional mutant receptors E209D, E209Q, and E209R. These mutant receptors were fully active and responded in a manner indistinguishable from wild-type receptors (Table I).

### Extracellular Loop 3

Beside Cys<sup>296</sup> (see above), five charged amino acids were investigated in EL3. The R285A, D289A, D300A, and R301A mutations had no effect on PLC activation, whereas the R287A mutant receptor was activated at more than 1000-fold higher agonist concentrations than wild-type receptors, and the profile of the concentration-response curves was surprisingly similar to that of the E209A mutant receptor (see Fig. 5). Thus, Arg<sup>287</sup> was targeted for a more detailed analysis. The R287K, R287Q, and R287E mutant receptors were generated and tested for PLC activation. The R287Q mutant receptor required >1,000-fold higher agonist concentrations for activation of PLC compared with the wild-type receptor, whereas the R287E mutant receptor was not significantly activated by agonist concentrations up to 100  $\mu$ M. In contrast to these findings, receptor activity could be partially to fully retained by changing Arg<sup>287</sup> to lysine. However, the concentration-response curves for the nucleoside diphosphate and triphosphate were shifted to the right (35- and 17-fold, respectively, compared with wild-type receptors), whereas the concentration-response curve for the nucleoside monophosphate was not shifted.

Since the E209A and R287A mutant receptors exhibited very similar activation patterns when stimulated with 2-MeSADP (Fig. 5), we tested the hypothesis that a direct interaction between these residues might be required for stabilization of the EL. Molecular modeling studies of the EL (33) suggested that Glu<sup>209</sup> and Arg<sup>287</sup> might form an ionic interaction. The double mutant E209R/R287E and the double deletion mutant E209A/R287A were constructed and investigated. The double mutant E209R/R287E was not activated by agonist concentrations up to 100  $\mu$ M, indicating that the amino acids at these positions were not interchangeable. The double mutant E209A/R287A exhibited an additive shift in concentration-response curves for receptor activation (Fig. 5) compared with the single mutants, suggesting that Glu<sup>209</sup> and Arg<sup>287</sup> affect receptor activation independently.

## DISCUSSION

In the present study we have identified both charged residues and Cys residues within the ELs of the human P2Y<sub>1</sub> receptor that are critical for the activation of the receptor. We have shown that the highly conserved disulfide bridge between the EL2 and TM3 is required for proper functioning of the receptor. Since the double cysteine mutant receptor (C42A/C296A, see Fig. 2) showed PLC activation similar to the single cysteine mutants (C42A and C296A), we deduced the existence of an additional, critical disulfide bridge between the EL3 and the N-terminal domain, which seems to be involved in receptor activation as well as proper receptor trafficking to the cell surface. A similarly situated disulfide bridge was found in the angiotensin AT<sub>1</sub> receptor (26, 27), and it was concluded that this disulfide bridge functions to position properly extracellular amino acids that are involved in the binding of AT II. Sequence comparisons show that the angiotensin AT<sub>1</sub> receptor is one of

the most closely related receptor to the family of P2Y receptors (21). This novel disulfide bridge would covalently constrain the helical bundle in a circular arrangement (33).

Moreover, each of the charged residues of EL2 and EL3 regions has been mutated, leading to the observation that three of these residues (Asp<sup>204</sup>, Glu<sup>209</sup>, and Arg<sup>287</sup>) appear to be involved in receptor activation and/or ligand recognition. Unfortunately, due to the lack of a high affinity radioligand (34), we could not distinguish between effects on agonist binding or on G protein coupling efficiency. At present the function of Asp<sup>204</sup> remains to be clarified, but this position appears to be very sensitive to even minor structural changes. Glu<sup>209</sup> was substituted by different amino acids, and all mutant receptors (except E209A) were fully active and responded in a manner indistinguishable from wild-type receptors (Table I). This indicates that this carboxylate group is more likely involved in hydrogen bonding rather than an ionic interaction. The conformational requirements of this hydrogen bonding would be largely flexible, since deletion of one methylene group (E209D mutant receptor) or change of the charge (E209R mutant receptor) had no further influence on receptor activation.

Arg<sup>287</sup> was substituted by various amino acids. Removal (R287Q mutant) or change (R287E mutant) of the charge of the amino acid side chain was not tolerated. Substitution of arginine by lysine (R287K mutant) could partially to fully retain receptor activity, depending on the length of the phosphate chain of the agonist used for receptor stimulation. Hence, Arg<sup>287</sup> appears to participate in a direct ionic interaction with the phosphate group of an agonist, which is crucial for receptor activation.

The possible existence of an ionic bridge between EL2 and EL3 via the side chains of Glu<sup>209</sup> and Arg<sup>287</sup> could not be clarified by mutagenesis. Both residues might have a combined function in maintaining the overall conformation of the receptor and/or through direct interaction with the ligand, as indicated by the different response of the R287K mutant receptor to different agonists (Table I). This issue is being explored in more detail through molecular modeling and molecular dynamics simulations (33). An energetically sound conformational hypothesis for the receptor has been calculated that includes transmembrane (TM) domains (using the electron density map of rhodopsin as a template), extracellular loops, and a truncated N-terminal region. ATP may be docked in the receptor, both within the previously defined TM cleft and within two other regions of the receptor, termed meta-binding sites, defined by the extracellular loops. The first meta-binding site is located outside of the TM bundle, between EL2 and EL3, and the second meta-binding site is positioned immediately underneath EL2 (33). In meta-binding site I, the side chain of Glu<sup>209</sup> (EL2) is within hydrogen bonding distance (2.8 Å) of the ribose O-3', and Arg<sup>287</sup> (EL3) coordinates both  $\alpha$ - and  $\beta$ -phosphates of the triphosphate chain, consistent with the insensitivity in potency of the 5'-monophosphate agonist, HT-AMP, to mutation of Arg<sup>287</sup>. Additional experiments showed a selective reduction of potency for 3'-NH<sub>2</sub>-ATP in activating the E209R mutant receptor (33). This is consistent with the hypothesis of direct contact between Glu<sup>209</sup> in EL2 and the nucleotide ligands, because the 3'-NH<sub>2</sub> group is positively charged and expected to have a repulsive interaction with the positively charged arginine in the E209R mutant receptor.

Our data have identified two or three stable, steric constraints on the conformation of otherwise highly flexible peptide loops suggesting a preferred three-dimensional arrangement of these ELs (33). These constraints might be responsible for proper positioning of amino acids involved in ligand recognition and/or receptor activation. Fig. 6 visually summarizes the currently available mutagenesis data for the human P2Y<sub>1</sub> receptor, obtained in the present and previous studies (5).

Unlike the transmembrane domains in GPCRs, the length and amino acid composition of the extracellular loops is highly variable. However, it is possible that the position rather than the actual amino acid within the extracellular loops, especially the second loop, is important for ligand recognition. For the human P2Y<sub>1</sub> receptor, two amino acids, Asp<sup>204</sup> and Glu<sup>209</sup>, were identified as being critical for receptor activation. These amino acids are located two and seven residues, respectively, beyond the Cys that forms the critical disulfide bridge between EL2 and TM3 (Fig. 6 and 7). Previous studies have shown the involvement of EL2 of other GPCRs in ligand recognition (Fig. 7). The amino acid in position Cys+2<sup>2</sup> was found to be involved in ligand binding for the human Y1 neuropeptide Y receptor (35), the thyrotropin-releasing hormone receptor of rat and mouse (14, 36), and subtype-specific agonist binding in bombesin receptors (37) and cholecystokinin receptor types A and B (38). For the substance P (NK-1) receptor from rat a six-amino acid portion of EL2, including the Cys+2 position, was found to be labeled by a photoreactive analogue of substance P (39). Almost the same region of the human NK-1 receptor was found to be responsible for a loss of agonist binding affinity, when substituted with the corresponding sequence of the human NK-3 receptor (40). A study of chimeric adenosine A<sub>1</sub>/A<sub>3</sub> receptors identified an 11-amino acid portion of EL2, including the critical cysteine, to be responsible for subtype specificity of ligand binding (13). Interestingly, in this chimeric receptor 8 amino acid residues were altered, but the amino acid in position Cys+2 was conserved. In the human A<sub>2A</sub> receptor, a glutamate residue three amino acids C-terminal to the cysteine in EL2 was found to be involved in ligand binding (12). Interestingly, the position Cys+3 was not conserved in the study of chimeric adenosine A<sub>1</sub>/A<sub>3</sub> receptors (13). For the AT1 angiotensin receptor, the position Cys+3 was also found to interact with the agonist (41). For the α<sub>1</sub>-adrenergic receptor, three amino acids beyond the Cys were found to be responsible for a subtype-specific antagonist binding profile (15).

Although there exist various examples in the literature for the importance of position Cys+2, there are only a few reports that correlate with the importance of the glutamic acid at the Cys +7 position. The only report showing the identical position to be important for ligand recognition was found for the human Y1 neuropeptide Y receptor (35). Studies on thrombin receptors have found a glutamic acid at the Cys+6 position to be important for agonist specificity (42). The same group published a report on chimeric constructs of human and *Xenopus* thrombin receptors, showing that an exchange of the C-terminal portion of the EL2, including position Cys+7, results in a robust constitutive activity (43). Hence, it appears that this portion of the EL2 might be important for maintaining the receptor in a resting conformation. None of the positions that were found to be important in our study were found to be important in the human interleukin-8 type A receptor (44). Nevertheless, six amino acids in EL2 play an important role in agonist binding and/or subsequent Ca<sup>2+</sup> mobilization.

The situation is different for EL3, because there is no conserved disulfide bridge in this loop that could be used as a reference point for a sequence alignment. A smaller number of studies reported amino acids in this loop to be important for proper receptor function. In most cases the reported amino acids were located in vicinity to the TMs. In various GPCRs, amino acids in the EL3 close to the top of TM6 (within 4 amino acids) are important for receptor function or subtype specificity (30–32, 35, 40, 45). This region would correspond to Arg<sup>287</sup>, which we found to be important for receptor function.

In conclusion, our data so far are consistent with the presence of two critical disulfide bridges and involvement of charged residues in ligand recognition. As suggested by mutagenesis and molecular modeling, Glu<sup>209</sup> and Arg<sup>287</sup> serve bifunctional roles. In the

<sup>2</sup>The amino acids in positions Cys+2, Cys+3, and Cys+7 are located 2, 3, and 7 residues, respectively, beyond the cysteine.



model Glu<sup>209</sup> interacts with the 3'-OH group of the ribose moiety, whereas Arg<sup>287</sup> interacts with the  $\alpha$ -phosphate of ATP (33). In the resting state of the receptor, these residues are likely to form an ionic bridge between EL2 and TM6, thus acting as a gate for ligand entry to the binding site within the helical bundle. The function of Asp<sup>204</sup> still remains to be clarified, but since the shift in concentration-response curves was consistent among mono-, di-, or triphosphates, this result most likely excludes a direct involvement of this amino acid in possible Mg<sup>2+</sup> coordination.

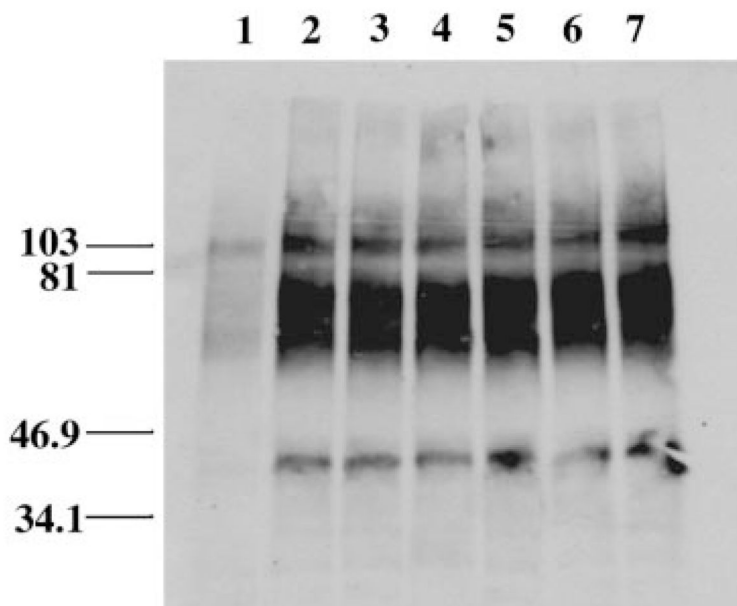
## Acknowledgments

We thank Dr. Ivar von Kügelgen for helpful discussions and Dr. Yong Chul Kim for synthesis of HT-AMP (Laboratory of Bioorganic Chemistry, NIDDK, National Institutes of Health). Furthermore we would like to thank the Information system for G-protein-coupled receptors (<http://swift.embl-heidelberg.de/7tm/>) and the G-protein-coupled receptor mutant data base (<http://www-grap.fagmed.uit.no/GRAP/homepage.html>) for valuable information.

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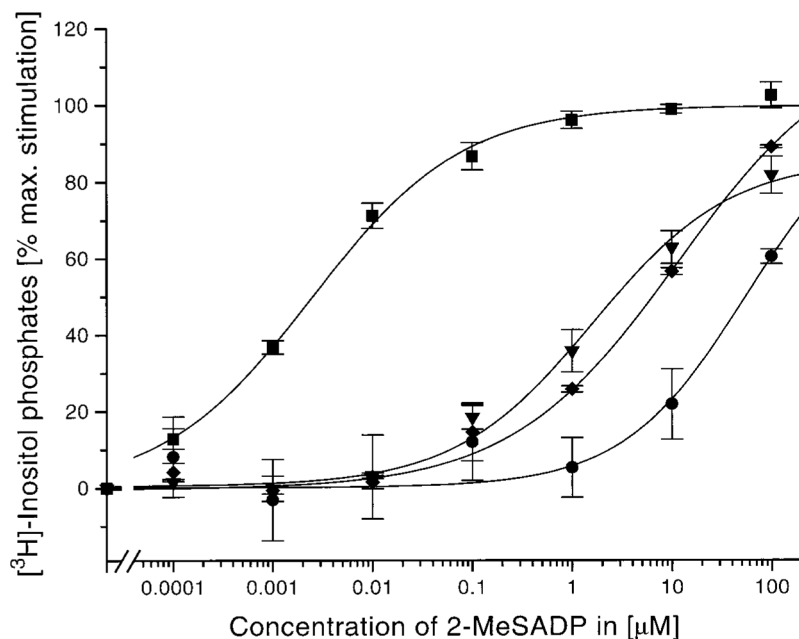
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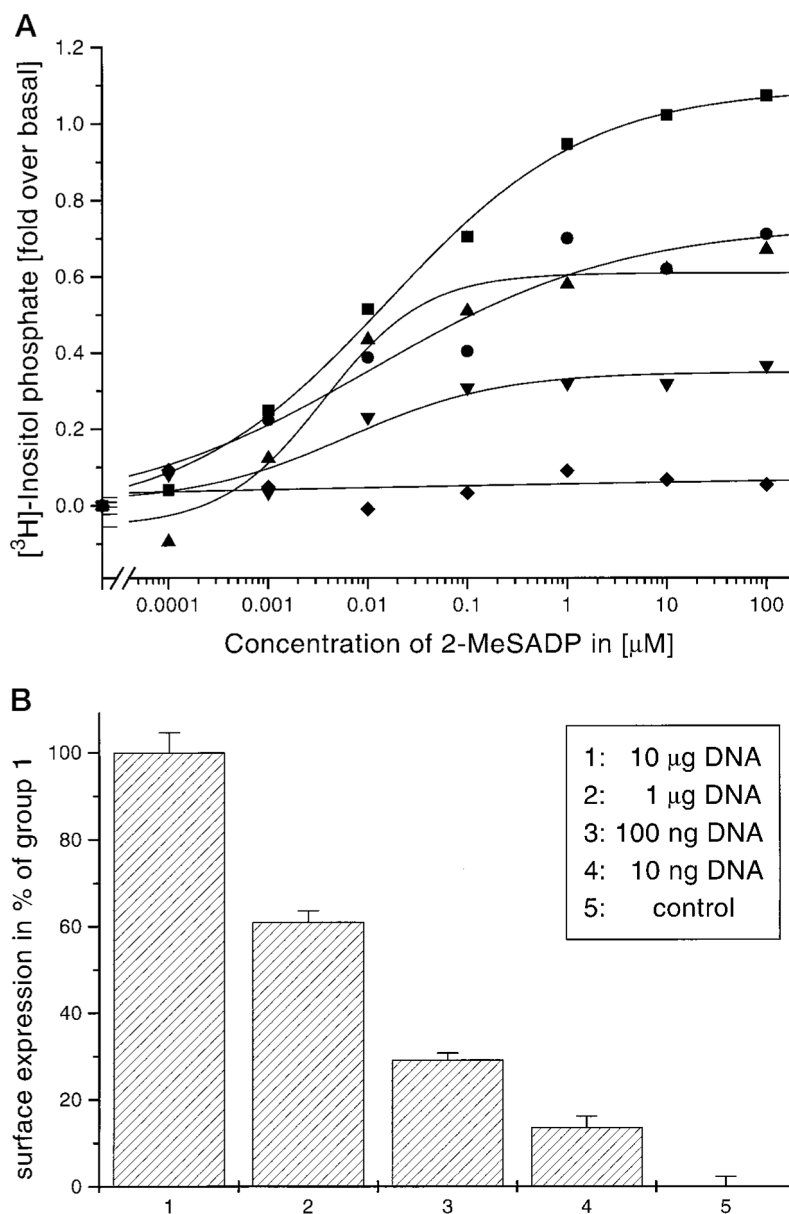
**Fig. 1. Western blot analysis of receptor mutant expression**

Wild-type and all cysteine mutant receptors were transiently expressed in COS-7 cells. Samples containing 50  $\mu$ g of solubilized membrane proteins prepared from whole transfected COS-7 cells were applied to each lane and resolved by SDS-polyacrylamide gel electrophoresis. Proteins were detected with a polyhistidine monoclonal antibody directed against the hexa-His tag at the C-terminal domain of all receptors and a horseradish peroxidase-conjugated sheep anti-mouse antibody. Lanes 1–7 correspond to control (transfected with PCD-vector, lane 1), wild type (lane 2), C42A (lane 3), C124A (lane 4), C202A (lane 5), C296A (lane 6), and C42A/C296A (lane 7) mutant receptors. Lanes 2–7 show an additional band at approximately 42 kDa, which is the size expected for the wild-type P2Y1 and mutant receptors. All bands are of similar intensity, indicating equal expression levels for wild-type and mutant receptors.

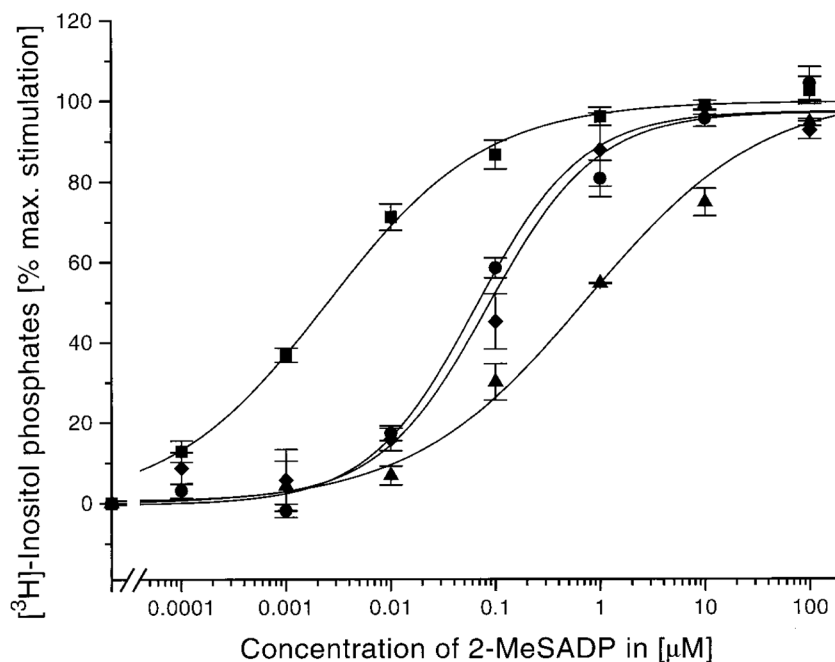


**Fig. 2. Concentration-response curves of P2Y<sub>1</sub> receptors with mutated cysteine residues in the N-terminal domain and the third extracellular loop**

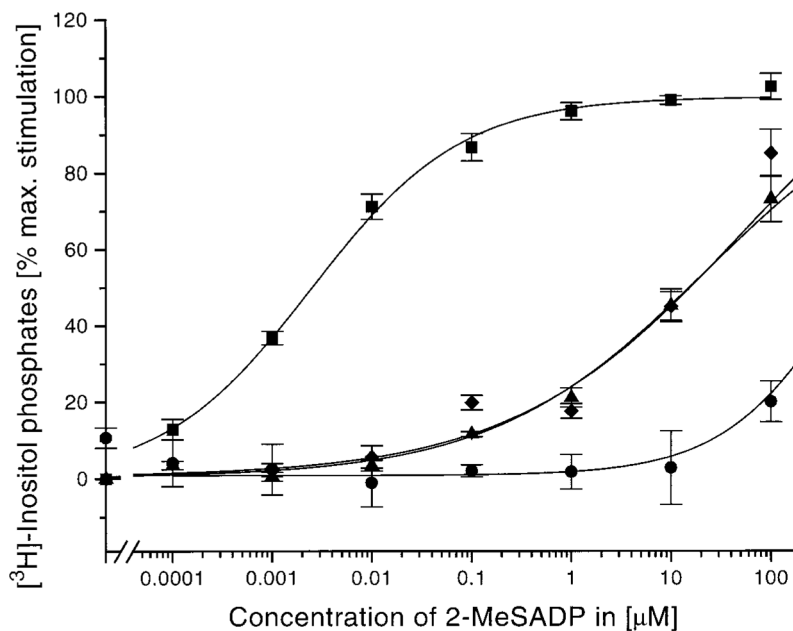
Wild-type human P2Y<sub>1</sub> receptor (*squares*) or mutant receptors in which Cys<sup>42</sup> (C42A, *circles*) and/or Cys<sup>296</sup> (C296A, *diamond*, and C42A/C296A, *inverted triangle*) were converted to alanine and transiently expressed in COS-7 cells. [<sup>3</sup>H]Inositol phosphate accumulation was measured following a 30-min incubation with increasing concentrations of 2-MeSADP in the presence of 10 mM LiCl (see “Experimental Procedures” for details). Maximal responses ranged from 2.5- to 4-fold increases in [<sup>3</sup>H]inositol phosphate accumulation over basal. Concentration-response curves represent the mean values  $\pm$  S.E. of 2 to 6 replicate experiments.



**Fig. 3. Stimulation of PLC in COS-7 cells transiently expressing wild-type human P2Y<sub>1</sub> receptors (A, concentration-response curves; B, surface expression of receptor)**  
 COS-7 cells were transfected with varied amounts of plasmid DNA coding for human P2Y<sub>1</sub>, supplemented with the PCD-PS vector DNA to keep the amount of total DNA constant at 10 μg/dish. A, concentration-response curves for activation of PLC by stimulation with 2-MeSADP. Transfections were done with the following amounts of P2Y<sub>1</sub> plasmid DNA: 10 μg (squares), 1 μg (circles), 100 ng (triangle), 10 ng (inverted triangle), and none (diamonds). B shows receptor surface expression of the same transfected cells as determined by ELISA (see “Experimental Procedures” for details). Surface expression is presented as percent expression relative to the expression observed in cell transfection with 10 μg of plasmid coding for human P2Y<sub>1</sub>.

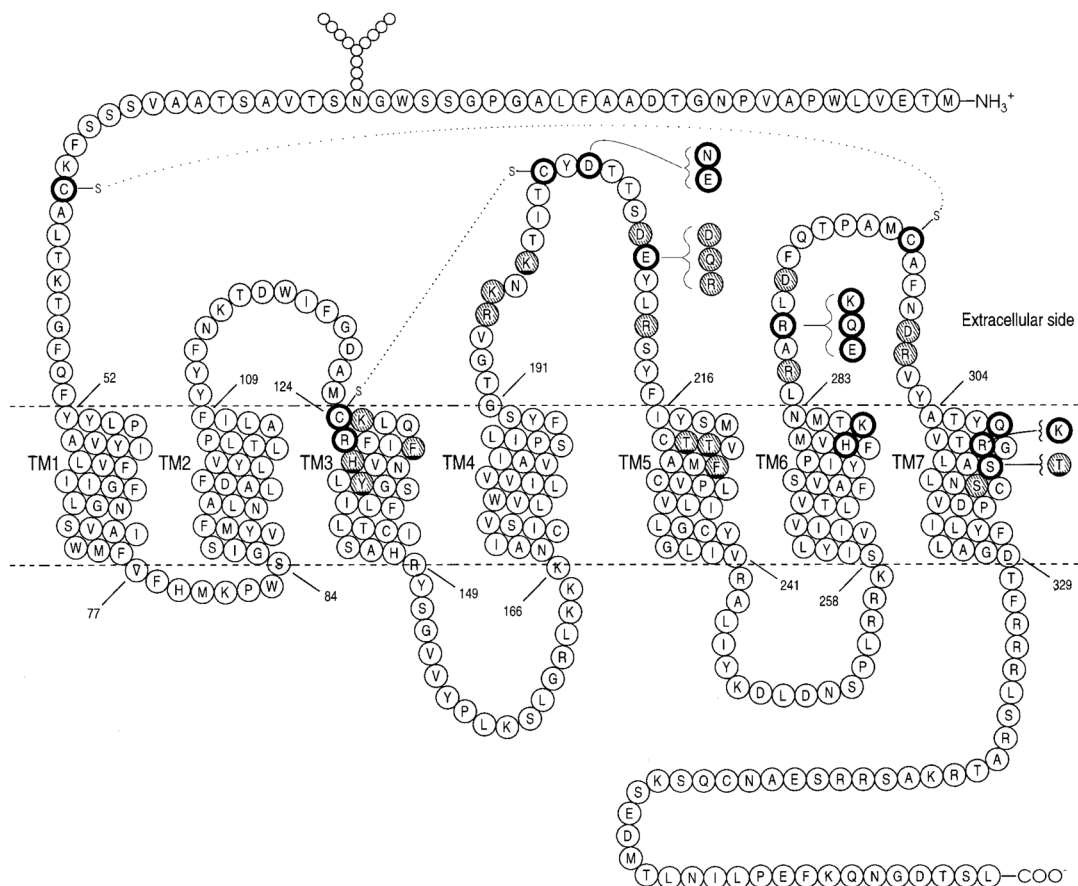


**Fig. 4. Concentration-response curves of P2Y<sub>1</sub> receptors with mutated Asp<sup>204</sup> residues in the second extracellular loop**  
 Wild-type human P2Y<sub>1</sub> receptor (*squares*) or mutant receptors in which Asp<sup>204</sup> was mutated to Ala (D204A, *circles*), Glu (D204E, *diamond*), or to Asn (D204N, *triangle*) were transiently expressed in COS-7 cells. [<sup>3</sup>H]Inositol phosphate accumulation was measured following a 30-min incubation with increasing concentrations of 2-MeSADP in the presence of 10 mM LiCl (see “Experimental Procedures” for details). Maximal responses ranged from 2.5- to 4-fold increases in [<sup>3</sup>H]inositol phosphate accumulation over basal. Concentration-response curves represent the mean values ± S.E. of 2 to 6 replicate experiments.



**Fig. 5. Concentration-response curves of P2Y<sub>1</sub> receptors with mutated residues in the second (Glu<sup>209</sup>) and third extracellular loop (Arg<sup>287</sup>)**

Wild-type human P2Y<sub>1</sub> receptor (*squares*) or mutant receptors in which Glu<sup>209</sup> (E209A, *triangle*) and/or Arg<sup>287</sup> (R287A, *diamond*, and E209A/R287A, *circle*) were mutated to alanine and transiently expressed in COS-7 cells. [<sup>3</sup>H]inositol phosphate accumulation was measured following a 30-min incubation with increasing concentrations of 2-MeSADP in the presence of 10 mM LiCl (see “Experimental Procedures” for details). Maximal responses ranged from 2.5- to 4-fold increases in [<sup>3</sup>H]inositol phosphate accumulation over basal. Concentration-response curves represent the mean values  $\pm$  S.E. of 2 to 6 replicate experiments.



**Fig. 6. Hypothetical human P2Y<sub>1</sub> receptor topology**

All information is given for receptor stimulation upon application of 2-MeSADP. *Shaded* amino acids exhibited 5-fold change in EC<sub>50</sub> when mutated to alanine. *Shaded and underlined* amino acids displayed a 5- to 20-fold shift when mutated to alanine or other amino acids as indicated. *Bold circled* amino acids showed >20-fold shift when mutated to alanine or other amino acids as indicated. The included data about residues in the transmembrane domain were taken from Jiang *et al.* (5). Only one (Asn<sup>27</sup>) of 4 possible glycosylation sites is indicated. The other possible glycosylation sites are Asn<sup>11</sup>, Asn<sup>113</sup>, and Asn<sup>197</sup>.



|                   |     |   | Ref. |
|-------------------|-----|---|------|
| P2Y1 human        | 194 | VRKNKTIITCY <b>D</b> TTSDEYLRS  |      |
| NPY human         | 179 | MTDEPFQNVTLDAYKDKYVCF <b>D</b> QFPSDSHRL  | [35] |
| TRH-R mouse       | 169 | STYKNAVVS <b>C</b> GYKISRNYYSPIYL   | [14] |
| TRH-R rat         | 166 | LNI <b>S</b> TYKDAI <b>V</b> IS <b>C</b> GYKISRNYYSPIYL                           | [36] |
| GRP-R mouse       | 180 | FSDLHPFHVDTNQTFIS <b>C</b> APYPHSNELHPKIHSM                                       | [37] |
| BRS-3 human       | 186 | FSNVYTRDPNKNMTFES <b>C</b> TSYPVSKKLLQEIHSL                                       | [37] |
| CCKBR rat         | 195 | VQPVGPRV <b>L</b> Q <b>C</b> MHRWPSARVQQ  | [38] |
| α1a AR rat        | 168 | PAP <b>E</b> DE <b>T</b> IC <b>Q</b> INEE   | [15] |
| AT1 rat           | 163 | AVIHRNVYFI <b>E</b> NTNITVCAF <b>H</b> YESRNSTLP                                  | [41] |
| A2a human         | 144 | NNCGQPKEGKNHSQGC <b>G</b> EVACL <b>F</b> EDVVP                                    | [12] |
| A1 bovine         | 147 | NNLSAVERDWLANGSV <b>G</b> EPV <b>E</b> <u>CQFEKVISME</u>                          | [13] |
| A3 rat            | 151 | WNRKVTLELSQNS <b>S</b> TL <u>SCHFRS</u> VVGLDTM                                   | [13] |
| NK1 rat           | 170 | TTETMP <b>S</b> <u>RVVCM</u> IEWPEHPNRTYEKA                                       | [39] |
| NK1 human         | 170 | TTETMP <b>S</b> <u>RVVCM</u> IEWPEHPNKIYEKV                                       | [40] |
| IL-8-type A human | 176 | QAYHPNNS <b>S</b> PV <b>C</b> Y <b>E</b> V <b>L</b> GN <b>D</b> TAKWRMVL <b>R</b> | [44] |
| THR-R human       | 245 | QVPGLNIT <b>T</b> CHDVL <b>N</b> ETL <b>L</b> EGY <b>A</b>                        | [42] |
| THR-R xenopus     | 244 | KIPRLDI <b>T</b> TTCHDVL <u>DLKDLKDFYI</u>  | [43] |

**Fig. 7. Alignment of extracellular loop 2 amino acid sequences from various GPCRs**  
Residues in *boldface* were identified by point mutations and shown to be important for receptor function, and *underlined* sequences were important for receptor function in chimeric receptors (see “Discussion” for details). All sequences were aligned with respect to the cysteine forming a disulfide bridge between EL2 and TM3. The numbers on the *left* indicate the position of the starting amino acid in the receptor sequence.

**Table I**  
**Receptor-stimulated PLC activation ( $[^3\text{H}]$ inositol phosphate accumulation) and surface expression assays (ELISA) of wild-type and mutant human P2Y<sub>1</sub> receptors**

EC<sub>50</sub> values are mean values  $\pm$  S.E. of two to seven independent experiments each done in duplicate. Maximal stimulation for each receptor was between 2.5- and 4.5-fold over basal. Determination of surface expression represents the average of six to eight experiments.

| Construct            | EC <sub>50</sub>     |                      |                      | Expression<br>% |
|----------------------|----------------------|----------------------|----------------------|-----------------|
|                      | 2-MeSATP             | 2-MeSADP             | HT-AMP               |                 |
|                      | <i>n<sub>M</sub></i> | <i>n<sub>M</sub></i> | <i>n<sub>M</sub></i> |                 |
| Wild-type            | 4.9 $\pm$ 3.3        | 2.2 $\pm$ 0.5        | 1,420 $\pm$ 580      | 100 $\pm$ 3     |
| N-terminal domain    |                      |                      |                      |                 |
| C42A                 | 21,200 $\pm$ 6,500   | 48,000 $\pm$ 500     | >100,000             | 12 $\pm$ 4      |
| Extracellular loop 1 |                      |                      |                      |                 |
| C124A                | NA <sup>a</sup>      | NA                   | NA                   | 10 $\pm$ 1      |
| K125A                | 12.9 $\pm$ 1.9       | 5.9 $\pm$ 7.8        | 6,600 $\pm$ 2,850    | 44 $\pm$ 3      |
| Extracellular loop 2 |                      |                      |                      |                 |
| R195A                | 17.6 $\pm$ 14.6      | 4.7 $\pm$ 0.6        | 2,100 $\pm$ 680      | 218 $\pm$ 3     |
| K196A                | 4.0 $\pm$ 0.6        | 2.5 $\pm$ 0.8        | 2,660 $\pm$ 540      | 175 $\pm$ 4     |
| K198A                | 5.0 $\pm$ 2.8        | 17.5 $\pm$ 2.1       | 2,720 $\pm$ 1,350    | 241 $\pm$ 3     |
| C202A                | NA                   | NA                   | NA                   | 0 $\pm$ 7       |
| D204A                | 119 $\pm$ 17         | 66.5 $\pm$ 11.5      | 22,800 $\pm$ 3,200   | 183 $\pm$ 5     |
| D204N                | 1,000 $\pm$ 70       | 595 $\pm$ 5.0        | >100,000             | 29 $\pm$ 2      |
| D204E                | 267 $\pm$ 3.4        | 145 $\pm$ 15.4       | 24,400 $\pm$ 5,700   | 81 $\pm$ 2      |
| D208A                | 27.0 $\pm$ 11.6      | 3.6 $\pm$ 0.7        | 1,990 $\pm$ 720      | 109 $\pm$ 4     |
| E209A                | 17,000 $\pm$ 8,500   | 17,200 $\pm$ 7,400   | >100,000             | 28 $\pm$ 3      |
| E209D                | 3.1 $\pm$ 0.2        | 3.2 $\pm$ 0.6        | 9,310 $\pm$ 5,200    | 127 $\pm$ 8     |
| E209Q                | 5.1 $\pm$ 1.5        | 8.6 $\pm$ 2.1        | 6,410 $\pm$ 1,880    | 135 $\pm$ 13    |
| E209R                | 2.7 $\pm$ 0.2        | 4.9 $\pm$ 1.3        | 4,180 $\pm$ 320      | 77 $\pm$ 3      |
| R212A                | 3.9 $\pm$ 2.9        | 7.8 $\pm$ 5.5        | 1,810 $\pm$ 780      | 93 $\pm$ 3      |
| Extracellular loop 3 |                      |                      |                      |                 |
| R285A                | 33.2 $\pm$ 24.5      | 9.2 $\pm$ 5.4        | 2,440 $\pm$ 1,530    | 129 $\pm$ 3     |
| R287A                | 52,100 $\pm$ 35,100  | 14,800 $\pm$ 2,600   | >100,000             | 161 $\pm$ 4     |
| R287K                | 80.9 $\pm$ 3.1       | 74.9 $\pm$ 5.5       | 4,210 $\pm$ 610      | 193 $\pm$ 1     |
| R287Q                | 9,670 $\pm$ 5,310    | 5,290 $\pm$ 360      | >100,000             | 77 $\pm$ 1      |
| R287E                | >100,000             | >100,000             | >100,000             | 76 $\pm$ 2      |
| D289A                | 10.5 $\pm$ 7.1       | 3.9 $\pm$ 0.4        | 3,940 $\pm$ 280      | 79 $\pm$ 13     |
| C296A                | 19,600 $\pm$ 5,700   | 5,160 $\pm$ 920      | >100,000             | 20 $\pm$ 5      |
| D300A                | 25.7 $\pm$ 12.2      | 9.8 $\pm$ 0.5        | 10,900 $\pm$ 770     | 124 $\pm$ 3     |
| R301A                | 9.0 $\pm$ 7.3        | 2.6 $\pm$ 0.3        | 3,480 $\pm$ 690      | 111 $\pm$ 4     |
| Double mutants       |                      |                      |                      |                 |
| E209R/R287E          | >100,000             | >100,000             | >100,000             | 117 $\pm$ 4     |
| E209A/R287A          | >100,000             | >100,000             | >100,000             | 77 $\pm$ 3      |

| Construct  | EC <sub>50</sub>     |                      |                      | Expression |
|------------|----------------------|----------------------|----------------------|------------|
|            | 2-MeSATP             | 2-MeSADP             | HT-AMP               |            |
|            | <i>n<sub>M</sub></i> | <i>n<sub>M</sub></i> | <i>n<sub>M</sub></i> |            |
| C42A/C296A | 2,250 ± 160          | 3,420 ± 1,380        | >100,000             | 8 ± 5      |

<sup>a</sup>NA, no activation detectable for 100 μM agonist.