Emerging structures and ligands for P2X₃ and P2X₄ receptors—towards novel treatments of neuropathic pain

Christa E. Müller

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Kawate T, Michel JC, Birdsong WT, Gouaux E (2009) Crystal structure of the ATP-gated P2X₄ ion channel in the closed state. *Nature* 460:592–598

Article summary

The paper by Kawate et al. describes the X-ray structure of the zebrafish P2X₄ receptor in the closed state at 3.1 Å resolution. This presents the very first crystal structure of a P2X receptor subtype. It was obtained by the following strategy: 35 different P2X receptor orthologues were transiently transfected into HEK293 cells and subjected to fluorescence-detection size exclusion chromatography in order to screen for stability and monodispersity of the trimeric receptor. The zebrafish P2X₄.1 receptor was found to show the most promising properties. It was then further modified in order to improve its crystallization behavior by removing amino acids from the N- as well as from the C-terminus but preserving functionality of the receptors. In addition, three-point mutations were introduced (Cys51Phe, Asn78Lys, and Asn187Arg) in order to avoid unnatural disulfide formation and glycosylation, which may lead to

heterogeneity. Expression in Sf9 insect cells using a baculovirus expression system yielded large amounts for crystallization, which was performed after solubilization of the membranes with *n*-dodecyl-β-D-maltoside followed by metal-ion affinity and size exclusion chromatography. The X-ray structure confirmed that the receptor was formed of three subunits and contained six transmembrane α-helices, two of each subunit. The large extracellular domain protruded ca. 70 Å above the membrane. Subunit–subunit interactions were mainly mediated by the extracellular domains. The X-ray structure, although in the closed state without bound ligand, showed the localization of the central ion pore and the gating mechanism (by hydrophobic amino acid residues) and provided an explanation for the cation selectivity (several acidic amino acid residues above the channel). One crystal structure was obtained with the antagonist Gd³⁺ bound to the receptor occupying four different sites: one in the center of the extracellular domains coordinated by Glu98 of the three subunits, and three in the periphery of the extracellular domain, one in each subunit. Unfortunately, the ATP-binding sites were not identified, but hypotheses as to where agonists and antagonists might bind were discussed in the manuscript. Surprisingly, the 3D structure was found to be similar to that of the chicken ASIC1 channel structure despite extremely low sequence similarity.

Commentary

The P2X₄ receptor is a member of the nucleotide-activated P2 receptor family. P2X receptors are ligand-gated ion channels consisting of three subunits. Functional homomeric (e.g., P2X₃ or P2X₄) as well as heteromeric (e.g., P2X_{2/3}) receptors have been described. Their structure is unique and unrelated to any ion channel proteins of known

C. E. Müller
PharmaCenter Bonn, Pharmaceutical Institute,
Pharmaceutical Chemistry I, Pharmaceutical Sciences Bonn (PSB),
University of Bonn,
An der Immenburg 4,
53121 Bonn, Germany

C. E. Müller (✉)
Pharmazeutisches Institut, Pharmazeutische Chemie I,
An der Immenburg 4,
53121 Bonn, Germany
e-mail: christa.mueller@uni-bonn.de

structure. The P2 receptors are integrated into the cell membrane, and therefore, it has been difficult to obtain crystals suitable for X-ray crystallography. The P2X₄ receptor like all other P2X subtypes is activated by the physiological agonist ATP. Neither potent nor selective antagonists for P2X₄ receptors have been described so far, while for some other P2X receptor subtypes (P2X₃ and P2X₇), potent and selective antagonists have been developed. P2X receptors in general and the P2X₄ receptor in particular are very promising novel drug targets. Several P2X receptor subtypes (P2X₃, P2X_{2/3}, P2X₄, and P2X₇) have been proposed to play a role in pain sensation and/or transmission. For example, P2X₄ receptors are upregulated in activated microglia in the central nervous system (CNS), and the receptor has been proposed as a novel target for the treatment of chronic neuropathic pain, brain and spinal cord injury, and cerebral ischemia. P2X₄ receptor knockout mice showed a strong reduction in mechanical allodynia and hyperalgesia in the spinal nerve ligation model of neuropathic pain. Hyperactive microglia is also critical for the pathogenesis of neurodegenerative disorders and stroke. Therefore, P2X₄ receptors may also be a potential therapeutic target for neuroprotection.

The obtained crystal structure is the very first X-ray structure of a P2X receptor, and therefore, not only important with regard to the P2X₄ receptor subtype but will help in understanding the structure and function of all P2X receptor subtypes. In particular, it will contribute to obtain improved molecular receptor models, which will be crucial for the development of new receptor ligands and, for example, allow the application of virtual screening approaches.

This study confirms and explains previous findings and assumptions, which had only been speculative so far. For example, it finally proves that the receptor is trimeric. It also gives a good idea about the ion channel pore and the potential mechanism of cation binding and the transduction mechanism. Furthermore, although only speculative, the ATP-binding sites can be more accurately postulated as being localized between subunits containing basic as well as aromatic amino acid residues. The described structure will contribute to the design of—urgently required—ligands for P2X₄ receptors, as well as for other P2X receptor subtypes. Furthermore, the developed method for obtaining the first crystal structure of a P2X receptor will guide the way to obtain more structures from other subtypes, from heteromeric receptors from activated receptor conformations, and from antagonist-bound receptors. This manuscript is therefore an important milestone in the field of P2X receptor research and will contribute to their exploitation as novel therapeutic targets.

Nagata K, Imai T, Yamashita T, Tsuda M, Tozaki-Saitoh H, Inoue K (2009) Antidepressants inhibit P2X₄ receptor function: a possible involvement in neuropathic pain relief. *Molecular Pain* 5:20–31

Sim JA, North RA (2010) Amitriptyline does not block the action of ATP at human P2X₄ receptor. *Br J Pharmacol*. doi:10.1111/j.1476-5381.2010.00683.x

Combined article summary

In two new studies, antidepressant and antiepileptic drugs have been investigated for their effects on P2X₄ receptors in order to clarify whether P2X₄ blockade might explain their effects on neuropathic pain. Nagata et al. investigated 16 antidepressant and antiepileptic drugs, including serotonin reuptake inhibitors (paroxetine, fluoxetine, fluvoxamine, citalopram, and milnacipran), tricyclic antidepressants (clomipramine, maprotiline, nortriptyline, desipramine, doxepine, amitriptyline, and mianserine), and antiepileptic drugs (gabapentin, zonisamide, and carbamazepine). All drugs were initially investigated for their inhibition of ATP-induced calcium influx in 1321N1 astrocytoma cells transfected with the rat P2X₄ receptor. The cells were pretreated for 10 min with the respective drug (10 μM) before stimulation of the P2X₄ receptors with ATP (30 μM). The weak, non-selective P2X₄ antagonist 2',3'-O-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate (TNP-ATP) was used as a control. The most potent antagonist was paroxetine (**1**), which was significantly more potent than the control antagonist TNP-ATP. Fluvoxamin was much weaker, while citalopram and amitriptyline (**2**) showed only low inhibitory potency. Subsequently dose–response curves were recorded for paroxetine, and IC₅₀ values at the rat and the human P2X₄ receptor were determined to be 2.45 (rat) and 1.87 μM (human), respectively. Concentration–response curves for ATP in the presence of three different concentrations of paroxetine appeared to indicate a non-competitive inhibition mechanism at both, the rat and the human P2X₄ receptor, since the maximal stimulation was reduced with increasing concentration of inhibitor. In addition, electrophysiological experiments were performed to show that paroxetine (10 μM) significantly inhibited ATP-induced currents in rat P2X₄ receptors expressed in 1321N1 cells as well as in primary microglia. As a next step, paroxetine was investigated in a rat model of neuropathic pain. Intrathecal administration showed an antiallodynic effect when applied 7 or 14 days after spinal nerve injury. Fluvoxamine showed a weaker effect, while citalopram was inactive consistent with the rank order of potency determined in vitro. The authors showed that the effect was not due to effects on serotonin levels since 5-HT_{1A},

5-HT_{2A}, and 5HT₃ antagonists or spinal 5-HT deprivation did not change the antiallodynic effect of paroxetine. Paroxetine also blocked P2X₇ receptors, but the non-selective P2X₇ receptor antagonist PPADS had in previous experiments not shown any effect on allodynia in an animal model of neuropathic pain. The authors conclude that P2X₄ receptor blockade may contribute to the antiallodynic effect of some antidepressant drugs, such as paroxetine.

In the study by Sim and North, the antidepressant drug amitriptyline (**2**), which is frequently applied for the treatment of neuropathic pain in the clinic, was investigated for its inhibitory potency at rat, mouse, and human P2X₄ receptors. In addition, the compound was investigated at P2X₂ and P2X₇ receptors. All receptors were expressed in HEK cells, and ATP-evoked inward currents were measured. The drug was applied at 10 μM concentration and either co-administered with ATP or applied 2–6 h prior to ATP stimulation. Full dose–response curves were determined for ATP in the presence or absence of the drug. Amitriptyline did not show any effect on human P2X₄ receptors and was only weakly active at rat and mouse receptors. No effects of amitriptyline were found on rat P2X₂ and rat or human P2X₇ receptors. The authors conclude that P2X₄ receptor blockade does not contribute to the antihyperalgesic activity of amitriptyline in humans.

Commentary

Neuropathic pain, which may, for example, be due to nerve injury, diabetes, herpes infections, or cancer, is characterized by severe allodynia (tactile hypersensitivity) and is chronic in nature. It is typically resistant to standard pain treatment, such as non-steroidal anti-inflammatory drugs and opioids. Some antidepressant and antiepileptic drugs have been found to be active in patients with neuropathic pain. However, their mechanism of action in the treatment of neuropathic pain is unknown. As described above, the P2X₄ receptor is a potential new drug target for the treatment of neuropathic pain. This is due to the fact that

P2X₄ receptors are upregulated on activated microglia after spinal nerve injury, and blockade of P2X₄ receptors, reduction (by RNA antisense oligonucleotide), or disruption of P2X₄ receptor expression prevented the development of allodynia.

Nagata et al. identified the serotonin reuptake inhibitor paroxetine as a relatively potent P2X₄ receptor antagonist with similar potency (IC₅₀ ca. 2 vs. 30 μM ATP) at human and rat P2X₄ receptors. The authors claim a non-competitive mechanism of inhibition. Their data appear to be in favor of this interpretation. Paroxetine showed activity in a rat model of neuropathic pain after intrathecal administration. It would have been interesting to see pharmacological activity after i.p. or oral administration as well.

The results obtained by Sim and North are well in accordance with the results described by Nagata et al.: amitriptyline is only a very weak P2X₄ antagonist and only active in rats and mice.

The final question remains unanswered: Does P2X₄ receptor blockade contribute to the antiallodynic effect of any of the antidepressant drugs used for the treatment of neuropathic pain? For amitriptyline, the answer is clearly “no.” For paroxetine or others, inhibition of P2X₄ receptors might contribute to their therapeutic effects, but this has still to be finally proven. The main result from the study by Nakata et al. is the identification of paroxetine as a novel lead structure for the development of P2X₄ receptor antagonists. Medicinal chemists are now asked to modify the structure and optimize it with respect to improved P2X₄ affinity and selectivity vs. serotonin transporters and P2X₇ receptors.

Carter DS, Alam M, Cai H, Dillon MP, Ford APDW, Gever JR, Jahangir A, Lin C, Moore AG, Wagner PJ, Zhai Y (2009) Identification and SAR of novel diaminopyrimidines. Part 1: the discovery of RO-4, a dual P2X₃/P2X_{2/3} antagonist for the treatment of pain

Article summary

The study describes the identification and optimization of novel antagonists for homomeric P2X₃ and heteromeric P2X_{2/3} receptors. A high-throughput screening (HTS) campaign was performed at Roche company, Palo Alto, using rat recombinant P2X₃ receptors expressed in CHO cells. Inhibition of agonist-induced calcium influx was fluorimetrically determined. The goal was to identify molecules for a drug development program. The hit rate of the HTS was very low (only 0.01% hits), and many of the hits had several acidic functions, which may prevent peroral bioavailability and/or CNS penetration. One weaker hit was selected for optimization: compound **3**, a

diaminopyrimidine derivative related to the antibacterial drug trimethoprim. Replacement of the ethyl by an isopropyl residue on the phenyl ring was important for increasing potency (**4**, RO-3). Systematic optimization of the bridge between the aromatic rings (oxygen was found to be optimal) and the substitution pattern of the phenyl ring led to a very potent P2X₃ antagonist, which also showed inhibitory potency at the human P2X_{2/3} receptor

expressed in astrocytoma cells in a fluorescence assay (compound **5**, RO-4). RO-4 was selected for further evaluation. It was selective versus P2X₁, P2X₂, P2X₅, P2X₇ (IC₅₀ > 10 μM), as well as a wide range of other receptors and enzymes (CEREP profile). Furthermore, RO-4 showed drug-like properties including Caco-2 permeability and good peroral bioavailability in dog with a half-life of 1.5 h.

Commentary

Homomeric P2X₃ and heteromeric P2X_{2/3} receptors are localized on small to medium diameter sensory afferent neurons. P2X₃ expression is increased in dorsal root ganglionic neurons after ligation of the sciatic nerve in the chronic constriction injury model. P2X₃ knockout mice showed reduced pain behavior, and reduction of P2X₃ expression by oligonucleotides or siRNA showed the same effect. Only very few P2X₃ antagonists have been described, including the ATP derivative TNP-ATP, which also blocks heteromeric P2X_{2/3} receptors, as well as P2X₄ receptors at higher concentrations (see above), the peptide spinorphin, and the small molecule A-317491. The latter blocks homomeric and heteromeric receptors and has shown activity in a number of pain models. However, due to its polar nature (three carboxylate function), it shows poor peroral and CNS bioavailability.

The present study describes the first P2X₃/P2X_{2/3} antagonists with high affinity and selectivity and without acidic functions rendering them more drug-like than all other P2X₃ antagonists described to date. Since the study was published as a (short) letter, it has some shortcomings: for example, the test systems are not well described, and the criteria for defining a “hit” are not provided. The synthetic access to the molecules is described, although not very detailed, but no

analytical data are provided for the final products. Most importantly, the authors did not describe any efforts to determine the mechanism of antagonistic activity, competitive or allosteric. On the other hand, the structural optimization of the initial hit **3** has been systematically performed, and the structure–activity relationships are well discussed. The authors indicate that a second study will be published which is to describe the modification and optimization of the diaminopyrimidine part of the molecule.

The development of RO-4 (**5**) from **3** via RO-3 (**4**) is definitely an important step towards a broader evaluation of drugs targeting P2X receptors in animal models and in clinical trials and may eventually contribute or lead to novel treatments of chronic pain based on inhibition of P2X receptors.

About the author

Christa Müller is Full Professor of Pharmaceutical/Medicinal Chemistry at the University of Bonn, Germany. Her research interests are focused on the medicinal chemistry of purinergic receptors (adenine, adenosine, and P2 nucleotide receptors) and ectonucleotidases, including the development of novel ligands, screening assays, receptor expression and mutagenesis, and in vitro pharmacology (<http://mueller-group.pharma.uni-bonn.de>).