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ORIGINAL PAPER

Defining the nucleotide binding sites of P2Y receptors using rhodopsin-based homology modeling

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Abstract Ongoing efforts to model P2Y receptors for extracellular nucleotides, i.e., endogenous ADP, ATP, UDP, UTP, and UDP-glucose, were summarized and correlated for the eight known subtypes. The rhodopsin-based homology modeling of the P2Y receptors is supported by a growing body of site-directed mutagenesis data, mainly for $P2Y_1$ receptors. By comparing molecular models of the P2Y receptors, it was concluded that nucleotide binding could occur in the upper part of the helical bundle, with the ribose moiety accommodated between transmembrane domain (TM) 3 and TM7. The nucleobase was oriented towards TM1, TM2, and TM7, in the direction of the extracellular side of the receptor. The phosphate chain was oriented towards TM6, in the direction of the extracellular loops (ELs), and was coordinated by three critical cationic residues. In particular, in the $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors the nucleotide ligands had very similar positions. ADP in the $P2Y_{12}$ receptor was located deeper inside the receptor in comparison to other subtypes, and the uridine moiety of UDP-glucose in the $P2Y_{14}$ receptor was located even deeper and shifted toward TM7. In general, these findings are in agreement with the proposed binding site of small molecules to other class A GPCRs.

Keywords GPCRs · P2Y receptors · Homology modeling · Binding mode · Ligand recognition · Nucleotides

Introduction

Eight subtypes of P2Y nucleotide receptors have now been defined and are under intense investigation for their roles in the immune, cardiovascular, and central nervous systems [1]. There is a wide diversity of agonist molecules within this family of G protein-coupled receptors (GPCRs). For some subtypes ($P2Y_1$, $P2Y_{12}$) extensive reports have been published on the structure activity relationship (SAR) of selective ligands, while the medicinal chemistry of other subtypes is less explored ($P2Y_2$, $P2Y_4$, $P2Y_6$) or nearly unexplored ($P2Y_{11}$, $P2Y_{13}$, $P2Y_{14}$). Molecular modeling of P2Y receptors is playing an increasing role in the design of new agonists and antagonists.

This paper summarizes and correlates the results of ongoing efforts to model the eight P2Y receptors. It is to be noted that these models are subject to modification and refinement as more pharmacological data are obtained.

The structural comparison of the known subtypes of the P2Y receptors was performed based on sequence analysis, mutagenesis data (mostly data available for the $P2Y_1$ subtype), and molecular modeling. A comprehensive alignment was reported by Costanzi et al. [2]. The phylogenetic analysis indicated that the P2Y

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receptors are subdivided into two main subfamilies. The first subfamily ($P2Y_1$–like receptors) consists of G_q-coupled subtypes $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$, while the $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ G_i-coupled receptors belong to the second subfamily ($P2Y_{12}$–like receptors). It was clearly demonstrated that the sequence similarity was high within a given subfamily, while receptors from different subfamilies show less conservation in their sequences (Fig. 1).

To facilitate the comparison among receptors, throughout this paper we use the GPCR residue indexing system as explained elsewhere [3].

The ligand binding site of the $P2Y_1$ receptor

Principal binding site in the transmembrane region

The $P2Y_1$ receptor is the most studied subtype of the P2Y receptors. The first molecular model of any P2Y receptor was built based on the 2D structure of bovine rhodopsin for the chicken homologue of the $P2Y_1$ receptor by van Rhee et al. and published in 1995 [4]. Molecular docking studies in conjunction with available pharmacological data indicated that K269 (6.55) and R299 (7.39) play a key role in nucleotide binding due to the electrostatic interactions with phosphate groups of the ligand (Fig. 2). Additionally, an interaction of Q296 (7.36) with the N^6-amino group of the adenine ring of ATP was suggested, while S303 (7.43) and S306 (7.46) were proposed to interact with the ribose moiety, and H121 (3.33), H266 (6.52), and Y125 (3.37) with the phosphate chain.

Later these findings were supported experimentally by mutational analysis performed at the human $P2Y_1$ receptor [7]. In that study 2–methylthioadenosine-5′-triphosphate (2-MeSATP) was used as a potent $P2Y_1$ receptor agonist, although the corresponding diphosphate is more potent. Another cationic residue (R3.29), previously not identified, was found critical for the activity of 2-MeSATP. In fact, 2-MeSATP had no activity in cells expressing the R128(3.29)A, R310(7.39)A, and S316(7.43)A mutant receptors. Also, the potency of the ligand was reduced in the K280(6.55)A and Q307(7.36)A mutant receptors. However, the potency of 2-MeSATP was unchanged in the S317(7.46)A mutant receptor. A 7 to 18-fold reduced potency of the ligand was observed with the Ala replacement of F131 (3.32), H132 (3.33), Y136 (3.37), F226 (5.47), or H277 (6.52) demonstrating a lesser role for these residues in ligand binding. Also, T221 (5.42) and T222 (5.43) were proposed to be involved in the recognition of the γ-phosphate group of the ligand.

Rhodposin-based homology modeling of the complex of $P2Y_1$ with its agonists was refined in our laboratories in recent years [2, 5, 8]. It indicated that the ribose moiety of ATP and ADP was situated in a hydrophilic pocket between transmembrane domain

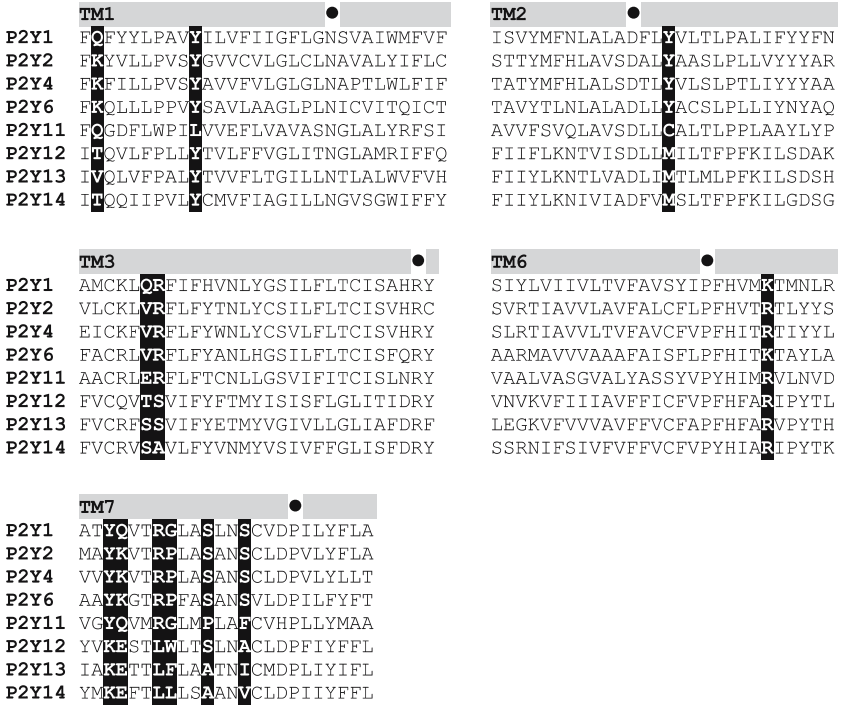

Fig. 1 Sequence alignment of selected regions of the P2Y receptor sequences, indicating (•) The most conserved residue in each TM. This alignment is based on a published alignment of 68 related protein sequences [2]. Residues highlighted in black are discussed in the section Comparison of functional amino acid residues within the two subfamilies of P2y receptors

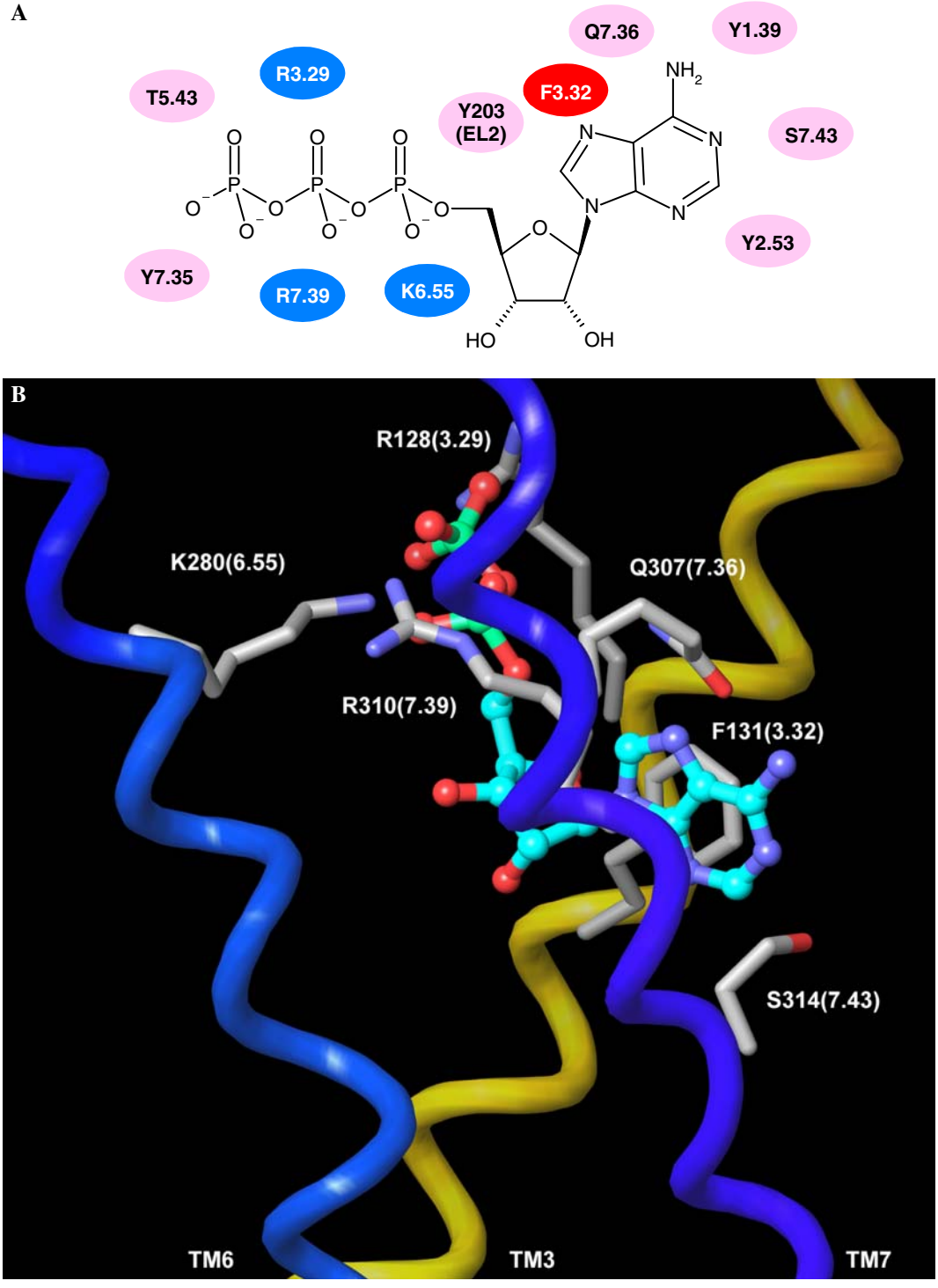

Fig. 2 (**A**) Key residues of the human $P2Y_1$ receptor involved in ligand recognition, as indicated by molecular modeling [2, 5, 6]. The ligand shown is ATP, which is less potent than ADP at this subtype, but was docked to the receptor by Moro et al. [5, 6]. The residues are colored as follows: pink—polar, uncharged residues; blue—positively charged residues; red—non-polar residues. The involvement in receptor activation of all residues shown, except for Y1.39 and Y2.53, has also been shown using mutagenesis. (**B**) Representation of the $P2Y_1$–ADP complex [2]. The sugar is accommodated between TM3 and TM7; the phosphate chain is oriented toward TM6 and is coordinated by three cationic residues located in TM3, TM6, and TM7; the nucleobase is oriented in the opposite direction and is coordinated by two residues located in TM7. (**C**) The superimposition of the $P2Y_{1, 2, 4, 6, 12, 14}$ receptors and their ligands (the $P2Y_1$ receptor is represented by colored tubes). ADP from the $P2Y_1$ receptor is colored in red; UTP–$P2Y_2$ is in yellow; UTP–$P2Y_4$ is in light blue; UDP–$P2Y_6$ is in dark blue; ADP–$P2Y_{12}$ is in green; UDP–glucose–$P2Y_{14}$ is in magenta

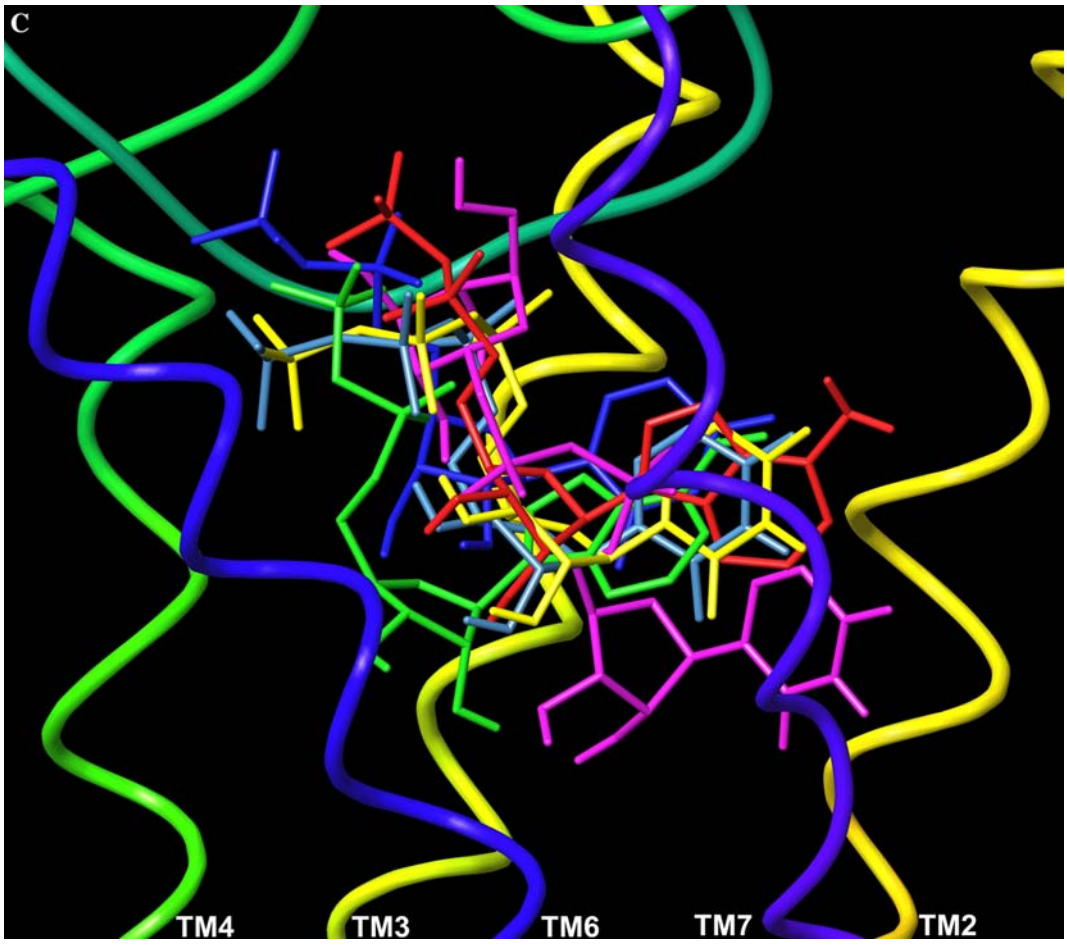

Fig. 2 continued

(TM) 3 and TM6 (Fig. 2). The studies also indicated that the adenine ring of the ligands interacts with residues from TM7 and points in the direction of TM1 and TM2. Q7.36 and S7.43 were found to form critical interactions with the nucleobase: the first was H-bonded with the N^6-amino group and the *N*7-nitrogen, the second with the *N*1-nitrogen. The three critical cationic residues (R3.29, K6.55, and R7.39) appeared in the model near the triphosphate chain of ATP. In particular, R3.29 was close to the β- and γ-phosphates, K6.55 was near the α-phosphate, and R7.39 interacted with the β-phosphate group. More recently, further molecular modeling analyses combined with new site-directed mutagenesis data [2] suggested the involvement of Y203 of extracellular loop 2 (EL2) and Y306 (7.35) in the binding of agonists and antagonists at $P2Y_1$ receptor. In our models, both Tyr appear close to the phosphate chain of the ligands. Residues fundamental for the nucleotide recognition by $P2Y_1$ are summarized in Fig. 2A. Major et al. obtained a similar arrangement of the $P2Y_1$ receptor agonists inside the putative binding site after a molecular dynamics simulation of the $P2Y_1$ receptor in the phospholipid bilayer [9, 10]

Site directed mutagenesis data were also used to identify the residues involved in the recognition of $P2Y_1$ antagonists [5, 11]. The ability of the nucleotide competitive antagonist MRS2179 (N^6-methyl-2′-deoxyadenosine-3′,5′-bisphosphate) to block stimulation of phospholipase C was lost in F5.27, K6.55, or Q7.36 alanine mutants of the $P2Y_1$ receptor. In contrast, alanine mutations of F3.32, T5.42, H6.52, or S7.46 had no effect on the activity of MRS2179.

The three cationic residues were also shown by modeling to be involved in recognition of bisphosphate groups of $P2Y_1$ antagonists [2, 5, 12]. In particular, it was suggested that R3.29 and R7.39 can interact with the 5′-phosphate group, while K6.55 can directly interact with both 3′- and 5′-phosphate groups of MRS2179 and its (N)-methanocarba (fused cyclopentane and cyclopropane ring system in place of ribose) analogues. Also, Y3.37 was found near the 5′-phosphate and was suggested to be involved in H-bonding

with this group. More recently, conformational analyses of five bisphosphate-containing $P2Y_1$ receptor antagonists (the (N)-methanocarba analogues MRS2279 and MRS2500, the carbocyclic locked nucleotide MRS2584, the anhydrohexitol analogue MRS2283, and the L-α-threofuranosyl analogue MRS2457) confirmed the findings and shed light into the conformational requirements for $P2Y_1$ binding. Additionally, two Tyr residues located in TM1 and TM2, namely Y1.39 and Y2.53, were proposed to be involved in recognition of the antagonists by interacting with the N^6-Me and 2-halogen substituents, respectively [13].

Thus, in our models the principal nucleotide binding site of the $P2Y_1$ receptor was located inside the transmembrane domain, near its extracellular region, similar to small molecule binding sites of other class A GPCRs. The adenine ring seemed to be oriented toward the intracellular region and is located near TM7. The triphosphate chain was oriented toward the ELs and interacts with amino acid residues from TM3, TM6, and TM7 (Fig. 1).

Meta binding sites

The binding of nucleotides to P2Y receptors closely resembles the pattern associated with the interactions of small molecules with class A GPCRs, i.e., most of the coordinating residues are in the transmembrane cleft, with the predominant involvement of TMs 3, 6, and 7. Nevertheless, evidence emerged for a role in ligand recognition of residues of the ELs, principally EL2. In some cases these residues are hypothesized to be in contact with the ligand, in other cases they appear to form secondary ligand binding sites. The latter are also known as meta-binding sites and are generally less energetically stable than the principal binding site [6].

Indeed, it is more challenging to model the flexible, nonconserved extracellular regions than the more conserved TMs. However, site-directed mutagenesis and molecular modeling were used to identify residues in the extracellular domains of the $P2Y_1$ receptor critical for ligand binding [6, 14]. Two putative disulfide bridges in the extracellular regions, forming at C124-C202 (conserved in class A GPCRs) and at C42-C296, appear to be essential for $P2Y_1$ activation in mutagenesis studies. Furthermore, sequence analysis revealed a preponderance of charged residues in EL2 and EL3 of the $P2Y_1$ receptor. Thus, both negatively and positively charged residues in this region of the $P2Y_1$ receptor have been probed through mutagenesis. Some residues appeared critical for the receptor function. For example, mutation of D204 in EL2, even to Glu, resulted in the inability of 2-MeSADP to stimulate the receptor. Moreover, it was shown that the EC_{50} of 2-MeSATP increases 1000-fold in the E209A (EL2) receptor, while the E209D, E209Q, or E209R mutants responded like the wild-type $P2Y_1$ receptor. R287 (EL3) is another charged residue that was found to be important for receptor activation. Similarly to E209, the mutation R287 to Ala, as well as Lys, Gln, or Glu, resulted in a large loss of potency of 2-MeSATP. Replacement of other charged amino acid residues located in the extracellular loops produced only minor changes in receptor activation.

These findings, combined with molecular modeling results, suggested the presence of two extracellular meta-binding sites, which could be reached by a nucleotide ligand on its path to the principal TM binding site. A graphical representation of the model of the meta-binding sites of the $P2Y_1$ receptor was presented in Moro et al. [6]. According to the proposed hypothesis, initially the ligand binds to meta-binding site I, located outside of the transmembrane domain between EL2 and EL3. E209 formed a H-bond with the 3′ oxygen atom of the ribose ring, R287 coordinates the phosphate groups of the ligand, and T47, located in the *N*-terminal domain, interacted with the adenine ring. This site was closer in energy to the principal binding site than meta-binding site II. From meta-binding site I, the ligand could progress to meta-binding site II, located immediately underneath EL2. At this site, the adenine ring was close to Q307 (7.36), the ribose ring in interaction with K280 in TM6, and the triphosphate chain coordinated by S213 (EL2), D204 (EL2), R128 (3.29), and T222 (5.43). Finally, the ligand reached the principal binding site located inside the TM bundle, as described above.

Mutagenesis of EL2 combined with selective reduction of the potency of 3′-amino-3′-deoxy-ATP supported the direct interaction of the ligand with the extracellular region [6].

Comparison of functional amino acid residues within the two subfamilies of P2Y receptors

The data on the most important residues of the P2Y receptors and their proposed role in ligand recognition are summarized in Fig. 1 and Table 1. As mentioned above, three cationic residues located in TM3, TM6, and TM7 seem to be critical for nucleotide recognition by the $P2Y_1$ receptor. Among them, only residue 6.55 appears in all the subtypes of the P2Y receptors, either as Arg or Lys. In particular, residue 6.55 is an Arg in all of the $P2Y_{12}$-like receptors, while within the $P2Y_1$-like

Table 1 Structural role of individual residues of the human $P2Y_1$ receptor, deduced from rhodopsin-based molecular modeling and mutagensis, and comparison to other P2Y subtypes (see [2] and references therein)

Residue[a] in $P2Y_1$	Role in: ADP–$P2Y_1$ complex	Other P2Y complexes[a]	Putative Interactions, Pharmacological Role in $P2Y_1$ [1]	Potency Loss at $P2Y_1$ for Ala mutation (ratio for 2-MeSADP)[a]
TM1				
Y(1.39)	N^6-NH_2	=O4 ($P2Y_{2,4}$),[b] 3-NH ($P2Y_6$),[b] N^6-NH_2 ($P2Y_{12}$)	H-bond donor to S314[c,d,e]	NA
TM2				
Y(2.53)	*N*3	=O2 ($P2Y_{2,4}$)[f]		
TM3				
R(3.29)	α,*β*-phos	*γ*-phos ($P2Y_{2,4}$), α,*β*-phos ($P2Y_6$)	Essential[e,g]	>50,000
F(3.32)	*π* complex with A	*π* complex with U ($P2Y_{2,4,6}$), with A ($P2Y_{12}$)	Modulatory toward agonist, but not antagonist[g]	8
H132 (3.33)	NC		Modulatory[h]	7
TM6				
Y273 (6.48)	NC		Essential for receptor activation, but not binding[e,i]	>3,000
H277 (6.52)	NC		Modulates effect of agonist, but not antagonist[g]	45
K(6.55)	*β*-phos	*β*-phos ($P2Y_6$), α,*β*-phos ($P2Y_{12}$), glucose 3″ ($P2Y_{14}$)	Essential; action of PPADS[e,g,j]	810
TM7				
Y(7.35)	NC	α-phos ($P2Y_{12}$), glucose 3″, 6″ ($P2Y_{14}$)	Modulatory toward agonist effects only[e]	5
Q(7.36)	N^6–NH_2, N7		H-bond acceptor from exocyclic NH of ADP[e,g]	210
R(7.39)	α-phos	α-phos ($P2Y_6$)	Essential[e,g]	>50,000
S(7.43)	N1	3-NH ($P2Y_{2,4}$), = O2 ($P2Y_6$), N^6–NH_2 or N1 ($P2Y_{12}$)	Essential, H-bond donor to adenine N1 or N3;[e,g] H-bond acceptor from Y58[c]	>50,000
N(7.45)	NC	=O2 ($P2Y_{14}$)		
EL2				
D204	NC		Meta-binding site[g]	30
EL2.49	NA	*β*-phos ($P2Y_{12}$), α-phos ($P2Y_{14}$)		

[a]Residue identifier of Ballesteros and Weinstein format X.YZ refers to the TM X and residue YZ with respect to a highly conserved amino acid in each TM numbered 50 [2, 3]. Potency ratio shown is EC_{50} at mutant receptor divided by EC_{50} at the wild type receptor. The EC_{50} of 2-MeSADP at the wild type human $P2Y_1$ receptors was reported as 2.2 ± 0.5[e] or 3 ± 1[d] nM. Ligands at other subtypes: UTP ($P2Y_{2,4}$), UDP ($P2Y_6$), ADP ($P2Y_{12}$), UDP-glucose ($P2Y_{14}$)

[b]Although our studies suggest for $P2Y_6$ and $P2Y_{2,4}$ the two distinct configurations presented in this table, our models do not exclude the possibility for the $P2Y_6$ receptor to establish nucleobase interactions similar to those described for $P2Y_{2,4}$ and vice versa (unpublished data)

[c]Applies only to ground state of receptor

[d]Not mutated, prediction from modeling only

[e]Costanzi et al. 2004 [2]

[f]Our recent modeling studies indicated that Y2.53 of the $P2Y_2$ receptor is probably not directly involved in interactions with UTP, but can form a H-bond with the nitrogen atom at position 1 of ATP (unpublished data)

[g]Moro and Jacobson, 2002 [15], and references therein

[h]Modulatory toward effects of agonist and nucleotide antagonist

[i]Mutation precludes only activation by agonist, but not binding of agonist or antagonist

[j]Guo et al., 2002 [11]

NA, not applicable; EL, extracellular loop; NC, not proposed to be in direct contact with the ligand

receptors it is a Lys in the diphosphate-preferring subtypes (i.e., $P2Y_1$ and $P2Y_{11}$) and an Arg in the triphosphate-preferring subtypes (i.e. $P2Y_2$, $P2Y_4$, and $P2Y_6$). R3.29 and R7.39 are conserved only within the $P2Y_1$ receptor subfamily. To account for cationic coordination of the phosphate moiety in the second subfamily, it was proposed [2] that in the $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptors the role of the latter two residues can by played by two Lys residues, one located in EL2 (immediately before the conserved Cys residue involved in the formation of the conserved disulfide bridge), and the other located in TM7 at position 7.35.

In the $P2Y_1$-like receptors, the residue following the conserved Cys in EL2 also appeared to be involved in interactions with the phosphates. Our sequence alignment showed that this amino acid is conserved as a Tyr in the diphosphate-preferring subtypes and as a His in the triphosphate-preferring subtypes, with the exception of $P2Y_{11}$ where a Ser is present at this position.

The Ser residue at position 7.43 of TM7, crucial for $P2Y_1$ function, is present in all the $P2Y_1$-like receptors with the exclusion of $P2Y_{11}$, which, interestingly, shows a Pro at this position. However, a Ser also occurs at this site in the $P2Y_{12}$ receptor, while the $P2Y_{13}$ and $P2Y_{14}$ receptors show an Ala. The second Ser residue initially proposed to be involved in interactions with the ligand (i.e., S7.46) is present only in the $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors. The $P2Y_{12}$-like receptors show non-conserved, but chemically related nonpolar residues at position 7.46: Ala in the $P2Y_{12}$, Ile in the $P2Y_{13}$, and Val in the $P2Y_{14}$ receptor. There is also a nonpolar, but aromatic residue (F213) at this position in the $P2Y_{11}$ subtype.

Concerning the two Tyr residues proposed to be involved in nucleobase recognition in the $P2Y_1$ receptor, Y1.39 is conserved among all P2Y receptors, with the exception of $P2Y_{11}$, which shows a Leu at this position. Y2.53 is conserved in the $P2Y_{1,\,2,\,4,\,6}$ subtypes, while, at the same position, $P2Y_{11}$ shows a Cys, and $P2Y_{12,\,13,\,14}$ show a Met. Y7.35, proposed to interact with the phosphate moiety of the ligands in the $P2Y_1$ receptor, is conserved among all $P2Y_1$-like subtypes. As already mentioned, there is a key cationic residue (K7.35) in the members of the second subfamily of P2Y receptors.

It follows from the phylogenetic analysis obtained for P2Y receptors [2] that $P2Y_1$ and $P2Y_{11}$ receptors cluster together on the phylogenetic tree, indicating that these two receptors are more highly related to each other than to the other P2Y subtypes. This finding is also supported by a detailed analysis of the binding sites. As mentioned above, $P2Y_1$ shows a Gln at position 7.36, which is critical for the receptor function, and, according to our models, interacts with the nucleobase of the ligands. Interestingly, only $P2Y_{11}$ shows also a Gln residue at position 7.36. At the same site, a Lys is present in $P2Y_2$, $P2Y_4$, and $P2Y_6$ while a Glu is present in all of the $P2Y_{12}$-like receptors. Also, at position 7.40 of $P2Y_1$ and $P2Y_{11}$ there is a Gly, while in $P2Y_{2,4,6}$ there is a Pro. Furthermore, in TM1 there is Gln at position 1.31 of $P2Y_1$ and $P2Y_{11}$, where the other subfamily members show a Lys. In TM3 at position 3.28 (the residue directly before the crucial R3.29) there is a Gln in $P2Y_1$ and a Glu in $P2Y_{11}$ while in $P2Y_{2,4,6}$ there is a there is a Val.

The putative ligand binding sites of $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{12}$, and $P2Y_{14}$ receptors

Detailed homology models have been constructed for other members of the P2Y receptor family [2, 15–19]. The models were built using the program Modeler [20] implemented in InsightII [21]. The X-ray structure of bovine rhodopsin (1F88.pdb) was used as a template for modeling. Five models with different configuration of the loops were built for each receptor. Then, the models were capped with an acetyl group at the amino-terminus and with an *N*-methyl group at the carboxyl-terminus to prevent electrostatic interactions and were optimized by means of the Discover3 module of InsightII, with the AMBER force field. A harmonic restraint of 25 kcal/mol was applied to the backbone atoms of the TMs and EL2. The structures were minimized until an RMS of 0.5 kcal mol^{-1} $Å^{-1}$ was reached; an NVT (constantvolume/constant-temperature) molecular dynamic simulation of 50 ps at 300 K was carried out, with a time step of 1 fs; the average structures from the last 10-ps trajectory of MD were reminimized until an RMS of 0.5 kcal mol^{-1} $Å^{-1}$ was reached. The harmonic restraints were gradually lowered in steps of 5 kcal/mol, minimizing the structure at each step until an RMS of 0.5 kcal mol^{-1} $Å^{-1}$ was reached. When the restraints were finally removed, the structures were submitted to the final energy minimization until an RMS of 0.001 kcal mol^{-1} $Å^{-1}$ was reached.

Although the native ligands of P2Y receptors are structurally diverse nucleotides, the putative binding sites are similar in location and orientation of the docked ligand (Fig. 2C). For example, molecular modeling combined with SAR analysis was recently applied to study the binding modes of UTP and its analogues in the $P2Y_2$ and $P2Y_4$ receptors [16]. A conformational analysis of UTP in the binding pockets of the $P2Y_2$ and $P2Y_4$ receptors was performed by

means of the mixed MCMM/LMCS sampling method as implemented in MacroModel 9.0 [22] The search was performed on the ligand and the residues located within 5 Å of the ligand, while the remaining residues were conformationally frozen. The calculations were conducted with the MMFF force field, using water as implicit solvent and a molecular dielectric constant of 1. The Polak-Ribier Conjugate Gradient was used for the energy minimizations with a convergence threshold of 0.05 $kJ \cdot mol^{-1} \cdot Å^{-1}$.

Since the binding sites of the $P2Y_2$ and $P2Y_4$ receptors appeared to be highly conserved, the UTP binding modes obtained in our modeling studies were also very similar for the two receptors. In analogy with the $P2Y_1$ receptor, in both cases three cationic residues were found to be involved in interactions with the phosphate chain of the ligand. The 3-NH group of the ligand was found to be H-bonded to conserved S7.43. Also, in both receptors, Y1.39 was found to interact with the oxygen atom at position 2, while Y2.53 was located near the oxygen atom at position 4. However, our recent modeling studies indicated that Y2.53 of the $P2Y_2$ receptor is probably not directly involved in interactions with UTP but can form a H-bond with the nitrogen atom at position 1 of ATP (unpublished data). The phenyl ring of F3.32 was found to be involved in π-π interactions with the uracil ring, while F6.51 was found to be in proximity of the 2′-hydroxyl group.

The main difference between the putative binding sites of the $P2Y_2$ and $P2Y_4$ receptors was associated with two residues located in TM2 and EL2. In particular, V90 (2.61) of the $P2Y_2$ receptor corresponds to I92 (2.61) of the $P2Y_4$ receptor, while T182 located in EL2 of the $P2Y_2$ receptor corresponds to L184 of the $P2Y_4$ receptor. These two residues in our models were located near position 5 of the uracil ring of the ligand, and affected the size of this region of the nucleotide-binding pocket for $P2Y_2$ and $P2Y_4$. This difference in the size of the pocket seemed to be critical for the selective recognition of 5-substituted UTP derivatives. For example, 5-methyl UTP is 9.8-fold less potent than UTP at the $P2Y_2$ receptor, but 53-fold less potent than UTP at the $P2Y_4$ receptor [16].

Novel results were obtained in our modeling studies of the $P2Y_6$ receptor [17]. Monte Carlo conformational searches suggested that, as previously proposed for $P2Y_1$, the $P2Y_6$ receptor might have an additional meta-binding site located above the principal binding site. Our Monte Carlo studies and a subsequent molecular dynamics simulation of the $P2Y_6$–UDP complex in the phospholipid bilayer predicted that only the (S)-conformation of the ribose would be tolerated at the principal binding site. The requirement of the (S)-conformation of the ribose ring for the $P2Y_6$ receptor activation was successfully confirmed experimentally [17]. While (N)-methanocarbaUDP was completely inactive at the $P2Y_6$ receptor, 2′-deoxy (S)-methanocarbaUDP maintained full agonistic properties at the $P2Y_6$ receptor with a 10-fold higher potency than 2′-deoxy-UDP.

Our modeling studies also suggested that, in analogy with the $P2Y_1$, $P2Y_2$, and $P2Y_4$ receptors, the phosphate chain of UDP was coordinated by R3.29, K6.55, and R7.39. In addition, it was proposed that Y7.35 was H-bonded to the diphosphate chain. Similar to the complexes obtained for the $P2Y_{1,2,4}$ receptors, F3.32 of the $P2Y_6$ receptor was involved in interactions with the nucleobase. The 3′-hydroxyl group of UDP could form an H-bond with K6.55 and also appeared to be involved in intramolecular interactions with the 5′-O.

It was proposed that S7.43 of the $P2Y_6$ receptor was H-bonded with the oxygen atom at position 2 of the ligand, rather than with the 3-NH group as in the case of the $P2Y_2$ and $P2Y_4$ receptors. At the same time the 3-NH group of UDP in the $P2Y_6$ receptor donated a H-bond to Y1.39. Although our studies suggest the existence of these two distinct configurations for $P2Y_6$ and $P2Y_{2,4}$, our models do not exclude the possibility for the $P2Y_6$ receptor to establish nucleobase interactions similar to those that we have described for $P2Y_{2,4}$ and vice versa (unpublished data).

Consistent with the hypothesis of an energetic contribution to the ligand recognition of the 3-NH [18], the 3-*N*-Me analogue of UDP was only weakly active in activation of the human $P2Y_6$ receptor. Most likely the loss of potency was not due to steric hindrance exerted by the methyl group, since a 3-phenylacetyl analogue of UTP (although not the preferred native agonist) activated this receptor with an EC_{50} value of 70 nM, indicating a considerable bulk tolerance at this position [23].

As mentioned above, our molecular modeling studies [2] suggested that, in the case of $P2Y_{12}$, the diphosphate chain was also coordinated by three cationic residues, namely R256 (6.55), conserved in all subtypes of P2Y receptors as either Arg or Lys, and K174 (EL2) and K280 (7.35) conserved among all the $P2Y_{12}$-like receptors. The importance of R256 was recently demonstrated with its mutation to Ala resulting in a right shift of two orders of magnitude for activation by 2-MeSADP [24]. In the K280A mutant receptor, 2-MeSADP was completely inactive. The adenine ring of ADP was putatively involved in π–π interactions with the phenyl ring of F104 (3.32), which is conserved among all P2Y receptors. In analogy with results obtained for other P2Y receptors, Y1.39 and

S7.42 of the $P2Y_{12}$ receptor were found near the adenine ring. In particular, Y1.39 could form a H-bond with the N^6-amino group, while S7.43 was found near the nitrogen atom at position 1 of the adenine ring. Alternatively, recent $P2Y_{12}$ receptor modeling results (S. Costanzi, unpublished) suggest that S7.43 could be also involved in interactions with the N^6-amino group. In analogy with the $P2Y_1$ receptor [2], in $P2Y_{12}$ F6.51 was found to be far from the 2′-hydroxyl group but near the 3′-hydroxyl group of ADP.

A similar binding mode was proposed for the pseudonucleotide antagonist AZD6140 [2]. In particular the interaction of the 3,4-difluorophenyl ring of the N^6 substituent of AZD6140 with the nonconserved F77 (2.57) was proposed to contribute to the selectivity of this ligand.

Recently, a different configuration of the binding site was proposed for the $P2Y_{14}$ receptor [19]. In contrast with the $P2Y_{1,2,4,6,12}$ receptors, which show a conserved Ser at position 7.43, the $P2Y_{14}$ receptor shows an Ala at this position. Furthermore, a Met residue occupies position 2.53 in the $P2Y_{14}$ receptor, while at the same position a Tyr is conserved in $P2Y_{1,2,4,6}$. Due to the nonpolar character of the residues 2.53 and 7.43 in the $P2Y_{14}$ receptor, they cannot be involved in H-bond interactions with the uracil ring of the $P2Y_{14}$ agonists. Based on the molecular model of the $P2Y_{14}$ receptor, it was proposed that the uridine moiety of UDP-glucose (a native agonist of the $P2Y_{14}$ receptor) bound deeper inside the transmembrane domain than the ligands of other P2Y receptors (Fig. 2C). For this reason, the highly conserved Tyr residue at position 1.39 appeared to be located between the uracil ring and the α-phosphate group of the ligand and could interact with the phosphate chain. The preferred conformation of the ribose ring of the ligand for binding to the $P2Y_{14}$ receptor has not been determined experimentally. However, molecular modeling suggested that the two conformations would lead to two different sets of interactions [19]. In the case of the (N)-conformation, the 2′-hydroxyl group of UDP-glucose would interact with N104 (3.35), while in the case of the (S)-conformation, it would be involved in interactions with N287 (7.45) and the backbone oxygen atom of S284 (7.42). Also, N7.45 could interact with the oxygen atom at position 2 of the uracil ring. The hydroxyl groups of S7.42 and T7.38 seemed to be H-bonded with the α-phosphate group, which could also interact with one of three critical cationic residues, namely K171 located in EL2. Interestingly, R6.55 and K7.35, i.e., the two other cationic residues involved in interactions with the ligand phosphate chain in the other $P2Y_{12}$-like receptors, were found near the sugar moiety of UDP-glucose. The molecular model suggests that R6.55 and E174 (EL2) were located near the 3-hydroxyl group of the glucose ring, K7.35 was located between the 3- and 6-hydroxyl groups, and E166 (EL2) could interact with the 6-hydroxyl group of the glucose moiety of the ligand.

Comparing our molecular models of the P2Y receptors, nucleotide ligands appeared to occupy very similar positions in $P2Y_1$, $P2Y_2$, $P2Y_4$, and appeared to $P2Y_6$ receptors. ADP in the $P2Y_{12}$ receptor was located deeper inside the receptor in comparison to other subtypes, and the uridine moiety of UDP-glucose in the $P2Y_{14}$ receptor was located even deeper and shifted toward TM7. However, in all our models obtained for the P2Y receptors, the ribose moiety was accommodated between TM3 and TM7, the nucleobase oriented toward TM1, TM2, and TM7 in the direction of the extracellular side of the receptor. The phosphate chain was oriented toward TM6 in the direction of the ELs and was coordinated by three critical cationic residues. In general, these findings are in agreement with the proposed binding site of small molecules to other class A GPCRs.

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