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Key Sites for P2X Receptor Function and Multimerization: Overview of Mutagenesis Studies on a Structural Basis

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Abstract: P2X receptors constitute a seven-member family (P2X1-7) of extracellular ATP-gated cation channels of widespread expression. Because P2X receptors have been implicated in neurological, inflammatory and cardiovascular diseases, they constitute promising drug targets. Since the first P2X cDNA sequences became available in 1994, numerous site-directed mutagenesis studies have been conducted to disclose key sites of P2X receptor function and oligomerization. The publication of the 3-Å crystal structures of the zebrafish P2X4 (zfP2X4) receptor in the homotrimeric apo-closed and ATP-bound open states in 2009 and 2012, respectively, has ushered a new era by allowing for the interpretation of the wealth of molecular data in terms of specific three-dimensional models and by paving the way for designing more-decisive experiments. Thanks to these structures, the last five years have provided invaluable insight into our understanding of the structure and function of the P2X receptor class of ligand-gated ion channels. In this review, we provide an overview of mutagenesis studies of the pre- and post-crystal structure eras that identified amino acid residues of key importance for ligand binding, channel gating, ion flow, formation of the pore and the channel gate, and desensitization. In addition, the sites that are involved in the trimerization of P2X receptors are reviewed based on mutagenesis studies and interface contacts that were predicted by the zfP2X4 crystal structures.

Keywords: mutational P2X receptor analysis, P2X assembly domains, P2X ATP binding pocket, P2X quaternary structure, P2X receptor function.

SCOPE OF THIS REVIEW

This review aims to provide a structure-interpreted overview on the mutational analysis of members of the P2X receptor family of trimeric ligand-gated ion channels (tLGIC). An exciting milestone in the P2X receptor field is the 3-Å resolution of the crystal structure of the zebrafish P2X4 (zfP2X4) receptor in both the unliganded apo-closed state and the ATP-bound open state [1, 2]. These crystal structures provide an invaluable framework to place data that were obtained by probing P2X receptor function through site-directed mutagenesis and pharmacological interventions into a three-dimensional context. The application of the zfP2X4 crystal structure for the interpretation of mutagenesis data has enormously advanced our understanding of the mechanisms of the molecular operation of the P2X receptor in almost every aspect, including ligand binding, channel gating, pore opening and ion permeation [3-11]. Based on these and other successful experiences, we feel confident in using the zfP2X4 crystal structures for the visualization of the mutated residues and, whenever reasonable, for the interpretation of the mutagenesis data. In addition to function, emphasis is also placed on the domains and individual residues that are involved in the trimerization of P2X receptor subunits as identified by structure-interpreted mutagenesis studies.

To unequivocally assign amino acid residues that are mentioned in the text to the corresponding species-specific P2X homolog, the species homologs are indicated throughout the manuscript by superscripts on the numbered residues, with h, r and zf denoting human, rat and zebrafish, respectively.

SHORT HISTORY OF THE IDENTIFICATION OF P2X RECEPTORS AS LIGAND-GATED ION CHANNELS

The first evidence that micromolar concentrations of extracellular ATP exert a neurotransmitter-like action by directly exciting within milliseconds a large conductance in mammalian cells that is permeable to cations, including Ca^{2+}, and reverses near 0 mV is usually assigned to patch-clamping experiments that were published in 1983 [12-14]. Molecular cloning has disclosed that all of the P2 receptors with ligand-gated ion channel (LGIC) activity belong to the subclass of P2X receptors. The designation P2X was originally coined to subdivide P2 receptors into two subtypes, P2X and P2Y, based on two broad patterns of potency of ATP analogs as observed in functional assays in isolated organs [15]. This original subdivision into P2X and P2Y receptors from 1985 was found to fortunately fit with the structurally defined subclasses of (ionotropic) LGICs and (metabotropic) G protein-coupled receptors, respectively [16-18].

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The molecular identity of the P2X receptors was reported in 1994 *via* publication in two companion papers of the coding sequences of the first two members, P2X1 and P2X2, from rat vas deferens and rat pheochromocytoma PC12 cells, respectively [19, 20]. Notably, the expression of a single P2X cDNA was (and is) sufficient to encode the synthesis of functional, ATP-gated ion channels on the surface of *X. laevis* oocytes or transfected mammalian cells. The cloning of the other P2X subunits followed soon after: P2X3 [21]; P2X4 [22-26]; and P2X5 and P2X6 [27, 28]. The first human homolog, hP2X1, was cloned in 1995 and gene-mapped to the short arm of chromosome 17 [29]. By 1996, with the cDNA cloning of the P2X7 receptor from rat brain [30], formerly the P2Z receptor [31], the entire vertebrate P2X multigene family of seven members, P2X1-P2X7, had been identified. In microorganisms, only the green alga *Ostreococcus tauri* and the amoeba *Dictyostelium discoideum* have been found to contain P2X receptor-homologous genes [32, 33]. The genome of *Dictyostelium discoideum* includes a total of five genes encoding proteins (P2XA-P2XE) that share weak sequence similarity with vertebrate P2X receptors and operate as exclusively intracellular ATP-gated ion channels [34].

PHYSIOLOGICAL, PATHOPHYSIOLOGICAL AND PHARMACOLOGICAL IMPORTANCE OF P2X RECEPTORS

P2X receptors play key roles in signal transduction in numerous excitable and non-excitable mammalian cells [33, 35, 36]. Growing evidence had implicated P2X receptors in a broad variety of pathological settings, including inflammation, cancer, and neurological disorders, such as neuropathic pain and depression. Accordingly, P2X receptors hold great promise as therapeutic targets for various diseases. No drug that acts on P2X receptors has been approved [37, 38]; however, the orally bioavailable P2X3 antagonist AF-219 (a diaminopyridine derivative of undisclosed structure) has successfully completed phase 2 clinical trials for the indications of urinary frequency and chronic idiopathic cough. AF-219 was found, for instance, to dramatically reduce cough frequency and severity in refractory patients [38-40] and will be further developed for this indication. The clinical development of P2X7 antagonists against rheumatoid arthritis has stopped because of a lack of efficacy [41, 42]. However, the P2X7 receptor is independently considered to be a promising target for a number of other diseases, such as pain, Alzheimer disease, major depressive disorder, chronic-inflammatory bowel disease and chronic obstructive pulmonary disease [37, 38, 43, 44], and even cancer [45-49].

OUTLINE OF THE ZFP2X4 CRYSTAL STRUCTURE AND RELATED DOMAIN NOMENCLATURE

In this chapter, we briefly outline the overall atomic-resolution structure of the zfP2X4 receptor and describe the nomenclature of the various protein domains as determined by X-ray crystallography at a 2.8-3.1-Å resolution for both the apo-closed state and the ATP-bound open state of the zfP2X4 receptor [1, 2]. No other high-resolution structure of a P2X receptor is presently available. The crystal structure of the zfP2X4 receptor verified the homotrimer architecture of this receptor class that was initially revealed by PAGE approaches for virtually all rat or human P2X family members [50-53] except for P2X6, which forms aggregates rather than assembling as defined homotrimers [52].

From the lateral side, the crystallized homotrimeric zfP2X4 receptor resembles a chalice (Fig. **1**). The bowl of the chalice is represented by the large extracellular domains of the three subunits, which extend approximately 70 Å beyond the membrane plane (Fig. **1**). The ATP binding pockets are located at a distance of approximately 45 Å beyond the membrane plane, one at each of the three subunit interfaces of the homotrimer. Each ATP binding pocket is formed by pairs of complementary half-shells on the surface of adjacent subunits (see below).The foot of the chalice is represented by the six membrane helices, which orient themselves in an overall hourglass-like shape due to the strongly tilted orientation of the three TM helix pairs, with each helix pair having a tilt angle of ~45^{o} relative to the membrane (Fig. **1**).

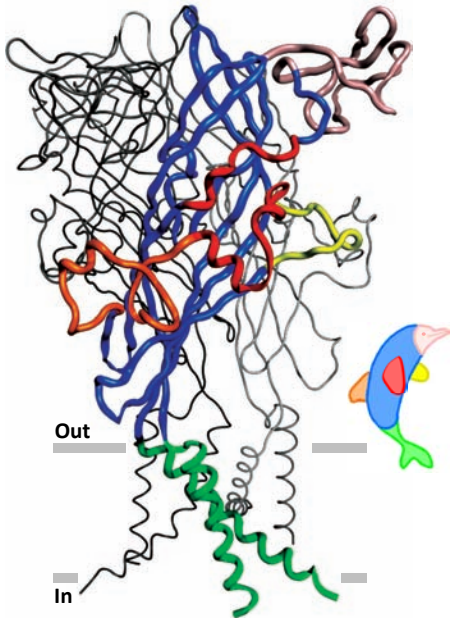

Fig. (1). Crystal structure of the zfP2X4 receptor. The apo-closed state homotrimeric zfP2X4 receptor structure is shown from the side in a wire representation with two subunits colored in light and dark gray. According to the dolphin-like shape of a single subunit, the third subunit is colored to illustrate the 'dolphin' domains:the body is blue, the fluke is green, the head is pink, the dorsal fin is orange, the right flipper is red, and the left flipper is yellow (Kawate and Gouaux, 2009; Hattori *et al.*, 2012). The zfP2X4 receptor structure was visualized by the molecular modeling program MOE2012.10 (Molecular Operating Environment 2012, Chemical Computing Group, Montreal, Canada) using the apo zfP2X4 crystal structure (PDB entry 4DW0, Hattori *et al.*, 2012).

Each single subunit within a homotrimeric zfP2X4 receptor has virtually the same overall shape that has illustratively been described to resemble a jumping dolphin [1] (Fig. **1**): the head domain (pink), the upper and lower body domains (blue) and the fluke (green) of the dolphin are represented by the ectodomain and the two TM helices of the zfP2X4 subunit, respectively. Other domains that have been assigned with anatomical terms are the dorsal fin (orange) and the right (red) and left (yellow) flippers [1]. Many of the muta-

tions reviewed here are shown in the analogous position in the zfP2X4 crystal structure (Fig. **5**).

FIRST EVIDENCE THAT P2X RECEPTORS ARE MULTIMERS

The ATP concentration dependence of ATP-activated native currents in sensory neurons fitted a model in which ATP must bind to each of three identical, non-interacting sites to open the channel [16, 54]. This finding was most likely the first to suggest that P2X receptors are composed of multiple subunits, akin to other LGICs, such as Cys-loop receptors. The first ATP concentration curve that was published for the cloned rP2X2 receptor showed strong cooperativity with a Hill coefficient of 2.0, also suggesting that more than one ATP molecule must bind before the channel opens [20]. Accordingly, the rP2X2 receptor must either be a monomer that is capable of binding more than one ATP molecule or, more likely given the general oligomeric nature of LGIC, an oligomer having one ATP binding site per monomer. Strong evidence for a multimeric nature of P2X receptors comes from the formation of a new receptor phenotype upon the co-expression of recombinant P2X2 and P2X3 subunits. This phenotype includes properties that are akin to both P2X2 and P2X3 receptors by being non-desensitizing and αß-meATP sensitive, respectively [21]. The most plausible explanation is that P2X2 and P2X3 subunits are capable of heteropolymerizing into a heterooligomeric P2X2/3 receptor that faithfully reproduces the receptor phenotype of the native ATP-gated channels that are found in sensory neurons.

The formation of the P2X2/3 heterooligomer was biochemically verified by co-immunoprecipitation experiments in *Spodoptera frugiperda* (SF9) insect cells that were baculovirally infected with the cDNAs for two differently epitope-tagged P2X2 and P2X3 subunits. Following solubilization with 1% Triton X-100, the P2X2 and P2X3 subunits could be cross-immuno-precipitated with epitope-tag-specific antibodies (anti-179 or anti-EE), showing that P2X2 and P2X3 subunits must be incorporated in a stable heteromeric complex [55]. Patch-clamp analysis confirmed the phenotype of a slowly desensitizing response to αß-meATP in the SF9 insect cells.

P2X RECEPTOR STOICHIOMETRY: TRIMERS OR TETRAMERS THAT WAS THE QUESTION

Altogether, these findings left no doubt that P2X receptors must be oligomeric proteins similar to the vast majority of ion channels. Additionally, a monomer with just two transmembrane domains appeared unlikely to account for a functional ion channel. Because the lack of homology with known ion channels provided no clue to the possible subunit stoichiometry, the topology with only two membrane-spanning domains and intracellular N- and C-termini was considered a possible structural hint. Indeed, based on the global membrane topology, P2X receptors appeared structurally more closely related to the inward rectifier K^+ (Kir) channels, the degenerin/epithelial Na^+(DEG/ENac) channels (which also include FMRF amide-gated channels of mollusks and acid-sensing ion channels, ASICs, also designated ACCNs) and the bacterial mechanosensitive channels (mscLs) than to Cys-loop receptors or ionotropic glutamate receptors [56].

The possible relationship with Kir channels gained initially support from hydropathy and homology analyses that indicated that the P2X1 and P2X2 subunits contain in addition to the two membrane-spanning domains a short hydrophobic sequence resembling the highly conserved pore-forming motif (P or H5 segment) of Kir channels. In particular, the eight-residue strand ^{331}TMTTIGSG^{rP2X1}(Fig. **5C**) is strikingly sequence-homologous to the H5 signature segment of K^+ channels [19]. Kir channels have been shown by a concatamer approach [57] as well as chemical cross-linking, immunoprecipitation and sedimentation analyses [58] to share a tetrameric architecture with voltage-gated K^+ (Kv) channels. Similarly, the αßγ-ENac channels and the related FMRF amide-gated channels were initially characterized as tetramers (however, see below) by functional or biochemical analysis, respectively [59, 60]. The implicated suggestion that P2X receptors are organized as tetramers was emphasized by the observation that the H5-like rP2X1 domain exhibits homology with the so-called motif 1 of class II aminoacyl-tRNA synthetases that leads to the formation of a tetramer (from two αß dimers) [61].

The quaternary structure of a recombinant P2X receptor was assessed for the first time from a bacterially expressed rP2X2 ectodomain (K53-K308^{rP2X2}) that was purified in urea on a metal affinity column and, following sulfitolysis, refolded *in vitro* by the gradual removal of urea through dialysis [62]. Equilibrium centrifugation gave a molecular size of 132 kDa for the refolded rP2X2 ectodomain, which fitted to a homotetramer of four protomers of a predicted size of 29 kDa each (31 kDa by SDS-PAGE). A tetrameric form was plausibly consistent at that time with the supposed tetrameric stoichiometry of the other ion channels of the same membrane topology, namely Kir, DEG/ENac/ASIC and mscL [62]. Higher molecular masses of 144 kDa and 160 kDa of the refolded rP2X2 ectodomain as determined by dynamic light scattering and gel filtration chromatography, respectively, were not further discussed [62], although indicative of a pentamer. Our own attempts to obtain oligomers of a defined oligomeric state by expressing rP2X1 and rP2X2 ectodomain constructs (with a cleavable N-terminal signal peptide of a type-I membrane protein) in *X. laevis* oocytes were unsuccessful (G.S. unpublished results).

We have assessed the subunit stoichiometric structure of the full-length homomeric rP2X1 and rP2X3 receptors following their synthesis in *Xenopus laevis* oocytes. To this end, we purified [^{35}S] methionine-labeled, fully functional rP2X1 and rP2X3 receptors under non-denaturing conditions from *X. laevis* oocytes by metal affinity chromatography via N-terminal hexahistidine tags. Shortly after their purification, the digitonin-solubilized rP2X1 and rP2X3 receptors were resolved in the non-denatured and partially urea-denatured or SDS-denatured state by blue native PAGE (BN-PAGE). Non-denatured rP2X1 and rP2X3 receptors were found to migrate quantitatively as non-covalently homotrimers [50]. The use of digitonin as a mild non-ionic detergent for receptor solubilization [63] turned out to be a lucky choice because digitonin aids proteins of distinct oligomeric states in migrating as distinct rather than smeared bands.

BN-PAGE was initially developed for the analysis of mitochondrial protein complexes [64], and its suitability as a method for the determination of the oligomeric state of ion

channels was untested. To validate the trimeric state of rP2X1 and rP2X3 receptors by an independent method, we used bifunctional derivatives of pyridoxalphosphate-6-azophenyl-2', 4'-disulphonic acid (PPADS) as chemical cross-linkers. PPADS is a non-selective P2X receptor antagonist [65, 66]. Two of these cross-linkers, designated DIPS and CL II, are formally dimers of two PPADS molecules that are linked by differently long spacers. PPADS is unique among the P2X antagonists in forming a Schiff base via its aldehyde group with the ε-amino group of a lysine residue [67]. Evidently favored by the local enrichment at the PPADS binding site, P2X1 and P2X3 receptors could be covalently cross-linked by micromolar concentrations of CL II and DIPS. These results fully support the homotrimeric organization of P2X1 and P2X3 receptors as suggested by BN-PAGE [50].

An efficient cross-linking of the P2X1 receptor was also achieved by PPAPA, which differs from PPADS solely by bearing at the azophenyl group a second reactive aldehyde function instead of the two sulfonic acid groups [50]. While CL II has a very long and flexible spacer of ~34 Å between the two reactive sites, PPAPA is a rigid molecule that bridges only 11 Å. Due to this short span, PPAPA can best be conceived to link two P2X subunits at an intersubunit position that plausibly may constitute the intersubunit ATP binding pocket. Following docking into the ATP binding pocket, the two aldehyde groups of PPAPA could then react with two lysine residues, one in each of the adjacent subunits, thus forming an intersubunit cross-link.

To directly test whether BN-PAGE is capable of correctly displaying the oligomeric state of proteins, we replicated the experiments with the prototypic rat α_2ßγδ nicotinic acetylcholine receptor [68, 69], which migrated as the expected heteropentamer in the BN-PAGE gel [50, 70]. Since then, BN-PAGE has been demonstrated as an extremely reliable method for the determination of the quaternary structure of membrane proteins of various oligomeric states, including dimeric anion exchangers and anion channels [71, 72], trimeric glutamate transporters [73], pentameric Cys-loop receptors [70, 74-78] and the hexameric scaffolding protein gephyrin [79]. By BN-PAGE, we could further demonstrate that a trimeric architecture also applies for the P2X2, P2X4, P2X5 and P2X1/2 receptor channels [52] as well as hP2X7 receptors [53]. In contrast, endoplasmic reticulum-retained tetramers and higher aggregates instead of defined homotrimers were resolved by BN-PAGE when hP2X6 subunits were expressed from a single cRNA in *X. laevis* oocytes [52]. This observation indicated that the inability of singly expressed P2X6 subunits to form ATP-activatable ion channels in *X. laevis* oocytes [28, 80] resulted from a combined folding and assembly defect [52].

In addition, imaging by atomic force microscopy (AFM) revealed that P2X6 subunits are unable to form stable trimers [81]. The detection of the rP2X6 subunits as monomers [81] as opposed to tetramers and higher aggregates [52] may be attributed to the use of distinct detection methods and solubilization detergents, AFM and CHAPS *versus* blue native PAGE and digitonin, respectively. By blue native PAGE, "free" P2X monomers were rarely, if ever, observed in the absence of denaturing agents, such as SDS or urea (G.S., unpublished observations).

Notably, the deletion of a stretch of 14 uncharged residues immediately following the initiating methionine or replacing serine residues in positions 3 and 11 of the rP2X6 subunit by charged residues (lysines or aspartates) permitted homotrimeric assembly and appearance at the cell surface [82]. However, despite the trimeric assembly of the rP2X6 mutants, no measurable current responses to ATP could be recorded [82]. Because the intracellular N-terminal tail was not required for the homotrimeric assembly of the crystallized N- and C-terminally truncated ΔzfP2X4 constructs (see below), it will be interesting to learn how exactly the N-terminal tail impairs the assembly of the rP2X6 subunit. In fact, the zfP2X4 crystal structure provided a different explanation for the assembly defect of wt P2X6 subunits: a comparison of the structure with a sequence alignment revealed that intersubunit contacts between P2X6 receptor subunits are compromised in the ectodomain due to nine missing residues in the left flipper [1] (Fig. **1**). This suggestions focuses attention on the interaction of the wild-type (wt) P2X6 subunits with ER-resident folding factors as the main mechanism for the retention of the nonnative P2X6 protein in the endoplasmic reticulum [83].

The trimeric organization of functional P2X receptor channels was supported by functional analysis of concatenated P2X2 subunit cDNAs carrying functional reporter mutations (Stoop *et al.*, 1999), atomic force microscopy [81], electron microscopy [84] and single-particle analysis [85]. Disulfide-bonded heterodimers formed between rP2X2 and rP2X3 when each subunit contained one substituted cysteine near the extracellular end of the TM1 and TM2 helices, $V48C^{rP2X2}$ and $I319C^{rP2X3}$, respectively, or *vice versa*, $V42C^{rP2X3}$ and $I328C^{rP2X2}$ [86]. These data showed for the first time that the outer ends of TM1 and TM2 must be in close proximity to each other and that the P2X subunits thus must be arranged in head-to-tail fashion. Disulfide cross-linking did not occur when the V48C, $I328C^{rP2X2}$ double mutant was co-expressed with wt rP2X3, suggesting that the subunit stoichiometry must be rP2X2/rP2X3/rP2X3(P2X2$(3)_2$) if the channel is a trimer [86]. Any residual doubts about the trimeric structure were cast away by the first crystal structure of the zfP2X4 receptor [1]. The trimeric quaternary architecture clearly defines the P2X receptors as a unique class of LGICs that are distinct from the tetrameric ionotropic glutamate receptors and the pentameric Cys-loop receptors (also designated as pLGICs).

REQUIEM FOR A SHARED ARCHITECTURE OF PROTEINS WITH TWO TM HELICES FLANKING AN ECTODOMAIN

The hypothesis that two membrane-spanning helices flanking a differently sized ectodomain represent a general motif that defines the stoichiometry of an oligomeric protein was gradually rejected. Kir channels have been crystallographically confirmed to be tetramers [87]. The αßγ-ENac was initially been suggested [59] and later confirmed to be organized as a tetramer [88, 89]. However, actual data convincingly indicate that αßγ-ENacs are trimers based on quantitative fluorescence analysis [90], AFM imaging of the ENac itself [91] and the trimeric crystal structure of the related channel ASIC1 [92]. The ASICs and P2X receptors are unrelated in amino acid sequence but share significant structural similarities, including the trimeric structure [93].

Fascinatingly odd is the variability in the oligomeric state that has been observed for the most spartan channel with two membrane-spanning domains, the bacterial mscL [94], which has a species-dependent monomer size of only 13-15 kDa (120-151 residues). Chemical cross-linking and two-dimensional crystallography initially suggested that the functional mscL complex from *Escherichia coli* is a homohexamer [95, 96]. The 3.5-Å crystal structure of an mscL homolog from *Mycobacterium tuberculosis* verified the two TM helices topology and disclosed a homopentamer with the subunits surrounding a central aqueous pore [97]. Surprisingly, when the same lab resolved the crystal structure of an mscL homolog from *Staphylococcus aureus* at 3.8 Å, a tetrameric state was found [98]. The pentameric state appears to constitute the predominant form under normal expression levels. So far, the available data do not permit the assignment of a given oligomeric state to defined domains of this so far uniquely plastic mscL protein family [99].

THE INTRACELLULAR N- AND C-TERMINAL TAILS ARE DISPENSABLE FOR P2X SUBUNIT ASSEMBLY

Co-immunoprecipitation with monoclonal antibodies (anti-FLAG, anti-HA) in non-ionic detergent (1% Nonidet P-40) was also used to probe for domains that are involved in P2X receptor assembly in HEK293 cells by examining the ability of P2X2 deletion mutants and chimeric constructs to associate with full-length P2X2 and P2X3 subunits in HEK293 cells [100]. Neither the N- nor C-terminal intracellular domain (until residue 28^{rP2X2} and beyond residue 362^{rP2X2}, corresponding to 30^{zfP2X4} and 370^{zfP2X4}, respectively) was required for cell surface expression or assembly with the full-length rP2X2 or rP2X3 subunits, as judged by co-immunoprecipitation. In addition, the replacement of rP2X2 residues 1-50 (which include the entire TM1 helix) by the cleavable signal peptide of the α7nAChR did not prevent co-immunoprecipitation with the full-length rP2X2 or rP2X3 subunit. These data largely ruled out a sequence-specific contribution of the cytoplasmic N- or C-terminal domains or the TM1 helix in subunit recognition [100].

The dispensability of the intracellular C-terminal domain for homotrimerization was directly demonstrated by blue native PAGE, showing that an hP2X5 construct containing inserted exon-10 residues ($hP2X5^{+exon10}$) but lacking almost the entire intracellular C-terminal tail (residues $R365^{hP2X5+exon10}$ onwards) assembled efficiently as a homotrimer [101]. The three basic residues capping the extreme C-terminal end of this mutant, $363KKR365^{hP2X5+exon10}$ (corresponding to $365KKR367^{zfP2X4}$), were most likely required as a topogenic signal to satisfy the "positive-inside rule" [102]. The homotrimeric crystal structure that was obtained with an N-and C-terminally truncated ΔzfP2X4 construct (residues $27\text{-}381^{zfP2X4}$ corresponding to $25\text{-}379^{hP2X5+exon10}$) corroborated the dispensability of the intracellular N-terminal tail and at least part of the C-terminal tail for assembly.

TM2 FOSTERS ASSEMBLY BY RESTRICTING THE FOLDING SPACE OF THE ECTODOMAIN

A critical role of the TM2 helix for homo- and heteropolymerization was deduced from the observation that C-terminal truncation at position $R304^{rP2X2}$ (corresponding to $R312^{zfP2X4}$ or $R310^{rP2X5}$, see arrow in Fig. **5A**) that also deleted the pre-TM2 region (equivalent to ß strand 14 of the zfP2X4 structure), and the TM2 helix prevented both assembly with full-length P2X2 or P2X3 subunits and self-assembly [100]. This view was further supported by co-immunoprecipitation experiments with two chimeras consisting of the P2X1 ectodomain flanked by the N- and C-terminal domains, including the transmembrane helices of rP2X3 (X3-X1-X3) and vice versa (X1-X3-X1) [100]. The rationale was that wt P2X6 can co-assemble with wt rP2X1 but not with rP2X3 when co-expressed in HEK293 cells [103]. Intriguingly, the X1-X3-X1 chimera co-precipitated with the wt rP2X6 subunit, whereas X3-X1-X3 and wt P2X3 did not [100, 103]. This finding was interpreted to support the view that a TM helix harbors a specific recognition motif that is critical for the assembly of P2X subunits.

To study the role of the TM2 helix in the assembly of P2X subunits, we exploited the fact that the wt P2X5 subunit occurs in humans merely as a natural deletion variant lacking the pre-TM2 region and much of the TM2 helix due to the splice-skipping of exon 10 (Fig. **5A**) designated as $hP2X5^{\Delta 328\text{-}349}$. While $hP2X5^{\Delta 328\text{-}349}$ does not express as a functional ATP-gated ion channel, the wt rP2X5 cDNA, which includes the exon-10 codons, resulted in the expression of typical P2X receptor-mediated currents [104]. By blue native PAGE, we could demonstrate that the full-length rP2X5 subunits were assembled efficiently into homotrimers, whereas the exon-10-lacking hP2X5 formed only higher-order aggregates that were entirely retained in the endoplasmic reticulum [101].

While these results confirmed that the TM2 helix was absolutely required for the efficient assembly of P2X subunits, the view that the TM2 helix provides specific subunit recognition information [100] turned out to be not tenable, at least for the P2X5 subunit [101]. This result occurred because extensive alanine and leucine block replacements in the TM2 helices impaired channel function, but neither disturbed the folding nor homotrimerization as probed by limited proteolysis and blue native PAGE, respectively. In the case of the exon-10-lacking hP2X5 subunit ($hP2X5^{\Delta 328\text{-}349}$), the insertion of an alanine stretch that was sufficiently long to eliminate the hydrophobic mismatch that was imposed by the splice-skipping half of the TM2 helix enabled the formation a membrane-spanning segment and concurrent homotrimeric assembly [101].

Altogether, these findings fit best with the view that the contribution of the full-length TM2 helix to homotrimerization is the provision of a second membrane anchor as a scaffold [101]. By tethering the C-terminal end of the ectodomain to the membrane, a loop-like structure is formed between TM1 and TM2 that constrains the spatial mobility of the ectodomain and thus restricts the folding space. This structural constraint may support the assembly by assisting in the correct positioning of the recognition surfaces, allowing more productive collisions to take place between neighboring ectodomains and thus preventing aggregation. All of the sequence manipulations that impaired the formation of a second membrane-spanning domain, including the lack of exon 10 (Fig. **5A**) in the hP2X5, also resulted in severe

subunit aggregation. The conclusion of this study that the specific information for homomeric subunit-subunit interactions must be located in the ectodomain [101] was supported by the zfP2X4 crystal structure, showing that inter-subunit contacts are largely provided by the ectodomains [1, 2].The three TM1 helices are in a peripheral position relative to the three TM2 helices that form and line the central ion channel [2]. There are a few intramembrane contacts between the TM2 helices (between Leu340^{zfP2X4}, Leu346^{zfP2X4} and Ala347^{zfP2X4}) that stabilize the closed channel. In response to ATP binding, these contacts break when the TM helices swing away from the central axis to open the channel. As a consequence, notable gaps arise, but new intrasubunit contacts also form (involving Leu346^{zfP2X4} and Ile355^{zfP2X4}) that stabilize the open channel [2].

D355^{rP2X5}, which is predicted by topology algorithms to belong to the TM2 helix, was the only hot spot residue that was identified to support the homotrimerization of the hP2X5 subunit to a relevant extent [101]. We considered D355^{rP2X5}, which is conserved across all seven P2X receptor subtypes, to be an interesting candidate of an assembly mediator given that polar residues in TM helices provide a strong driving force for dimer or trimer formation [105, 106]. However, according to the crystal structure of the zfP2X4 receptor the corresponding residue D357^{zfP2X4}is localized the inner end of the TM2 helix (Fig. **5B**). In this location, D357^{zfP2X4} is more likely to play a role in the proper positioning of the TM2 helix with respect to the membrane by interacting via its negatively charged site chain with the lipid head groups than in subunit assembly.

INTERACTIONS BETWEEN THE TM HELICES IN THE FUNCTIONAL LIFE OF THE P2X RECEPTOR

The fact that sequence-specific TM2 interactions are not needed to achieve the mature homotrimeric structure does not rule out that interactions between the TM helices play an important role later in the functional life of a P2X receptor as an ATP-gated channel. Indeed, there is indirect evidence that the loose packing of the TM helices displayed by the structure of zfP2X4 receptor is defined in part by detergent aggregate known to preferentially occupy the larger volume that surrounds the hydrophobic domain of membrane proteins with large water-soluble domains [107]. Support for this view comes from the observation that the highly tilted and kinked TM helices in the zfP2X4 structure result in a thin hydrophobic thickness (< 20 Å) that is much too short to span a native membrane. The suggestion that the inter-monomer fenestrations seen in the structure leave the channel pore open to the fatty acyl environment [107] could be demonstrated by a molecular dynamics simulation of the membrane-embedded zfP2X4 [108]. Relatively modest structural rearrangements of the TM helices were sufficient to improve the hydrophobic mismatch and resulted in the formation of intersubunit interfaces within the membrane by allowing for a much tighter packing of the TM helices [108]. The two following interfaces were identified: (i) TM2 residues L351^{rP2X2}, I355^{rP2X2} and W358^{rP2X2} of one subunit interact with L346^{rP2X2}, A347^{rP2X2} and V354^{rP2X2} on the adjacent TM2 helix; (ii) TM1 residue Y45^{rP2X2} interacts with L340^{rP2X2} in TM2 of the adjacent subunit [108].

Computational and experimental evidence for tighter helix packing within the membrane-embedded receptor in the open state was also inferred from functional analysis of metal bridges between S345C^{rP2X2}, C348^{rP2X2} (TM2) and H/C33^{rP2X2} (TM1) of the rP2X2 receptor within each individual subunit [108]. The close intrasubunit proximity of TM1 and TM2 was also evident from disulfide trapping experiments of an H33C/S345C^{P2X2}mutant [109]. Interestingly, Li and colleagues 2010 previously mentioned that within the closed-state zfP2X4 structure, subunit-subunit interactions within the pore region are limited to the gate region, indicating that upon pore expansion during channel opening, other subunit-subunit contacts are required to stabilize the open pore structure, and suggested that the TM1 residue H33 interacts with the S345 residues of the two adjacent TM2 helices in the open state of the pore [6].

MAPPING CONTACT SITES BASED ON THE ZFP2X4 CRYSTAL STRUCTURE

To identify contact patches that potentially contribute to the assembly of zfP2X4 subunits, we visualized the solvent-accessible van der Waals interaction surface according to the hydrophobicity and electrostatic potentials (Fig. **2**). By considering the van der Waals surface areas of atoms in a proximity of < 4.5 Å between two adjacent subunits, the subunit-subunit contact surface could be visualized in the closed and open state (Fig. **3**). The specific residues of adjacent subunits that interact electrostatically or via hydrogen bonds or hydrophobic (van der Waals bonds) are listed in Table **1**.

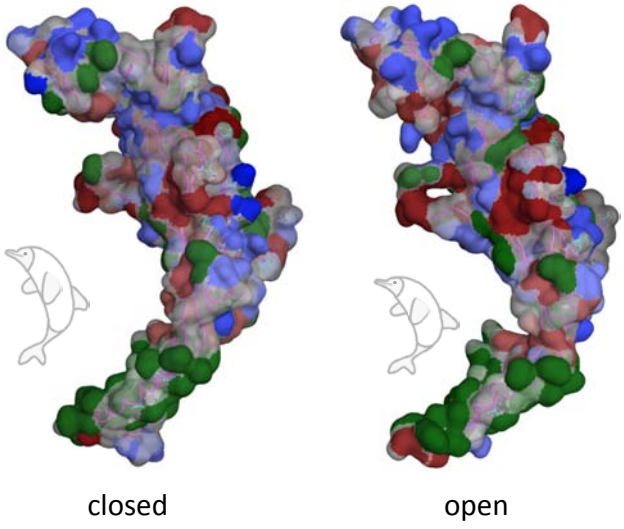

Fig. (2). Surface-exposed side chains available for zfP2X4 intersubunit interactions. Shown is a representation of surface-exposed side chains of a single subunit of the zfP2X4 receptor in the apo-closed state (left panel; PDB entry 4DW0[2]) and the ATP-bound open state (right panel; PDB entry 4DW1[2]). Surface areas that are highly prone to protein association and are therefore available for subunit-subunit interactions were determined based on the solvent-accessible Van-der-Waals interactions and colored according to hydrophobicity (green) and negative (red) and positive (blue) electrostatic potential. The electrostatic and hydrophobic potentials [221-223] were calculated using routines that were implemented in the protein patch analyzer in the MOE2012.10 program.

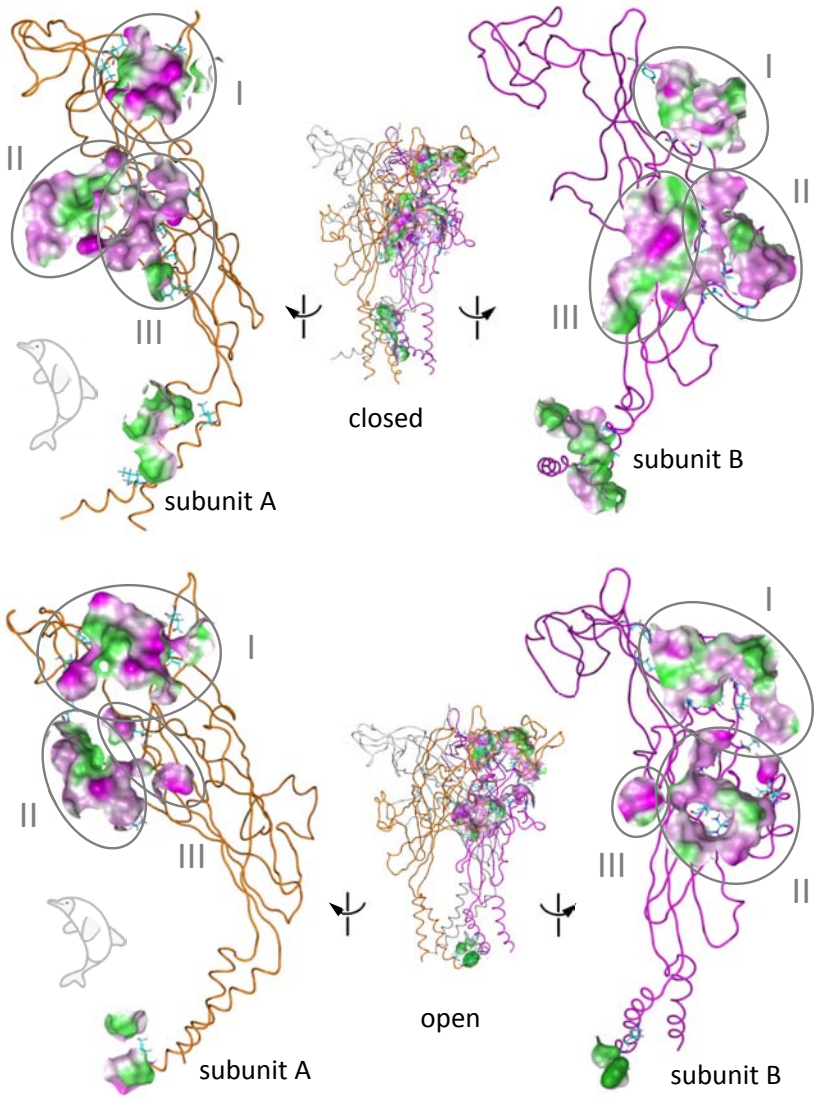

Fig. (3). Contact surfaces of two neighboring zfP2X4 subunits. Shown are the additive contact surfaces between the atoms of the residue side chains of two neighboring zfP2X4 subunits of the zfP2X4 receptor in the apo-closed state (upper panel; PDB entry 4DW0) and the ATP-bound open state (lower panel; PDB entry 4DW1). The contact surfaces were identified by calculating the Van-der-Waals surface areas of atoms in a proximity of < 4.5 Å between two adjacent subunits A and B (ribbon colored orange and pink, respectively). In the middle of the upper and lower panels, the entire zfP2X4 receptor is shown as a ribbon representation (3rd subunit in gray and with the depiction of the contact surfaces of the orange and pink subunits). Hydrophilic portions of the contact surfaces are colored gradually pink, while neutral and hydrophobic portions are colored gradually white and green, respectively. The residues that define these domains through interaction with adjacent subunits by means of hydrogen bonds and by hydrophobic and ionic interactions are specified in Table **1**. The gray ovals illustrate the extracellular interaction domains I-III of two adjacent subunits A and B (indicated by superscripts): I, head^{A}-to-body^{B}; II, left flipper^{A}-to-dorsal-fin^{B}; and III, body^{A}-to-body^{B} [1]. The identical numbering is used in Table **1**. The graphical representation was generated using MOE2012.10.

ATP BINDING SITE

The interested reader is referred to several excellent recent reviews on this subject that also contain extensive background information [110-115]. One of these review contains a particularly comprehensive and valuable tabular overview about the mutated residues that are involved in ATP binding [116].

Attempts to localize the ATP binding site in the pre-crystal structure period focused on ectodomain residues that are conserved among the seven P2X receptor subunits. The corresponding residues were mutated to alanine or cysteine, and the expressed mutants were electrophysiologically characterized with respect to the action of ATP. This approach identified a series of positively charged residues (species and P2X subtype as indicated by the superscripts), such as K68^{hP2X1}, K70^{hP2X1}, R292^{hP2X1} and K309^{hP2X1} (corresponding to K70^{zfP2X4}, K72^{zfP2X4}, R298^{zfP2X4} and K316^{zfP2X4}) (Fig. **4**, **5D**); the polar residues T186^{hP2X1} and N290^{hP2X1} (T189^{zfP2X4} and N296^{zfP2X4}) (Figs. **4**, **5D**), the aromatic residues F185^{hP2X1}and F291^{hP2X1} (F188^{zfP2X4} and F297^{zfP2X4}); and several others [34, 117-132]. Experimental evidence that ATP binding site(s) may

Table 1. ***In silico*** **prediction of subunit-subunit interaction sites based on the crystal structures of the zfP2X4 receptor. The data were calculated by applying proposed filter criteria [224, 225] at a cutoff of 4.5 Å to the crystal structures of the zfP2X4 receptor in the apo-closed state (PDB entry 4DW0) and the ATP-bound open state (PDB entry 4DW1) [2]. The interaction domains I-III of two adjacent subunits A and B (indicated by superscripts) refer to I, head^{A}-to-body^{B}; II, left flipper^{A}-to-dorsal-fin^{B}; and III, body^{A}-to-body^{B} [1] and are identically numbered in Fig. (3). The atom types that are involved in the interaction were taken from the coordinate section in the PDB files (http://www.wwpdb.org/docs.html) and classified corresponding to their type of interaction (HB, hydrogen bond; HYD, hydrophobic interaction; and ION, ionic interaction).**

Apo-Closed State						ATP-Bound Open State					
Inter-Action Domain	Residue Subunit A	Atom Subunit A	Residue Subunit B	Atom Subunit B	Type of Inter-Action	Inter-Action Domain	Residue Subunit A	Atom Subunit A	Residue Subunit B	Atom Subunit B	Type of Inter-Action
I	Q116	NE2	I86	O	HB	I	Q116	OE1	I86	N	HB
I	W167	NE1	D91	OD2	HB	I	N140	ND2	I74	O	HB
I	Y302	OH	D91	OD1	HB	I	N140	O	R187	NH2	HB
I	E310	OE1	R85	NH2	HB	I	W167	NE1	D91	OD1	HB
I	E310	OE2	Y303	OH	HB	I	Y302	OH	A90	O	HB
I	R312	NH2	D88	OD2	HB	I	Y302	OH	D91	OD2	HB
I	R312	NH1	D91	OD1	HB	I	E310	OE2	K301	NZ	HB
I	V147	CG2	I86	CD1	HYD	I	E310	OE2	Y303	OH	HB
I	L165	CD2	L76	CD1	HYD	I	R312	NH2	D88	OD2	HB
I	L165	CD2	I86	CG1	HYD	I	R312	NH1	D91	OD2	HB
I	E310	OE1	R85	NH2	ION	I	V147	CG2	I74	CG2	HYD
I	R312	NH2	D88	OD2	ION	I	V147	CG2	L76	CB	HYD
I	R312	NH1	D91	OD1	ION	I	V147	CG2	I86	CG2	HYD
II	E98	OE1	Q97	NE2	HB	I	L165	CDC	L76	CD1	HYD
II	L282	O	N195	ND2	HB	I	L165	CG	I86	CG2	HYD
II	L282	O	R206	NH2	HB	I	W167	CD1	I86	CD1	HYD
II	Y295	OH	Q97	OE1	HB	I	E310	OE1	R85	NH2	ION
II	R321	NH1	S66	OG	HB	I	E310	OE2	K301	NZ	ION
II	R321	NH2	V67	O	HB	I	R312	NH2	D88	OD2	ION
II	D323	OD2	S66	OG	HB	I	R312	NH1	D91	OD2	ION
II	M325	CB	L64	CD1	HYD	II	K285	N	N195	OD1	HB
II	F327	CD1	L64	CD1	HYD	II	N289	OD1	R206	NH2	HB
II	R321	NH1	D99	OD1	ION	II	V291	O	R206	NH2	HB
III	P287	O	S214	OG	HB	II	A292	O	K193	NZ	HB
III	D288	O	S214	OG	HB	II	A292	O	R206	NH1	HB
III	N289	ND2	P210	O	HB	II	G294	O	K193	NZ	HB
III	N290	O	S214	OG	HB	II	V291	CG1	I208	CG2	HYD
III	V291	O	K193	NZ	HB	II	V291	CG1	L217	CD1	HYD
III	A292	O	K193	NZ	HB	II	R321	NH1	D99	OD2	ION
III	N296	OD1	K70	NZ	HB	III	R143	NH2	L217	O	HB

(Table 1) contd….

Apo-Closed State						ATP-Bound Open State					
Inter-Action Domain	**Residue Subunit A**	**Atom Subunit A**	**Residue Subunit B**	**Atom Subunit B**	**Type of Inter-Action**	**Inter-Action Domain**	**Residue Subunit A**	**Atom Subunit A**	**Residue Subunit B**	**Atom Subunit B**	**Type of Inter-Action**
III	V291	CB	L191	CD2	HYD	III	R143	NH2	C220	O	HB
III	V291	CG2	I208	CD1	HYD	III	N290	OD1	S214	OG	HB
III	V291	CG2	L217	CB	HYD	III	N296	OD1	K70	NZ	HB
TM	V50	CG1	I336	CG2	HYD	TM	I355	CD1	L346	CD1	HYD
TM	L340	CD2	L340	CD2	HYD	TM	W358	CH2	V354	CG2	HYD
TM	L348	CD2	L339	CB	HYD						
TM	L351	CB	L346	CD1	HYD						

be formed at the interfaces of two adjacent subunits came from oxidative oligomerization via the intersubunit cross-linking of cysteines that were substituted for residues that are essential for high ATP potency. In the rP2X1 receptor when expressed in *X. laevis* oocytes, spontaneous intersubunit cross-links form between two substituted cysteines, K68C^{rP2X1} and F291C^{rP2X1} (Fig. **5E**), which are located in the ectodomain [133]. The pairwise cysteine mutations of six other residues (K70^{rP2X1}, F185^{rP2X1}, K190^{rP2X1}, R292^{rP2X1}, R305^{rP2X1}, and K309^{rP2X1}) did not result in intersubunit cross-linking.

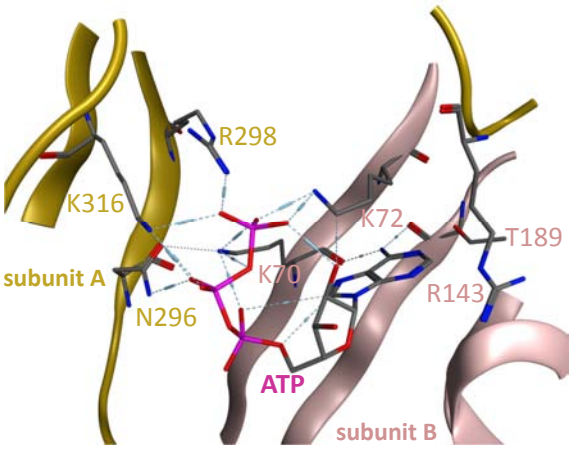

Fig. (4). ATP coordination within a zfP2X4 intersubunit agonist-binding site. The detailed intersubunit ATP-binding pocket of the open-state zfP2X4 receptor structure (PDB entry 4DW1[2]) in complex with ATP is shown. Selected parts of the polypeptide backbones of the two adjacent zfP2X4 subunits that contribute to the ATP binding site are colored in yellow and pink; specifically labeled residues are depicted as sticks. The ionic interactions that coordinate ATP are indicated by dashed cyan lines. The thickness of the center of the dashed lines indicates the strength of the ionic interaction, which was assessed using MOE2012.10.

In addition, in a study based on the alanine substitutions of lysine residues that were implicated in ATP binding and located near each end of the receptor ectodomain, K69^{rP2X2} and K308^{rP2X2}as well as K68^{rP2X3} and K299^{rP2X3}, which are essential for the ATP binding of the rP2X2 and rP2X3 receptor, it was concluded that the agonist binding site is located at the subunit interface between two adjacent subunits [129].

The definitive confirmation that these intersubunit grooves represent the ATP binding pockets was provided by the crystallization of the zfP2X4 receptor in the ATP-bound open-state [2]. The triphosphate chain and the adenine ring of the ATP molecule adopt a U-shaped configuration within the ATP-binding pocket. The phosphate oxygens of ATP are coordinated by the side chains of K70^{zfP2X4} and K72^{zfP2X4} of one subunit and N296^{zfP2X4}, R298^{zfP2X4} and K316^{zfP2X4} of the adjacent subunit (Fig. **4**). In agreement with the labeling of a cysteine mutant of the P2X2 receptor with the thiol-reactive NCS-ATP, hydrophobic interactions with L191^{zfP2X4} (see Fig. **5F**; corresponding to L186^{rP2X2}) and I232^{zfP2X4} coordinate the adenine base of ATP. The adenine base is further stabilized by hydrogen bonds with the side chain and backbone of T189^{zfP2X4} and the backbone of K70^{zfP2X4} [2] (Fig. **4**). The ribose moiety faces toward the solution [10] and is recognized solely by hydrophobic interactions with L217^{zfP2X4} [2]. Specific characteristics of the ATP-binding sites at the interface of different subunits as present in heteromeric receptors were recently disclosed by homology modeling [134].

STRUCTURAL INTERPRETATION OF MUTATIONAL DATA PROVIDES CRUCIAL INSIGHT INTO ATP BINDING

Cysteine-scanning mutagenesis of the ectodomain residues E52-G96^{hP2X1}combined with comprehensive pharmacological analysis, [^{32}P]2-azido-ATP cross-linking and cysteine modification by thiol-specific reagents identified the residues K68^{hP2X1}, K70^{hP2X1} and F92^{hP2X1}as most important for hP2X1 activation and inhibition by agonists and the antagonist suramin, respectively [135]. Mapping of the E52^{hP2X1}-G96^{hP2X1} data together with data from the former cysteine-scanning mutagenesis of flanking regions of the hP2X1 receptor [127, 128] and the *in silico* molecular docking of ATP and BzATP provided homology models of the ATP-binding site that are consistent with the experimental data [135].

The functional analysis of hP2X3 alanine mutants identified conserved residues that are important for the ATP bind-

ing to the hP2X3 receptor, including the basic residues $K63^{hP2X3}$, $K65^{hP2X3}$, $K176^{hP2X3}$, $K299^{hP2X3}$ and $R281^{hP2X3}$ (Fig. **5D**); the polar residues $G66^{hP2X3}$, $N177^{hP2X3}$, $N279^{hP2X3}$, and $T172^{hP2X3}$; and the aromatic residues $F171^{hP2X3}$ and $F280^{hP2X3}$. The projection of the identified residues on an hP2X3 receptor homology model showed that groups of residues that were organized in clusters at the subunit interface rather than individual residues are responsible for ATP recognition [136].

In an ingenious study using the 8-thiocyano-ATP derivative (NCS-ATP) with the sulfhydryl-reactive group at position 8 of the adenine ring of the ATP molecule, the ATP binding pocket of the P2X2 receptor was explored [3]. Cysteine residues that were substituted in two positions from adjacent subunits and separated ~18Å ($N140C^{rP2X2}$ and $L186C^{rP2X2}$; Fig. **5F**) were shown to be covalently labeled by NCS-ATP and trapped the receptor in a covalently agonist-bound state that, depending on the tethered position, decreased ($L140C^{rP2X2}$) or increased ($L186C^{rP2X2}$) the ability to gate the channel. These results demonstrated a dynamic ATP binding site at the subunit interface in which the orientation of ATP bound to the receptor modulates the gating probability of the channel [3]. In a subsequent study, NCS-ATP bound to $L186C^{rP2X2}$ induced a conformational change of the ATP binding site but did not result in channel opening, suggesting that intermediate or preactivation state(s) exist that prime(s) the channel gating of the P2X2 receptor [137].

A voltage-clamp fluorometry study of the P2X1 receptor used tetramethylrhodamine maleimide (TMRM)-labeled cysteine residues substituted in the stretch of residues between the first conserved disulfide bridge between $C117^{rP2X1}$/$C126^{rP2X1}$ (Fig. **5G**) localized at the base of the head domain to analyze ATP activation, channel gating and desensitization [10]. The interpretation of these experimental data by a P2X1 homology model and ATP-docking demonstrated that the base of the head domain faces the ATP-binding site and that the ribose moiety of ATP faces the solution. TMRM covalently attached to specific engineered cysteine residues was suggested to report ligand binding ($N120C^{rP2X1}$, $E122C^{rP2X1}$, and $G123C^{rP2X1}$), conformational changes associated with channel opening ($G115C^{rP2X1}$ and $G124C^{rP2X1}$) and desensitization ($P121C^{rP2X1}$ and $I125C^{rP2X1}$) [10]. The fluorescent ATP derivative Alexa-647-ATP was shown to be a valuable tool for visualizing ligand binding and unbinding. The long-lasting desensitization of the P2X1 receptor is characterized by the slow unbinding of the agonist and agonist-induced receptor internalization [138]. Interestingly, the treatment of the generally ATP-insensitive $K69C^{rP2X2}$ mutant P2X2 receptor with the thiol-reactive fluorescent dye Alexa-Fluor 546 C_5-maleimide resulted in a channel that gated (opened) in response to zinc or acidic pH and in the absence of ATP [139], which may enable the study of the mechanism of activation independently of ATP binding.

INHERENT DYNAMICS OF THE ATP BINDING SITE AFFECT ATP BINDING AND SUBSEQUENT ALLOSTERIC CHANGES

In a study of the hP2X3 receptor using molecular-dynamics simulations and oxidative cross-linking of cysteines substituted at positions of close proximity between intra- or intersubunit domains, such as the head-domain and dorsal-fin or the dorsal-fin and left-flipper, it was shown that the ATP binding pocket undergoes spontaneous conformational changes even in the absence of ATP [140]. Interestingly, the spontaneous cross-linking of $K201C^{hP2X3}$ (dorsal fin) and $V274C^{hP2X3}$ (left flipper) (Fig. **5H**) prevented the binding of ATP to the receptor as analyzed by the fluorescent APT-derivative BODIPY-TR ATP, which was equipotent to αβ-meATP. It was concluded that the high conformational flexibility of domains constituting the ATP binding pocket is a prerequisite for agonist binding and receptor activation [140]. In addition, in the zfP2X4 receptor, ATP recognition is determined by the inherent dynamics of the head domain [141]. Inherent dynamics in the absence of ATP and ATP-induced coordinated allosteric changes in the left flipper and dorsal fin domains were also revealed by molecular dynamics and normal-mode analysis of the zfP2X4 receptor and verified by cysteine cross-linking and a engineered zinc bridge between the left flipper and dorsal fin [142].

NUMBER OF ATP MOLECULES REQUIRED TO OPEN A P2X RECEPTOR

An ATP concentration-response analysis of whole-cell currents mediated by the cloned rP2X2 receptor yielded a Hill coefficient of 2.0, suggesting that activation requires the binding of more than one agonist molecule [20]. A similar Hill coefficient of 2.3 for ATP activation was estimated from the ATP concentration dependence of single-channel recordings of rP2X2 receptor-mediated currents [143]. The Hill coefficient is not a reliable indicator of the number of functionally important ligand binding sites. Even in cases of strong positive cooperativity, only a minimum estimate of the number of binding sites that were involved is provided [144]. Therefore, a Hill coefficient of 2.3 indicates that there are at least three ATP binding sites in a functional P2X2 receptor. Kinetic modeling of the data suggested that the binding sites are not independent and that only fully agonist-liganded channels open. Spontaneous openings in the absence of ATP were not observed [143].

Experiments with mutants of homo- and heterotrimeric P2X receptors combined with kinetic modeling provided a different view by demonstrating that the binding of two ATP molecules per P2X receptor is sufficient to gate the channel [86, 145-149]. The occupancy of only one ATP binding site per homotrimer did not evoke a detectable inward current but induced conformational changes of the unoccupied ATP-binding sites [3, 137, 150]. This conformational change mostly facilitates the binding of the second and third ATP molecule and is suggested to be the structural correlation of the positive cooperativity that was detected in early electrophysiological studies [143]. In addition, the photoaffinity labeling of an rP2X2-X1 chimera and the rP2X2 receptor and analysis by voltage-clamp fluorometry have demonstrated that agonist-primed channels exhibit an increased gating efficiency in subsequent agonist binding [151]. Together, these data strongly suggest the existence of the aforementioned intermediate (also termed primed or flipped) state that precedes the activation of the channel in terms of channel opening [137, 150, 152]. In the intermediate closed state, which is induced by conformational changes upon ATP

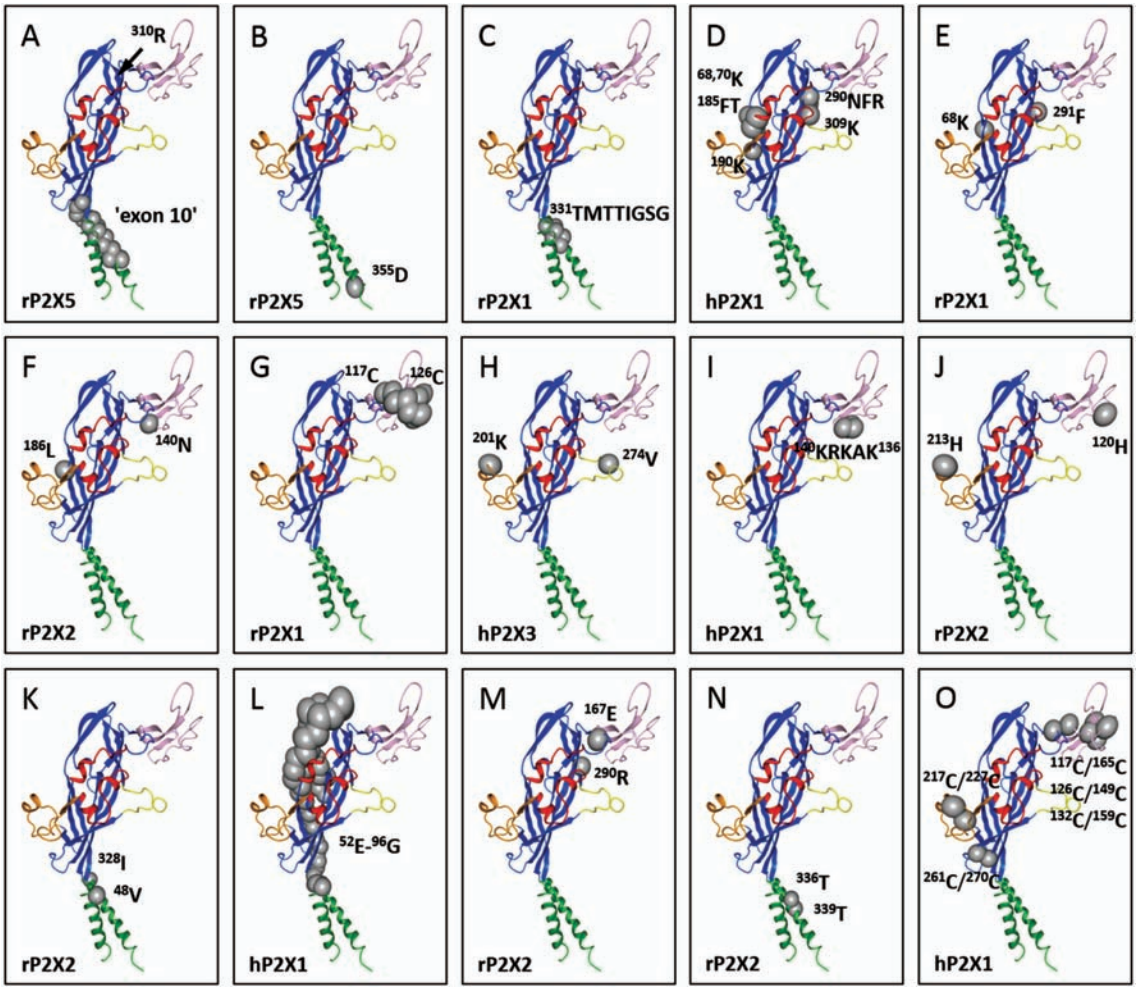

Fig. (5). Visualization of the position of selected residues in the zfP2X4 structure. A-O, the selected residues that are discussed in the text were aligned to the homologous zfP2X4 residues using the alignment of Kawate *et al.* (Kawate *et al.*, 2009) and mapped to the apo-closed-state structure of the zfP2X4 receptor (PDB entry 4DW0 (Hattori and Gouaux, 2012)). The 'dolphin' domains are colored as in Fig. (1). The selected residues are highlighted by a gray ball representation of the corresponding C_α-atom. The numbers refer to the residue of the appropriate P2X isoform as mentioned in the text and as indicated in the lower left corner of each panel.

binding to one of the three binding sites, pyrimidine and diphosphate nucleotide analogs, such as ADP, UTP and CTP, become effective [150].

BINDING OF COMPETITIVE ANTAGONISTS AS REVEALED BY HOMOLOGY DOCKING AND MUTAGENESIS

The hP2X1 receptor mutants $K68A^{hP2X1}$ and $K309A^{hP2X1}$, which caused the largest decrease of the ATP potency, did not affect the antagonism by suramin [117]. These and similar results with the mutants of aromatic residues of the hP2X1 receptor raised the question of whether the binding sites for ATP and suramin binding are different [120].

The *Dictyostelium discoideum* P2X receptor (DdP2X) was insensitive to the common P2X receptor blockers suramin, PPADS and TNT-ATP [34]. DdP2X receptors lack a significant portion of the cysteine-rich head domain that is found in mammalian P2X receptors. Pharmacologic analysis of chimeras between the NF449-sensitive human P2X1 receptor and the NF449-insensitive DdP2X receptor and of hP2X1 and hP2X2 mutants identified a cluster of positively charged residues ($^{136}KAKRK^{hP2X1}$; Fig. **5I**) that contributes to suramin and NF449 sensitivity [153]. Because this motif is absent in the P2X2 receptor, these data provide a plausible explanation for the weak potency of NF449 [153, 154] and some related derivatives [155-157] at the P2X2 receptor.

Evidence for the view that the ATP-binding pocket of P2X receptors may accommodate at least part of the suramin-derivative molecule comes from its competitive interaction with ATP and the observed crucial role of the number and position of acidic groups for the high inhibitory potency of suramin derivatives [155]. It was thus reasonable to assume that the sulfonic acid groups of suramin or its derivatives interact electrostatically with the lysine and arginine residues that are physiologically involved in attracting the phosphate groups of ATP [155, 158] as well as with non-conserved basic residues of the ATP-binding pocket. In agreement with this view, a structure-activity relationship analysis of anthraquinone derivatives demonstrated the importance of negative charges for high-potency P2X2 antago-

nism. The most potent antagonist of this series, PSB-1011, contains two negatively charged sulfonic acid moieties and preferentially blocks the rP2X2 receptor with nanomolar potency [159].

In silico docking of the nanomolar potent P2X2 receptor antagonist, the suramin derivative NF770, into the ATP binding site of the rP2X2 receptor revealed that NF770 fits into the ATP binding pocket and was suggested to enter into strong electrostatic interactions with basic residues that are important for ATP binding [37, 160]. Furthermore, the residues $G72^{rP2X2}$ and $E167^{rP2X2}$, which are important for the optimal coordination of NF770 in the ATP binding pocket, play an important role in the ATP activation of the rP2X2 receptor [160].

SUPERIMPOSED CLOSED AND OPEN CRYSTAL STRUCTURES REVEAL GATING MOVEMENTS

The resolution of the ATP-bound open-state structure of the zfP2X4 receptor [2] and its comparison and superimposition to the apo-closed-state zfP2X4 structure revealed the conformational changes underlying the ATP-induced closed-to-open transition of the zfP2X4 receptor (channel gating and pore opening) [2]. After ATP binding, the intersubunit cleft between the head and the dorsal fin domains closes, and an outward flexing and rotation of the subunits occurs. This flexing results in the substantial expansion of the extracellular vestibule outside of the channel pore, and an iris-like motion of the membrane-spanning helices subsequently occurs that eventually opens the ion channel pore [2].

ATP-INDUCED TIGHTENING OF THE ATP BINDING SITE FAVORS OPENING OF THE ION CHANNEL

Several of the early mutagenesis studies also revealed amino-acid residues involved in the coupling of ATP-binding to channel opening (channel gating). For instance, the $T339S^{rP2X2}$mutation that destabilizes the closed state of the receptor and the simultaneous $K308A^{rP2X2}$ mutation revealed a role in the channel gating of the $T339^{rP2X2}$ and $K308^{rP2X2}$ residues [161]. Later, the $T339S^{rP2X2}$ mutation of the P2X2 receptor, which has been suggested to alter the equilibrium between the closed- and open-state in favor of the latter, in combination with native ($H120^{rP2X2}$ and $H213^{rP2X2}$ (Fig. **5J**)) [162-165] and engineered Zn^{2+}binding sites was instrumental in demonstrating that following ATP binding, the head and dorsal fin domains move closer to each other. The resulting tightening of the ATP binding sites correlates precisely with channel opening [4]. Thus, the ATP-induced tightening of the ATP binding sites represents an essential early step in the coupling of ATP binding to channel gating and favors the opening of the ion channel. The competitive antagonist TNP-ATP prevents gating by preventing the tightening of the ATP-binding pocket [4].

The substantial movement of the head domain upon ATP binding was also demonstrated by the voltage-clamp fluorometric analysis of TMRM-labeled engineered cysteine residues in the P2X1 receptor (Fig. **5G**) [10].

SUBSTANTIAL GATING MOVEMENTS AT THE INTERFACE OF ADJACENT SUBUNITS AS REVEALED BY MUTATIONAL STUDIES

The need of significant conformational movements at the interface of adjacent subunits during channel gating was previously concluded from disulfide-bond-formation studies between introduced cysteine residues at the extracellular subunit interfaces of several P2X isoforms: the homomeric rP2X1 receptor ($K68C^{rP2X1}$ and F291CrP2X1 (Fig. **5E**)) [133], the heteromeric rat P2X2/3 receptor ($V48C^{rP2X}$ and $I328C^{rP2X2}$(Fig. **5K**), and $V42C^{rP2X3}$ and $I319C^{rP2X3}$) [86, 166]; and the heteromeric rat P2X1/2 receptor ($K68C^{rP2X1}$and $F289C^{rP2X2}$) [167]. Later, a significant movement of the subunit interface at the level of the lower body domain was also found in disulfide trapping experiments of cysteines when introduced in position $E63^{rP2X2}$ of one subunit and $R274^{rP2X2}$ of the adjacent subunit in the rP2X2 receptor [168]. ATP binding was suggested to induce movements of the adjacent subunits at the level of $E63^{rP2X2}$/$R274^{rP2X2}$, resulting in the spatial separation of these residues and a disruption of the putative intersubunit salt bridge between $E63^{rP2X2}$/$R274^{rP2X2}$ [168].

Extensive conformational changes of the ectodomain of the hP2X1 receptor as induced by ATP binding were also evident from mapping MTSEA-biotinylation data of cysteine mutants ($E52C^{hP2X1}$ to $G96C^{hP2X1}$ (Fig. **5L**)) to a homology model and by electron microscopy of the purified hP2X1 receptor protein in the presence and absence of ATP [5].

Introduced disulfide bonds (disulfide locks) at the interface of adjacent subunits at various ectodomain or TM levels significantly inhibited the channel function by restricting intersubunit movements of the hP2X1 [5] or the rP2X2 receptor [169]. Channel gating is also inhibited by intersubunit disulfide bond formation between the rP2X2 receptor mutants $E167C^{rP2X2}$ and $R290C^{rP2X2}$ [11]. In this study, the analysis of mutants of $E167^{rP2X2}$ and $R290^{rP2X2}$ (Fig. **5M**) revealed that $E167^{rP2X2}$ and $R290^{rP2X2}$ interact electrostatically within the ATP binding site. The comparison of the closed and open-state model of the rP2X2 receptor revealed a significant rearrangement of $E167^{rP2X2}$ and $R290^{rP2X2}$ during the closed-to-open transition of the receptor. The release of the $E167^{rP2X2}$/$R290^{rP2X2}$ salt bridge during ATP activation of the P2X2 receptor enables $R290^{rP2X2}$ to undergo a strong ionic interaction with a γ -phosphate oxygen of ATP. This switching of the electrostatic interaction of $R290^{rP2X2}$ from $E167^{rP2X2}$ to ATP seems to represent a crucial early event in the gating of the rP2X2 receptor by linking the ionic coordination of ATP within the ATP binding pocket with a simultaneous destabilization of the closed state of rP2X2 via the ATP-induced breaking of the $E167^{rP2X2}$/$R290^{rP2X2}$ salt bridge [11].

GATING OF P2X RECEPTORS BY OPTOGATING OR LIPOPHILIC ATTACHMENTS TO THE TM2 DOMAINS

Two ingenious studies were published that used *cis-trans* isomerization to engineer a light-gated rP2X2 receptor. One study made use of cysteine substitution mutants of the outer TM helices of the P2X2 receptor, which were previously

shown to modify channel gating when chemically modified [170]. Attachment of the sulfhydryl reactive maleimide ethylene azobenzene trimethyl ammonium derivative (MEA-TMA), which shows strong photo isomerization from trans-to-cis- or cis-to-trans-isomers using 365 nm or 525 nm of light, respectively, to $V48C^{rP2X2}$ or $I328C^{rP2X2}$ (Fig. **5K**), resulted in a P2X2 channel that could be turned on and off by light. The simultaneous mutation of $I328C^{rP2X2}$ and of $K69A^{rP2X2}$, which makes the P2X2 receptor ATP insensitive, revealed that the light gating of the channel does not require the natural ligand ATP [170]. The expression of the $K69A^{rP2X2}/I328C^{rP2X2}$ P2X2 mutant in dissociated hippocampal neurons endogenously expressing the P2X2 receptor enabled the optical control of P2X2-like currents in the absence of ATP that evoked the neuronal activity (action potential firing) of the neurons. These data indicate that this approach will enable the dissection *in vivo* of the physiological and pathological roles of P2X2 receptors at an unprecedented level of precision by the rapid and reversible control of neuronal activity [170].

Simultaneously with the former study, it was demonstrated that the covalent attachment of the bifunctional azobenzene 4,4'-bis(maleimido)azobenzene to cysteine residues when substituted at position 329 of the rP2X2 receptor ($P329C^{rP2X2}$) resulted in a P2X2 receptor channel that could be light-dependently gated by *cis-trans* isomerization [171]. The TM2 $P329^{rP2X2}$ residue is oriented toward other $P329^{rP2X2}$ residues in adjacent subunits. Thus, the bridging of two adjacent engineered cysteine residues by the bifunctional azobenzene derivative enabled channel opening by the *cis-to-trans* isomerization-induced separation of the residues $P329^{rP2X2}$ of adjacentTM2s. Interestingly, this study also demonstrated the light-gating of homomeric rP2X3 and heteromeric rP2X2/3 receptors via a similar chemical modification of the $P320C^{rP2X3}$ mutant of the rP2X3 subunit and of hASIC1a channels ($G430C^{hASIC1a}$ mutant) [171]. These findings suggest that the opening mechanism of homo- and heteromeric P2X channels and of ASIC1a channels at the level of the transmembrane domain is functionally similar [171]. Another study used the $V48C^{rP2X2}$ or $I328C^{rP2X2}$ mutants (Fig. **5K**) of the P2X2 receptor together with hydrophilic and lipophilic methanethio sulfonates (MTS) [172]. The TM1/TM2 interaction of $V48^{rP2X2}/I328^{rP2X2}$stabilizes the closed state, and the attachment of a larger lipophilic moiety (e.g., propyl-MTS) to $I328C^{rP2X2}$ breaks this interaction, most likely by steric hindrance, which results in the destabilization of the closed state and the facilitation of channel opening [172]. Together, these findings suggest that the intersubunit movement at the level of the outer TMs can be modulated by surrounding lipids that may directly affect the open channel stability.

EARLY MUTAGENESIS STUDIES OF THE PORE REGION

The modification of the cysteine residues that were introduced into the first transmembrane domain 1 (TM1) by MTS reagents, alanine or tryptophan substitution and the analysis of fractional calcium currents indicated that TM1 residues do not directly contribute to the transmembrane permeation pathway [166, 173-178]. However, when substituted by cysteine, the residue $V48^{rP2X2}$ of the outer TM1 of the P2X2 receptor or the corresponding valine residue of the P2X3 receptor was shown to build an inhibitory cross-link with $I328C^{rP2X2}$ or $I319C^{rP2X3}$ [166]. This finding led to the conclusion that the valine residue at the outer end of TM1 moves with channel opening [166].

Later, analog experiments were performed at the heteromeric P2X2/3 receptor and revealed the head-to-tail subunit arrangement and the $P2X2(3)_2$ stoichiometry of the P2X2/3 receptor [86]. Several early studies that were mainly performed by the substituted cysteine accessibility method (SCAM) in combination with MTS reagents and Ag^+ or Cd^{2+} modification or by alanineor tryptophan scanning demonstrated that several TM2 residues line the permeation pathway of the P2X2, P2X4 or P2X7 receptor [179] Li [6, 174, 175, 177, 178, 180-186].

EARLY MUTAGENESIS STUDIES OF THE CHANNEL GATE

Already, early SCAM studies of the rP2X2 receptor addressed the precise localization of the channel gate [179, 180]. However, due to the different accessibility of $L338^{rP2X2}$, the location of the gate was slightly different in these studies. While Rassendren and colleagues reported the gate between $L338^{rP2X2}$ and $D349^{rP2X2}$, Egan *et al.* concluded that the gate is located more extracellularly (proximal) of TM2 (between $I328^{rP2X2}$ and $L334^{rP2X2}$) and that TM2 crosses the membrane in a non-helical manner because $G342^{rP2X2}$ was accessible by $MTSEA^+$ from both sides of the membrane. Later, a SCAM study of the rP2X2 receptor using $MTSET^+$, Ag^+ and Cd^{2+} as modification reagents located the gate in the external (helical) half of TM2 between $I332^{rP2X2}$ and $T341^{rP2X2}$ [177]. This study also suggested that TM1 is positioned peripherally to TM2. Considering previous results [181, 182], the gate region itself may also function as a selectivity filter [177].

CRYSTAL STRUCTURE-BASED MUTATIONAL ANALYSIS OF THE PORE REGION AND CHANNEL GATE

In the post-crystal structure era, two further SCAM studies using MTS-reagents, Ag^+ and Cd^{2+}[6], or only Cd^{2+} [186] as modification reagents revealed the gates between $I332^{rP2X2}$ and $T339^{rP2X2}$, and $T336^{rP2X2}$ and $T339^{rP2X2}$, respectively. The mapping of these residues to P2X2 receptor homology models based on the closed-state crystal structure of the zfP2X4 receptor showed that the narrowest part of the closed-ion conducting pathway is formed by $T336^{rP2X2}$ and $T339^{rP2X2}$ [187] (Fig. **5N**), which is in general agreement with the latest SCAM and mutagenesis studies [6, 9, 177, 186]. The $T339K^{rP2X2}$ mutant of the P2X2 receptor was shown to increase anion permeability and reduce unitary conductance gradually when mutated in one, two or three subunits of the P2X2 receptor, indicating that each subunit contributes symmetrically to the open-channel permeation pathway upon the iris-like separation of the three TM2 helices [188]. The slight deviations in the accessibility of one or two residues between these studies of the P2X2 receptor can most likely be attributed to the use of cysteine-reactive reagents of different molecular sizes and differences in the experimental setup.

For further details regarding the individual TM residues forming the pore and channel gate as revealed by mutagenesis, the reader is referred to the reviews of Browne *et al.* [112] and Jiang *et al.* [111] and the comprehensive review of the principles and properties of ion flow in P2X receptors by Samways and colleagues [187].

EXTRACELLULAR ION ACCESS PATHWAY

Poisson-Boltzmann calculations along with thiol-reactive modifications of introduced cysteine residues in the surrounding of the lateral portals, which were evident from the zfP2X4 receptor structure and homology models of other P2X members, identified the three lateral ion-access-pathways [7, 8, 189]. This finding has significantly improved our understanding of how ions access and flow through P2X receptors. Interestingly, the fractional and relative Ca^{2+} permeability through the ivermectin-sensitive hP2X4 receptor was diminished by ivermectin, which is the first report of an allosteric modulation of the Ca^{2+} current of a LGIC by an exogenously applied drug [190]. The $E51Q^{hP2X4}$ mutation located at the entry of the lateral portals significantly attenuates the effect of ivermectin on the Ca^{2+} permeability, indicating a key role of the negative charge at the lateral ion entrance to control Ca^{2+} access to the pore [190]. In addition, the permutation of several other residues, such as $E56^{r/hP2X4}$, $F324^{rP2X4}$, $G325^{rP2X4}$, $K329^{rP2X4}$, and $D331^{hP2X4}$, was shown to affect the permeation properties of the P2X4 lateral portals or central vestibule [8, 175, 189-191]. The existing data regarding extracellular access to the transmembrane channel pore were comprehensively reviewed by Samways and colleagues [187].

DESENSITIZATION

Based on current whole-cell recordings, heterologously expressed P2X receptors can be divided into rapidly desensitizing (P2X1 and P2X3) and slowly desensitizing (P2X2, P2X4, P2X5, and P2X7) receptors [192]. The time course of desensitization of specific P2X receptors is of significant physiological relevance because desensitization determines the time course of P2X receptor signal transduction.

The molecular mechanisms controlling desensitization are not yet fully understood and apparently involve various domains of the receptor (intracellular, TM, and extracellular domains), and interactions with other proteins or intracellular messengers are possibly involved [193, 194]. An analysis of chimeras that were composed of parts of desensitizing and non-desensitizing P2X receptors, P2X2 splice variants and mutant P2X receptors indicated that the desensitization rate of P2X receptors is primarily determined by the first and second transmembrane domain (TM1 and TM2) and the cytoplasmic N- and C-terminal domains [155, 195-210]. Substitution of solely the intracellular N- or C-terminal parts of desensitizing P2X receptors by corresponding parts of non-desensitizing receptors and even such point mutations within the cytoplasmic domains were shown to be sufficient to slow desensitization and to obtain at least partially desensitizing or non-desensitizing receptors [199, 201, 204, 207]. For instance, the desensitization of the human P2X1 receptor or the rat and human P2X3 receptor was markedly delayed by an N-terminal $D17E^{hP2X1}$ [201] or $S15V^{r/hP2X3}$mutation [204], respectively. The $S15V^{r/hP2X3}$ substitution of the P2X3 receptor was sufficient to delay desensitization of the P2X3 receptor to permit the reliable assessment of the mechanism of the antagonism of compounds, which was formerly hampered by the rapid desensitization of the P2X3 receptor [204].

The acceleration of desensitization of the P2X2 receptor was demonstrated for the $T18A^{rP2X2}$ or $K20T^{rP2X2}$ mutations, which disrupted, among mammalian P2X receptors, the conserved N-terminal $^{18}TXK^{20}$ sequence [211, 212]. However, mutations of corresponding threonine residues in the P2X1 or P2X3 receptors result in rudimentary functional or non-functional receptors, respectively [211, 213, 214]. Further desensitization-accelerating mutations include $V21S^{rP2X2}$/$I22V^{rP2X2}$ in the N-terminal tail [204]; $G342C^{rP2X2}$, $S345C^{rP2X2}$, $L352C^{rP2X2}$, $L353C^{rP2X2}$, and $T354C^{rP2X2}$ in the C-terminal half of the TM2 [177, 180]; and $K365Q/N^{rP2X2}$, $K369Q/N^{rP2X2}$ [215] and $K373A/R/C^{hP2X4}$ [199] in the C-terminal endodomain. Interestingly, the penultimate C-terminal charge of the arginine residue of the P2X receptor from the cattle tick *Boophilus microplus* was responsible for the slow desensitization kinetics, but not for the current run-down during repetitive ATP applications, indicating that run-down and desensitization are controlled by distinct mechanisms [207].

In conclusion, these data suggest that desensitization is determined mostly but not exclusively by the N- and C-terminal P2X receptor segments. However, the structural interpretation of the desensitization process as mediated by the N- and C-terminal domains is hampered by the fact that the crystal structures of zfP2X4 are lacking these intracellular termini to aid the crystallization behavior of the zfP2X4 constructs [1, 2].

FURTHER KEY SITES OF P2X RECEPTORS AS IDENTIFIED BY SITE-DIRECTED MUTAGENESIS

The functional importance of the 10 conserved cysteines (Fig. **5O**) and the disulfide connectivity of each subunit were explored by mutagenesis studies [216, 217]. Disulfide bonds between the conserved cysteine residues were also shown to contribute substantially to the structure of the ligand binding pocket and channel gating [218]. Mutagenesis of the intracellular C-terminal cysteine residue $C430^{rP2X2}$ revealed that $C430^{rP2X2}$ functions as a redox sensor enabling the functional modulation of the rP2X2 channel by the redox state of the cell [219]. Functional analysis of the wt and mutant human and rat P2X3 receptor revealed that the position of non-conserved C-terminal tyrosine residues controls in part the species-specific properties of the human and rat P2X3 receptors [220]. A recent study used chimeras between the human P2X1 and P2X2 receptors to assign several properties of these channels to distinct protein domains [200]. It was shown that the agonist potency and efficacy, open-channel properties and recovery from desensitization can be controlled independently by concerted interaction between the intracellular, transmembrane and extracellular domains of the receptor. For instance, the ectodomain determines the antagonist sensitivity, the C-terminus is involved in recovery from desensitization and the TMs control agonist sensitivity and efficacy, while the N-terminus can regulate agonist efficacy independently of agonist sensitivity [200].

CONCLUDING SUMMARY

The discovery of ATP-gated P2X receptors dates back more than 30 years. Soon after their isolation by DNA cloning, P2X receptors were demonstrated to assemble as trimers, which was a new structural motif for a ligand-gated ion channel at that time. Hence, each of the three major classes of ligand-gated ion channels is characterized by its own unique subunit stoichiometry, trimeric for the P2X receptors, tetrameric for ionotropic glutamate receptors and pentameric for Cys-loop receptors. Site-directed mutagenesis was instrumental in disclosing key sites of the function and oligomerization of P2X receptor. The event of the high resolution crystal structures of the zfP2X4 receptor in the homotrimeric apo-closed and ATP-bound open states provided and will continue to provide invaluable new insights into receptor mechanisms and subunit assembly. Beyond doubt, we are in a particularly exciting era of P2X receptor research.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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