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Visualization of the trimeric $P2X_2$ receptor with a crown-capped extracellular domain ☆

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Abstract

The $P2X_2$ purinergic receptor permeates cationic ions in response to stimulation by ATP and mediates fast synaptic transmission. Here, we purified the $P2X_2$ receptor using baculovirus-Sf9 cell expression system and observed its structure using electron microscopy. The FLAG-tagged $P2X_2$ receptor, which has intact ion channel function, was purified to be a single peak by affinity purification and gel filtration chromatography. It was confirmed to be a trimer by introducing cross-linking. Negatively stained $P2X_2$ protein images were homogeneous and picked up by automated pick-up programs, aligned, and classified using the modified growing neural gas network method. Similarly oriented projections were averaged to decrease the signal-to-noise ratio. These images demonstrate an inverted three-sided pyramid with the dimensions of 215 Å in height and 200 Å in side length. It is composed of a high-density trunk and a stain-permeable swollen extracellular domain of a crown-shaped structure. The internal cavities and constituent segments were clearly demonstrated in both the raw images and the averaged images. The threefold symmetrical top view demonstrates the first visual evidence of the trimeric composition of the P2X receptor family.

Keywords: P2X receptor; Purinergic; ATP; Ion channel; Single-particle analysis; Negative staining; Electron microscopy

Adenosine 5′-triphosphate (ATP) is a humoral signal transmitter both in neuronal and non-neuronal tissue. Extracellular ATP controls cell function via the cell-surface ATP receptors. P2X receptors are ATP-gated non-selective cation channels widely distributed in the peripheral and central nervous system [1]. It is known that they play critical roles in fast synaptic transmission [2,3] and in presynaptic modulation [4–6].

The primary structure of the P2X receptor was first determined by isolating the cDNA using the expression cloning method [7,8] and seven subtypes of P2X cDNAs have been cloned so far [9]. All of them have two transmembrane regions with a large extracellular loop containing multiple pairs of cysteins, which form disulfide bonds to determine the receptor conformation [10]. From the mutation analysis, an ATP binding region of the $P2X_2$ receptor was identified near the first transmembrane domain, which contains the two kinds of lysines (Lys-69, Lys-71) [11]. Extensive studies including electrophysiological research, native-gel electrophoresis, and cross-linking

☆ *Abbreviations:* ATP, adenosine 5′-triphosphate; DDM, *n*-dodecyl-β-D-maltoside; EM, electron microscopy; GNG, growing neural gas network; NN, neural network; PAGE, polyacrylamide gel electrophoresis; SA, simulated annealing; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline.

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experiments demonstrate that P2X receptors form trimers as their functional structure [12–14]. Direct observation of the $P2X_2$ protein using atomic force microscopy (AFM) was recently reported from two laboratories [15,16]. Although the resolution is not sufficient to identify each subunit of the $P2X_2$ receptor, the observations provide the volume of purified $P2X_2$ protein, which is in agreement with the size of the trimer [15] and evidence of molecular reassembly induced by ATP treatment [16].

Ligand-gated ion channels are now categorized to three groups. The first group is a Cys-loop superfamily which includes acetylcholine, glycine, $GABA_A$, $GABA_C$, and 5-HT_3 receptors. The nicotinic acetylcholine receptor (nAChR) has a pentamer structure assembled from homologous subunits. Its 3D structure was determined using cryo-EM images from cylindrical crystals [17,18]. The second group is an ionotropic glutamate receptor family encompassing the AMPA, kainate, and NMDA receptors. The AMPA receptor is a tetramer; its 3D structure was determined using single particle analysis and partly fitted by the structures of crystallized domains [19,20]. The ligand-binding core structure and the mechanisms of activation and desensitization were demonstrated by X-ray diffraction [21].

P2X receptors compose the third group of ligand-gated ion channels. Compared to the other two groups, however, the structures of the P2X receptors are poorly understood, due to the difficulty in purification and crystallization. Here, we isolated the $P2X_2$ protein as uniformly sized particles and observed the structure using electron microscopy. Statistical analysis [22] revealed the threefold symmetrical structure of the $P2X_2$ receptor, in which the overall shape is a crown-capped inverted three-sided pyramid. This is the first visual evidence of the trimer structure of the $P2X_2$ receptor, and could be applicable to other subtypes in the P2X receptor family.

Materials and methods

Molecular biology and current recording. A *Bam*HI–*Not*I fragment of the original rat $P2X_2$ receptor cDNA [7] was subcloned into pBluescript vector. A cDNA with a FLAG tag (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) immediately after the first Met was constructed by PCR, and the sequence was confirmed by DNA sequencing. For expression in *Xenopus* oocytes, cRNA was prepared from the linearized plasmid cDNA using an RNA transcription kit (Stratagene, La Jolla, CA). Isolated oocytes were treated with collagenase (2 mg/ml, type 1, Sigma–Aldrich, St. Louis, MO), after which oocytes of similar size at stage V were injected with approximately 50 nl cRNA solution. cRNA of 2.5 ng (w.t.) or of 50 ng (FLAG-tagged one) was injected to oocytes. The injected oocytes were incubated for 3 days at 17 °C in frog Ringer solution. All experiments are in compliance with the guidelines of the National Institute of Advanced Industrial Science and Technology, and the National Institute for Physiological Sciences. Macroscopic currents were recorded using the two-electrode voltage clamp technique with a bath-clamp amplifier (OC-725C, Warner, Hamden, CT) as described previously [23]. Stimulation, data acquisition, and data analysis were all done on a Pentium-based computer using Digidata 1322A and pCLAMP 8 software (Axon Instruments, Sunnyvale, CA). Recordings were obtained at room temperature (25 °C).

Infection of Sf9 cells and membrane preparation. The $P2X_2$ receptor cDNA was further subcloned to the pFastBac donor plasmid (Invitrogen, Carlsbad, CA) as a FLAG tagged at the N-terminus and transformed to the DH10Bac *Escherichia coli* strain to obtain the Bacmid DNA containing FLAG-tagged $P2X_2$ receptor. The Sf9 cells were infected with the recombinant Bacmid. After 96 h of infection, cells were harvested using Teflon cell scrapers, collected by centrifuge, immediately frozen at −80 °C until use.

Frozen cells were homogenized in ten volumes (v/w) of TBS buffer with a Teflon homogenizer. Homogenates were first centrifuged for 15 min at 10,000*g* to remove debris, and the supernatant was further centrifuged at 100,000*g* for 60 min to obtain membrane fraction. All the procedures were performed on ice or at 4 °C.

Protein preparation. The membrane fraction was homogenized with a Teflon homogenizer in 4 ml TBS buffer containing 50 mM *n*-dodecyl-β-D-maltoside (DDM) (Sigma–Aldrich), 100 μM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) hydrochloride, 80 nM aprotinin, 5 μM bestatin, 1.5 μM E-64 protease inhibitor, 2 μM leupeptin hemisulfate, 1 μM pepstatin, and 0.02% sodium azide. After centrifuging for 60 min at 100,000*g*, the supernatant containing the solubilized FLAG-tagged $P2X_2$ receptor was loaded onto a column containing 1 ml of anti-FLAG affinity gel (Sigma–Aldrich) pre-equilibrated with the same buffer. The column was then washed with 20 ml of wash buffer (TBS containing 5 mM DDM, 300 mM $MgCl_2$, and 0.02% sodium azide), and the bound protein was eluted with elution buffer containing 100 μg/ml FLAG peptide (Sigma–Aldrich). The elution was analyzed by silver staining and by Western blotting, and the mixture of two positive fractions (400 μl) was concentrated to 50 μl with a Microcon centrifuge filter unit YM-50 (Millipore, Bedford, MA). It was further purified by Superdex 200 (3.2/30) gel filtration chromatography in a SMART system (Amersham Biosciences, Piscataway, NJ) with a TBS buffer containing 5 mM DDM, 300 mM $MgCl_2$, and 0.02% sodium azide. The elution of the protein was monitored by measuring the absorbance at 280 nm. The column was eluted into 20 μl fractions with a flow rate of 40 μl/min. The protein concentrations were determined with the BCA method [24].

SDS-gel electrophoresis and chemical cross-linking of $P2X_2$. The standard method of Laemmli was applied for SDS–PAGE [25]. Samples were mixed with a sample buffer containing 62.5 mM Tris–HCl (pH 6.8), 2% SDS, 25% glycerol, 0.04 M dithiothreitol, and 0.01% bromophenol blue, and then incubated at 95 °C for 3 min. Proteins were separated in a 4–20% or 2–15% gradient acrylamide gel with an electrophoresis buffer containing SDS and visualized by silver staining. For Western blots, proteins in the electrophoresed gel were transferred to PVDF membrane and detected with anti-FLAG antibody (Sigma–Aldrich) and secondary antibody for chemiluminescent staining. For chemical cross-linking, the buffer of the solubilized protein was substituted at the gel filtration chromatography stage with a phosphate-buffered saline solution (136 mM NaCl, 1.4 mM KCl, 10 mM Na_2HPO_4, and 1.7 mM KH_2PO_4, pH 7.4) containing 5 mM DDM, 300 mM $MgCl_2$, and 0.02% sodium azide. Glutaraldehyde (Polysciences, Warrington, PA) was then added at 25 °C for 30 min. The cross-linking reaction was terminated by incubation with the SDS sample buffer containing dithiothreitol at 60 °C for 15 min, and the protein was analyzed by SDS–PAGE.

Transmission electron microscopy. Detergent solubilized particles of approximately 120 μg/ml were adsorbed by thin carbon films rendered hydrophilic by glow discharge in low pressure of air and supported by copper mesh grids. Samples were washed with five drops of double-distilled water, negatively stained with 2% uranyl acetate solution for 30 s twice, blotted, and dried in air. Micrographs of negatively stained particles were recorded in a JEOL 100CX transmission electron microscope (JEOL, Tokyo, Japan) at 40,000× magnification with 100 kV acceleration voltages. Images were recorded on SO-163 films (Eastman Kodak, Rochester, NY), developed with a D19 developer (Eastman Kodak), and digitized with a Scitex Leafscan 45 scanner (Leaf systems, Westborough, MA) at a pixel size of 2.5 Å at the specimen level.

Automated particle selection and image analysis. Image analysis was performed using our methods [26–29] and the Imagic V program [30]. Primary selection of the $P2X_2$ projection was performed automatically using the auto-accumulation method with simulated annealing (SA) [26]. Three hundred particles were extracted into 60×60 pixel subframes at a