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and Biogenesis:
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Molecular Shape, Architecture, and Size of P2X<sub>4</sub> Receptors Determined Using Fluorescence Resonance Energy Transfer and Electron Microscopy

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P2X receptors are ATP-gated nonselective cation channels with important physiological roles. However, their structures are poorly understood. Here, we analyzed the architecture of P2X receptors using fluorescence resonance energy transfer (FRET) microscopy and direct structure determination using electron microscopy. FRET efficiency measurements indicated that the distance between the C-terminal tails of P2X<sub>4</sub> receptors was 5.6 nm. Single particle analysis of purified P2X<sub>4</sub> receptors was used to determine the three-dimensional structure at a resolution of 21 Å; the orientation of the particle with respect to the membrane was assigned by labeling the intracellular C termini with lectin. We found that human P2X<sub>4</sub> is a globular torpedo-like molecule with an approximate volume of 270 nm<sup>3</sup> and a compact propeller-shaped ectodomain. In this structure, the distance between the centers of the gold particles was 6.1 nm, which closely matches FRET data. Thus, our data provide the first views of the architecture, shape, and size of single P2X receptors, furthering our understanding of this important family of ligand-gated ion channels.

P2X receptors, of which seven subtypes are found in mammals, display a diverse tissue distribution and play key roles in a variety of physiological processes such as neurotransmission, sensory transduction, inflammation, and cardiovascular regulation (1–3). They are found mostly on the plasma membrane, but in some species such as Dictyostelium may also fulfill physiological functions within intracellular membranes (4). They are the third major superfamily of ligand-gated ion channels; however, in contrast to other cationic channel superfamilies (5–7), very little is known about their three-dimensional structure.

The topology of P2X receptors is well defined; each monomer subunit is predicted to possess intracellular N and C termini, two transmembrane-spanning domains, and a large ectodomain (2). The functional channel unit is thought to be a trimer (8–11), a feature that is shared with acid-sensing ion channels (12). A low resolution structural study of the rat P2X<sub>2</sub> isoform has been published; electron microscopy of single particles gave rise to a structure with a volume of ~1200 nm<sup>3</sup> (13). However, the orientation of the molecule was not assigned, and there was no independent evidence to verify the dimensions. Moreover, atomic force microscopy (AFM) studies suggest a smaller molecular volume of 490 nm<sup>3</sup>; however, these studies modeled the channel as a flat spherical cap with a height of 2.9 nm (10, 14). Because the thickness of the plasma membrane is ~7 nm, this implies that the AFM studies reported on molecules in a squashed conformation (10, 14). Thus, the available data do not provide a consistent or satisfying view of the shape, architecture, or size of P2X receptors.

To further our structural knowledge of P2X receptors and to address the above discrepancies in estimates of molecular volume and size, we have explored two parallel experimental approaches. First, we measured the efficiency of fluorescence resonance energy transfer (FRET) between subunits within homomeric P2X receptors bearing yellow (YFP) or cyan (CFP) fluorescent protein tags at their C termini. Extensive previous studies by us and others have employed green fluorescent protein (GFP)-labeled P2X receptors (15–25), demonstrating that tagging P2X<sub>3</sub>, P2X<sub>4</sub>, and P2X<sub>7</sub> receptors on the C termini with GFP does not affect receptor function. Second, we overexpressed P2X<sub>4</sub> in human embryonic kidney 293 (HEK293) cells and purified the trimeric form to homogeneity. We used single particle analysis and domain-specific labeling to generate the three-dimensional structure of human P2X<sub>4</sub> at a resolution of 21 Å, oriented with respect to the lipid bilayer.

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<sup>3</sup>The abbreviations used are: AFM, atomic force microscopy; FRET, fluorescence resonance energy transfer; YFP, yellow fluorescent protein; CFP, cyan fluorescent protein; GFP, green fluorescent protein; HEK293, human embryonic kidney 293; DDM, β-D-decyl maltoside; PFO, perfluorooctanoic acid; Ni-NTA, nickel-nitrilotriacetic acid; EM, electron microscopy.
Molecular and Cell Biology—HEK293 or HEK293T cells transiently expressing the appropriate wild-type and fluorescently labeled P2X receptor subunits were used. Some cDNAs were available from previous work (18–20), and others were generated using standard cloning procedures. The resulting plasmids were verified by sequencing (P2X₁–P2X₆, all tagged with either CFP or YFP). The plasmids were propagated in Escherichia coli with a C-terminal EYMPME tag has been described previously (26). Human P2X₄ containing a C-terminal decahistidine tag was generated from this construct by PCR. HEK293 cells (obtained from American Type Culture Collection) were maintained in 75-cm² cell culture flasks (Corning) in Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 with Glutamax (Invitrogen) supplemented with 10% fetal bovine serum and penicillin/streptomycin. Cells were grown in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C in a cell culture incubator. The cells were split 1 in 10 when confluence reached 60–90%, which was generally every 4 days. Cells were prepared for transfection by plating onto 6-well plates at the time of splitting, 3–4 days before transfection. They were transfected at ~60% confluence. For transient expression in HEK293 cells, we used 0.5–1 μg of plasmid cDNA and Effectene transfection reagent (Qiagen Inc.) for each well of a 6-well plate. The manufacturer’s instructions were followed, with 4 μl of enhancer and 10 μl of Effectene were used for each transfection. The transfection efficiency was 40–60%. The HEK293 stable cell line constitutively expressing human P2X₄-His₁₀ was generated using electroporation and selection with 400 μg/ml G418 (Invitrogen) after 14 days in 15-cm culture dishes. Single colonies were isolated using sterile greased stainless steel rings, and penicillin/streptomycin. Cells were grown in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C in a cell culture incubator. The cells were split 1 in 10 when confluence reached 60–90%, which was generally every 4 days. Cells were prepared for transfection by plating onto 6-well plates at the time of splitting, 3–4 days before transfection. They were transfected at ~60% confluence. For transient expression in HEK293 cells, we used 0.5–1 μg of plasmid cDNA and Effectene transfection reagent (Qiagen Inc.) for each well of a 6-well plate. The manufacturer’s instructions were followed, with 4 μl of enhancer and 10 μl of Effectene were used for each transfection. The transfection efficiency was 40–60%. The HEK293 stable cell line constitutively expressing human P2X₄-His₁₀ was generated using electroporation and selection with 400 μg/ml G418 (Invitrogen, Paisley, UK) for 14 days in 15-cm culture dishes. Single colonies were isolated using sterile greased stainless steel rings, trypsin-treated, and transferred to 24-well plates. Expression of P2X₄-His₁₀ was verified by Western blotting according to standard protocols, and the highest expressing clone was selected for overexpression.

Epifluorescence FRET Microscopy—We have previously described the setup (19). We used an Olympus BX50 microscope equipped with a Peltier cooled (−15 °C) Imago CCD camera (640 × 480 pixels, each pixel 9.9 × 9.9 μm), epifluorescence condenser, control unit (containing ISC and PDC boards), and a Polychrome IV monochromator (all from Till Photonics). The light from the monochromator was led to the microscope through a quartz fiber light guide. The hardware was controlled by a personal computer, an appropriate frame grabber (TILL Photonics), and macros driven by TILLvisION Version 3.3 software. The cells were viewed with a 40× water immersion objective lens with a numerical aperture of 0.8 (Olympus). We used the following filters for acquiring CFP or YFP images (all from Glen Spectra, Stanmore, UK; order is dichroic, emitter in nanometers): CFP (455DRLP, 480AF30) and YFP (525DRLP, 545AF35). To photostable the YFP, we used 525 nm light from the monochromator and a 525DRLP dichroic.

Electrophysiology—HEK293 cells were used for recordings 24–48 h post-transfection. Cells were gently mechanically dispersed and plated onto glass coverslips 2–12 h before use. We included this step (27) to ensure adequate voltage clamp during reversal potential measurements from single spherical cells. The extracellular recording solution comprised 150 mM NaCl, 1 mM MgCl₂, 1 (or 0.1) mM CaCl₂, 10 mM HEPES, and 10 mM glucose (pH 7.4), and the pipette solution comprised 154 mM NaCl (or CsCl or CsF), 11 mM EGTA, and 10 mM HEPES. Whole-cell voltage-clamp recordings were made with ~4-megohm borosilicate glass electrodes (World Precision Instruments) using an Axopatch-1D or Axopatch 200 amplifier controlled by a computer running pCLAMP 8.1 or 10 software via a Digidata 1322 interface (Molecular Devices). Data were filtered at 0.5–2 kHz and digitized at 1–5 kHz. Drugs were applied to single cells by pressure ejection using a Picospritzer (Intracell Ltd.) or in the bathing medium, which flowed at 2–3 ml/min. For pressure application (~20 p.s.i., 1 p.s.i. = 6.89 kilopascals), we used 2–4-megohm pipettes as described previously (21); solution exchange occurred in ~10 ms.

Protein Purification—Membrane fractions were prepared from 2.5 × 10⁸ cells and solubilized in phosphate-buffered saline containing 1% (w/v) β-d-dodecyl maltoside (DDM; Merck, Nottingham, UK) and protease inhibitors (Complete-EDTA, Roche Applied Science). Samples were diluted into buffer A (150 mM NaCl, 20 mM sodium phosphate (pH 7.4), 150 mM imidazole, and 0.1% (w/v) DDM) and incubated overnight at 4 °C with nickel-Sepharose beads (Amersham Biosciences, Chalfont St. Giles, UK) to capture His-tagged protein. Beads were packed into columns and washed with 20 column volumes of buffer A; 50% pure His-tagged protein was eluted in buffer A containing 500 mM imidazole.

Purification of P2X₄ Trimers—1-ml protein-containing fractions from the nickel affinity column were concentrated using 50-kDa cutoff filters (Millipore, Watford, UK) and separated by perfluorooctanoic acid (PFO)-PAGE according to published methods (28). For preparative PFO-PAGE, samples were loaded onto 1.5-mm thick 8% (w/v) polyacrylamide gels and run at 4 °C at 10 mA. P2X₄ trimers (150–180 kDa) were excised from nonstained gels and placed into 2-mL D-tube dialysis units (Merck), and protein was eluted at 60 V for 2 h at 4 °C. Protein samples were diaffiltered into phosphate-buffered saline containing 0.1% (w/v) DDM.

Gold and Lectin Labeling—For gold labeling, purified P2X₄-His₁₀ in 0.1% DDM was incubated for 10 min at room temperature with a 10:1 molar excess of 1.8-nm diameter nickel-nitrol triatic acid (Ni-NTA)-Nanogold particles (Nanoprobes, Yaphank, NY). Samples were adsorbed onto grids and washed with Nanogold suspension to prevent unbinding of the protein-associated gold particles. For lectin labeling, purified P2X₄-His₁₀ in 0.1% DDM was mixed with Lenticulinaris lectin (Sigma, Poole, UK) at a molar ratio of 1:1 and immediately applied to grids. After transmission electron microscopy and image processing, 1966 gold-labeled P2X₄-His₁₀ particles (in 48 × 48 pixel boxes) and 2862 lectin-labeled particles (in 64 × 64 pixel boxes) were manually selected. Three-dimensional structures were generated using the refined P2X₄ structure as a starting model with eight rounds of iterative refinement.

Structure Determination by Electron Microscopy and Single Particle Analysis—Protein samples (20 μg/ml) were adsorbed onto glow-discharged carbon-coated copper grids and nega-
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To approximate distances, we used the Förster equation, \( e = R_0^6/(R_0^6 + R^6) \), where \( R_0^6 = (8.79 \times 10^{23})n^2 \kappa^2 e^2 \Phi_{DA} \), \( e \) is the experimentally determined FRET efficiency, and \( R \) is the distance between donor and acceptor fluorophores (30, 31). \( R_0 \) includes terms for the donor quantum efficiency (\( \Phi_D \)), the solvent refractive index (\( n \)), overlap of the donor emission and acceptor absorption spectra (\( I_{DA} \)), and the orientation factor (\( \kappa^2 \)). \( R_0 \) is the distance at which \( e \) is 50% for any given fluorophore pair. In the case of CFP and YFP, this is \( \sim 50 \) Å (5 nm). Notably, \( R_0 \) contains a term for the fluorophore dipole orientation factor (\( \kappa^2 \)), which at 2/3 assumes random tumbling of the fluorophores (30). This means that \( R_0 \) and the distance measurements reported here assume that the fluorophores adopt all spherically random orientations. Given that we do not know the relative orientation of the fluorophores on P2X$_4$ channels, assuming a \( \kappa^2 \) value of 2/3 seems appropriate (30). When interpreting FRET \( e \) measurements in terms of distance, it is important to consider that in the case of CFP and YFP the fluorophore is buried in the center of a β-can with a width of 30 Å and a length of 45 Å (32). A further consideration is that we do not know the absolute ratio of donors to acceptors during FRET at the single molecule level. We have previously considered these issues in depth (18, 19). For the FRET experiments, we chose plasma membrane regions of interest post hoc. Our criteria were that regions of interest (i) were bright in both CFP and YFP images; (ii) located at the edge, presumably plasma membrane of the cell; (iii) did not change or drift in intensity during the control period; and (iv) remained in focus during the entire experiment.

RESULTS

Tagged P2X Receptors Are Functional—In past work, it has been demonstrated that P2X$_2$, P2X$_3$, and P2X$_7$ receptors tagged on their C termini with GFP function like their wild-type counterparts in terms of ATP-evoked currents and ATP sensitivity (15–25). We started by recording ATP-evoked currents from HEK cells expressing CFP- and YFP-tagged P2X receptors and...
compared the responses with wild-type P2X receptors (Fig. 1). Receptor function was preserved for all receptors and the kinetics of their activation, desensitization, and deactivation were not detectably affected for some receptors. Below and in Fig. 2, we present a detailed characterization of these parameters for P2X4 receptors. Thus, as expected (3), YFP-tagged P2X2 and P2X3 receptors desensitized rapidly (<1 s); P2X2 and P2X4 receptors desensitized slowly (over tens of seconds); and P2X5 receptors expressed poorly (100 μM ATP used for activation). In the case of P2X7 receptors, we recorded large variability in 30 min photodestruction time point. D, time course of YFP photodestruction and CFP photorecovery for whole-cell and near membrane regions. Graphs such as these are used to determine FRET e. YFP photodestruction is plotted versus CFP photorecovery, and the data are fit with a straight line and extrapolated to 100% YFP photodestruction. The intersection with the y axis represents the FRET e (see Fig. 3).

functional properties and FRET for CFP- and YFP-tagged P2X8 receptors. A, average 100 μM ATP-evoked current traces for P2X8, P2X8-CFP, and P2X8-YFP receptors (n = 9), with bar graphs below showing the properties of the currents. (The kinetic values are time constants.) B, FRET values (picofarads). C, concentration-response curves for wild-type and CFP/YFP-tagged receptors. C, images of two HEK cells expressing P2X4-CFP and P2X4-YFP before and after photodestruction of the acceptor YFP. Note that the fluorescence intensity of the YFP channel decreases following photodestruction, whereas that of the CFP channel increases. The images were from the 30-min photodestruction time point. D, time course of YFP photodestruction and CFP photorecovery for whole-cell and near membrane regions. Graphs such as these are used to determine FRET values (see Fig. 3).

Functional Properties of CFP- and YFP-tagged P2X4 Receptors—In the remainder of this study, we focused on homomeric P2X4 receptors, and so we began by determining if CFP or YFP tagging affects the overall properties of P2X4 receptors (Fig. 2, A and B). Fig. 2A shows average 100 μM ATP-evoked currents from cells expressing wild-type P2X4, P2X4-YFP, or P2X4-CFP receptors. The traces are shown as picocamps/picofarads and clearly show that CFP or YFP tagging did not significantly affect the peak responses or the overall shape of the ATP-evoked current waveforms (n = 9). The bar graphs in Fig. 2A plot the average rise time (milliseconds), percent desensitization in 2 s ATP, and decay time (milliseconds) after ATP removal for wild-type and CFP/YFP-tagged P2X4 receptors. We next examined the ATP concentration dependence of the ATP-evoked responses for wild-type and CFP/YFP-tagged receptors. Fig. 2B shows that the concentration-response curves superimpose with EC50 values of ~3 μM and Hill slopes of ~1.5 (n = 8). These values are similar to previous work on wild-type P2X4 receptors (38). Overall, our data with CFP- and YFP-tagged receptors reproduce past work with GFP-tagged P2X4 receptors, where it was demonstrated that GFP did not affect P2X4 responses (15).

Receptor-specific FRET for Homomeric P2X Receptors—To measure FRET efficiency (e), we studied homomeric P2X receptors tagged with CFP and YFP and expressed in single living HEK cells. We focused on plasma membrane regions of interest and used donor dequenching (30) to estimate FRET e for P2X receptors in these regions (19, 39, 40). In Fig. 2 (B and C), we present representative images and graphs for P2X4-CFP/P2X4-YFP receptors. We measured a decrease in P2X4-YFP fluorescence intensity with a time course that followed that of the P2X4-CFP increase when the acceptor YFP was optically destroyed with 525 nm light (Fig. 2C). Maximum P2X4-CFP photorecovery when P2X4-YFP was 100% photodeestroyed cor-
regions is subunit-specific. In the case of P2X6 receptors, we cytosolic CFP and YFP and P2X6-CFP and P2X6-YFP receptors 20–70 Å within the cell. The measured FRET efficiency for arises as CFP and YFP randomly approach a distance of repeated these experiments to establish the level of FRET noise that could not measure FRET at the point of photodestruction and compared the estimated FRET $e$ values with those determined with extensive photodestruction (Fig. 2, C and D, and Fig. 3). Fig. 4 shows that FRET values agree to FRET $e$ for all homomeric P2X receptors from only one its tip (Fig. 3). Using this method, the distance between the C-terminal tails of functional P2X4 receptors in the plasma membrane of single living cells was calculated to be 5.6 nm. All of the FRET experiments shown in Fig. 3 were carried out using identical imaging/analysis methods and in parallel to reduce any variability between experiments. In the case of P2X5-P2X6 receptors, the data points were well fit by a straight line (Fig. 3). However, in the case of P2X4 and P2X7 receptors, the linear plots were less aesthetically pleasing. We could think of no precise scientific reason to discard these data and present them faithfully in Fig. 3. The relevant issue here is whether the dihedral angle $\gamma$ is taken as $\pi/2$ and assumes random tumbling as discussed by us and others (19, 30, aa amino acids). the receptors. From these data, a trend is readily apparent. The FRET $e$ for P2X receptors (and thus one assumes the interfluorophore distance) is related to the length of the C-terminal tail. For instance, the channels with the shortest C-terminal tails display the lowest FRET $e$ (e.g. P2X1, and P2X7, C-terminal tail lengths of 44 and 42 residues), whereas the channels with the longest C-terminal tails display the highest FRET $e$ (e.g. P2X2 and P2X7, C-terminal tail lengths of 118 and 238 residues). P2X5 sits intermediate in this trend, consistent with its C-terminal tail being of intermediate length (96 residues). P2X4, with a C-terminal tail length of 33 residues is the receptor that is slightly shifted in the overall trend. Overall, our data show a surprising trend between FRET $e$ for P2X receptors and the C-terminal tail length, implying that the distance between the tips of the C-terminal tails for P2X receptors near the membrane is larger than at the tips of those with longer tails. Put another way, the cytosolic domain of P2X2 and P2X7 receptors narrows from its origins at the plasma membrane to responds to FRET $e$, which in the case of P2X4-CFP/P2X4-YFP channels near the cell perimeter was $30 \pm 5\%$ ($n = 8$). We repeated these experiments to establish the level of FRET noise that arises as CFP and YFP randomly approach a distance of $20–70 \text{Å}$ within the cell. The measured FRET efficiency for cytosolic CFP and YFP and P2X4-CFP and P2X4-YFP receptors within HEK cells was $\sim 3\%$ (Fig. 3A). In these experiments, we used P2X4 receptors as a negative control because they do not express in the plasma membrane and are unassembled (41). We next extended the work by measuring FRET $e$ for all homomeric P2X receptors in plasma membrane regions. The donor quenching plots are presented in Fig. 3. These experiments clearly establish that FRET $e$ for receptors in plasma membrane regions is subunit-specific. In the case of P2X4 receptors, we could not measure FRET $e$ for membrane regions because these channels are retained within the cell in intracellular compartments (41).

We explored the relationship of the experimentally determined FRET $e$ for P2X receptors in the membrane to the dependence of FRET between CFP and YFP and interfluorophore distance (in angstroms). For this comparison, we used the Förster equation and an $R_0$ value of 50 Å. The graph in Fig. 3I plots FRET $e$ for all homomeric P2X receptors along with how FRET $e$ falls (as $1/R^6$) with distance between the chromophores of CFP and YFP. The numbers next to the data points indicate the length of the cytosolic C-terminal tail for each of its tip. We used this method to determine the FRET $e$ for all homomeric P2X receptors from only one point of photodestruction and compared the estimated FRET $e$ values with those determined with extensive photodestruction (Fig. 2, C and D, and Fig. 3). Fig. 4 shows that FRET values agree to between these methods. Thus, although there is some variability between P2X receptors in the extent to which the CFP photorecovery is fit by linear functions (compare plots in Fig. 3), this does not under- or overestimate FRET $e$ values. This is consistent with past work for a variety of FRET constructs, including P2X2 receptors, where we showed using three-cube

![Image](http://www.jbc.org/)
linear unmixing that FRET $e$ could be reliably estimated by donor dequenching (Figs. 3 and 4) (18).

We next sought to generate a three-dimensional structure of purified human P2X$_4$ protein. Our reasoning was 2-fold. First, because FRET is an ensemble measurement and makes a number of assumptions about the fluorophore orientations, a three-dimensional structure could be used to verify the FRET $e$ measurements. Second, the estimates of distance by FRET may be used as a “barometer” to ascertain that the P2X$_4$ structure was of a size appropriate for the channel in the membrane. This is important because the published single particle and AFM studies differ largely on the dimensions of P2X receptors (see the Introduction).

Functional Expression and Purification of Human P2X$_4$-His$_{10}$ from HEK Cells—The expression of human P2X$_4$-His$_{10}$ was verified by Western blotting and compared with that of P2X$_4$-EYMPME (26). Both the C-terminal P2X$_4$ epitope and the His tag were readily detected, and total protein expression levels appeared to be similar to those of P2X$_4$-EYMPME (data not shown). Whole-cell currents were evoked by a 2-s application of 1, 10, and 100 $\mu$M ATP in perforated patch-clamp configuration; peak current densities to 100 $\mu$M ATP were 40 ± 4 pA/picofarads for P2X$_4$-EYMPME and 45 ± 6 pA/picofarads for P2X$_4$-His$_{10}$ ($n = 8$). EC$_{50}$ values were 2.9 ± 0.2 $\mu$M for P2X$_4$-EYMPME and 2.7 ± 0.3 $\mu$M for P2X$_4$-His$_{10}$ ($n = 8$).

Following nickel affinity chromatography, the majority of P2X$_4$ eluted in the 500 mM imidazole fraction as a diffuse 55-kDa band. The size of the band was in agreement with that observed by Western blotting, and purity was estimated to be >50%. To purify P2X$_4$ to homogeneity, we used nondenaturing PFO-PAGE (28), followed by electrophoretic fractionation. When separated by PFO-PAGE, 50% pure P2X$_4$ presented as three bands corresponding to a monomer (55 kDa), a faint dimer (110 kDa), and a trimer (165 kDa) (Fig. 5A). No higher order oligomers were observed. We assumed that the observed monomers and dimers corresponded to immature nonassembled proteins recovered from internal membranes. Trimmers were selectively purified by excising bands from preparative nonstained PFO-polyacrylamide gels and eluting protein under an electric field.

SDS-PAGE analysis of eluted protein demonstrated the high purity obtained using this purification method (Fig. 5B); the only bands visible on the silver-stained gel corresponded to P2X$_4$. (SDS-stable dimeric and trimeric forms were also observed.) In addition, PFO-PAGE of purified trimers was used to demonstrate that the trimeric form was stable after purification.
C-terminal His tags of human P2X4 were labeled using 1.8-nm gold-NTA-Nanogold particles and washing our EM grids, and negatively stained with 2% (w/v) uranyl acetate. An image field for wild-type human P2X4 is shown in Fig. 5C, arrow. After diaphragulation into 0.1% (w/v) DDM, 40 μl of P2X4 was recovered at an approximate concentration of 1 mg/ml. Yield was estimated by comparing the band intensity of pure P2X4 on Coomassie Blue-stained SDS-polyacrylamide gels with known quantities of bovine serum albumin. In summary, 40 μg of pure P2X4 trimer was recovered from 2.5 × 10⁷ cells (equivalent to a confluent monolayer of 5000 cm² in area).

Electron Microscopy and Single Particle Analysis—Pure protein was diluted to 25 μg/ml, applied to electron microscopic (EM) grids, and negatively stained with 2% (w/v) uranyl acetate. An image field for wild-type human P2X4 is shown in Fig. 5D. Single particles of the P2X4 receptor were relatively homogeneous, with the majority (~70%) appearing either nearly circular, with a radius of ~8 nm, or nearly rectangular, with a short side of 8 nm and a long side of 12 nm. We interpreted these particles as either top/bottom views or side views, respectively. In total, 6826 particles were manually selected in 48 × 48 pixel boxes for image processing using the EMAN software package (29). Reprojections of the C3-symmetrized three-dimensional structure, paired with their corresponding unsymmetrized class averages, are shown in Fig. 5G. Representations of the structure of human P2X4 were generated and are shown in Fig. 5H. The resolution of the wild-type structure was ~21 Å as judged by Fourier shell correlation of structures calculated from sets of even- and odd-numbered particles (Fig. 5F).

Gold and Lectin Labeling—To orient our structure, the C-terminal His tags of human P2X4 were labeled using 1.8-nm diameter Ni-NTA-Nanogold particles and washing our EM grids in a Nanogold suspension to prevent unbinding during sample preparation. A high off-rate for this probe has recently been described (42). We selected 1966 particles with at least one gold particle attached. A sample field of view is displayed in Fig. 6A. Reprojections of the C3-symmetrized three-dimensional structure, paired with their corresponding unsymmetrized class averages, are shown in Fig. 6C. Gold particles were clearly visible, with some classes containing two attached gold particles per trimer. The gold-labeled particles were significantly smaller than the wild-type particles, most noticeably in areas near where the gold was bound, due to the strong negative density of the gold overshadowing the protein density within its vicinity.

The extensive glycosylation present on human P2X4 receptors enabled labeling of the ectodomain with L. culinaris lectin tetramers. The human P2X4 receptor ectodomain displayed cavities with lateral fins running alongside them. This volume threshold corresponds to a molecular mass of 220 kDa and a volume of 270 nm³, allowing for one P2X4 trimer in addition to a detergent micelle (assuming a protein density of 1.35 g/ml and no significant internal cavities within the structure). At 3σ above the mean density (Fig. 5H, dark blue outer core), the cavities disappeared, and the lateral fins were more pronounced. This density corresponds to a molecular mass of 410 kDa and a volume of 506 nm³. Our value for the molecular volume of human P2X4 of 270 nm³ is consistent with the value of 409 nm³ obtained for rat P2X2 using AFM imaging (trimer masses of 165 kDa for human P2X4 and 210 kDa for rat P2X2) (10). The transmembrane domains are most likely shrouded in a torus-shaped detergent micelle, which could explain the bulbous nature of the P2X4 three-dimensional structure in the region below the fins that we associate with the ectodomain. However, we cannot rule out the possibility that regions of the ectodomain re-enter the transmembrane region and contribute to the protein content of the transmembrane space.

**DISCUSSION**

The main results of this study are (i) we developed methods to overexpress P2X4 receptors for structural work; (ii) we determined the shape, architecture, and size of a single human P2X4 receptor; and (iii) we verified the dimensions with independent measurements using FRET. We initially chose to work on all homomeric P2X receptors using FRET microscopy and measured the proximity between their C-terminal tail tips at 5.3–6.4 nm. We then focused on P2X4 receptors for electron microscopy because of its favorable expression profile (43). The single particle studies provide data on the overall shape and size of a single P2X receptor and on the approximate distance between the C-terminal tail tips at 6.1 nm. The agreement for proximity between FRET and electron microscopy attests to the reliability of the methods. Förster theory of energy transfer makes several assumptions about the mutual orientation of the fluorophores (notably the κ² value). In the usual case, κ² is taken to equal 2/3, which implies adoption of all spherically random orientations...
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between the fluorophores (30). The agreement for distance estimates by FRET and single particle imaging suggests that FRET is useful as a molecular caliper for membrane-embedded P2X channels. This is consistent with previous pioneering FRET studies by Nashmi et al. (39) of neuronal nicotinic receptors and comparisons of distance to the structural model of the muscle nicotinic receptor (19, 39, 40).

One of the key issues in the P2X field is the need to obtain direct structural information for this novel class of membrane proteins. This is a nontrivial issue because no P2X receptors have been found in bacteria, and there are no other receptors with similar structure based on sequence analysis. This calls for the development of approaches that can be used for the expression and purification of P2X receptors. In this work, we have both developed an efficient expression and purification system for human P2X_4 and significantly improved the quality and resolution of existing structural data available for P2X receptors. Our methods should also enable relatively straightforward purification of larger quantities of human P2X_4 for higher resolution structure studies. Preparative electrophoresis has been used extensively for the purification of membrane protein complexes (44), and in at least one case, this method has yielded sufficient quantities for three-dimensional crystallization (45). The use of our method, by us and others, will allow high resolution structural information on P2X receptors.

Selective purification by preparative electrophoresis of a band corresponding in apparent molecular mass to a trimeric form of human P2X_4 strongly suggested the application of C3 symmetry in image processing and three-dimensional reconstruction. Since initial pioneering work with Blue native PAGE by Nicke et al. (8), virtually all recent evidence also indicates that trimers are the functional unit of P2X receptors (9–11). In addition, we were able to label the C-terminal His tags on each monomer with 1.8-nm gold particles. Although three gold particles attached to a single trimeric particle were only infrequently observed, probably because of a relatively weak binding or a rapid off-rate (42), many particles were doubly labeled, and after classification and averaging, a 120° angle was subtended between the two gold labels in the classes corresponding to “face-on” views along the C3 axis of rotational symmetry (Fig. 6C, arrows).

Labeling of the large, multiply glycosylated ectodomain with plant lectin confirmed that the “propeller”-like domain, previously identified in the low resolution structural analysis as being at the opposite end of the molecule to the Ni-NTA-gold label, probably corresponded to this extracellular region. A composite model based on all the EM data is shown in Fig. 5.

Note that the expected dimensions of a torus-shaped DDM micelle are indicated, which would leave a relatively small volume at its center corresponding to the transmembrane regions of P2X_4. The final structure is therefore expected to be torpedo-shaped, with a narrow transmembrane domain and compact ectodomain that does not display a central cavity or pore. Approximate maximum dimensions were 8 nm in diameter and 12 nm along the longest axis, coincident with the C3 symmetry axis. Under our experimental conditions, in which P2X_4 was solubilized in detergent, the position of the membrane-spanning domain could not be accurately determined; however, we have indicated its approximate position in Fig. 7. It is probable that the transmembrane domains were protected from the aqueous environment by a detergent micelle, which could possess a molecular mass of in excess of 50 kDa. However, our structure does not inform us about protein organization within the transmembrane region, and it is possible that regions of the ectodomain may form re-entrant loops, increasing the protein

![Diagram](http://www.jbc.org/ at UNIV WALES COLL OF CARDIF on February 27, 2014)
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content within the transmembrane space. In the model shown in Fig. 7, the C-terminal decahistidine tag is positioned close to the start of the membrane-spanning domain, a location that would place it close to the polar headgroup region of the inner leaflet of the plasma membrane.

Our work represents the first direct structural study of a human P2X receptor, but should be discussed in the light of previous work on rat P2X2. Mio et al. (13) purified rat P2X2 from an insect cell line and determined a structure by transmission electron microscopy and single particle analysis. Their structure resembled an inverted three-sided pyramid, 21.5 nm in height and 20 nm in side length, corresponding to a molecular volume of ~1200 nm^3; this is 4.5-fold greater than our measurement of 270 nm^3. The authors stated that their model contained a large, stain-permeable cavity; however, as the orientation of the particle was not assigned, it was not possible to state which portion of the molecule contained the cavity in their experiments. The authors assumed that this portion of their structure corresponded to the ectodomain by comparison with the topology map of the receptor. However, our data clearly show that the ectodomain of human P2X4 is a highly compact structure, such as has been shown in the recently published structure of the trimeric acid-sensing ion channel 1 (12). Given the high degree of sequence homology between rat P2X2 and human P2X4, it seems unlikely that the structures at the monomeric level would be so radically different in size and shape. A possible explanation for the discrepancy may be that the rat P2X2 particles were composed of a higher order quaternary structure. Four trimeric P2X2 complexes of the dimensions described here could potentially associate into a large complex with tetragonal symmetry. Such a tetramer of trimers, composed of 12 P2X2 polypeptides, would account for the ~4-fold greater volume of the rat P2X2 particles versus expectations and the very large (20-nm side length) overall dimensions of the pyramidal particles. Two additional studies of rat P2X2 structure have been published. Nakazawa et al. (14) analyzed single particles of rat P2X2 using AFM. They found that in the presence of 1 mm ATP, particles of ~10 nm in diameter were observed, with evidence suggesting the presence of a central pore. The diameter of these particles is more consistent with those observed for human P2X4 in our study (8-nm diameter along the C3 axis); however, at the resolution of our study, we could not observe a central pore. It is possible that AFM, which measures the surface topography of the specimen, is detecting an indentation in P2X2 that is not present in P2X4 or, alternatively, that the negative staining and EM method do not access this surface topography in P2X4. Barrera et al. (10) also imaged rat P2X2 using AFM. They provided evidence for the trimeric nature of rat P2X2 by measuring the average angle (120°) between peripheral particles interpreted as antibodies attached to the (three) C termini of their P2X2 specimen. Their estimate of the volume of the receptor was 409 nm^3, which is consistent with our measurement for human P2X4 of 270 nm^3, allowing for the difference in molecular mass between the two receptor subtypes (165 kDa for human P2X4 trimers versus 210 kDa for rat P2X2 trimers).

In summary, we have introduced an efficient method for purification of human P2X4 that has been overexpressed in human cells. This methodology may be readily applicable to other members of this family and could be of interest for the purification of other membrane proteins where isolation of a single oligomeric form is desirable. This method has allowed us to study the overall structure and topology of P2X4 and has helped to interpret existing structural data for this unique and intriguing family of nonselective cation channels. Our work has enabled visualization of some global structural features of these receptors and paves the way for future studies. This work should be viewed as an initial and necessary step toward the longer term goal of obtaining high resolution structural information for P2X receptors. From this perspective, it is worth considering that spectacular progress has been made using cryoelectron microscopy studies of nicotinic receptors since initial views of the quaternary structure published in 1985 (46) to a recent 4 Å resolution model (47).

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