Direct Observation of ATP-Induced Conformational Changes in Single P2X4 Receptors

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The ATP-gated P2X4 receptor is a cation channel, which is important in various pathophysiological events. The architecture of the P2X4 receptor in the activated state and how to change its structure in response to ATP binding are not fully understood. Here, we analyze the architecture and ATP-induced structural changes in P2X4 receptors using fast-scanning atomic force microscopy (AFM). AFM images of the membrane-dissociated and membrane-inserted forms of P2X4 receptors and a functional analysis revealed that P2X4 receptors have an upward orientation on mica but lean to one side. Time-lapse imaging of the ATP-induced structural changes in P2X4 receptors revealed two different forms of activated structures under 0 Ca2+ conditions, namely a trimer structure and a pore dilation-like tripartite structure. A dye uptake measurement demonstrated that ATP-activated P2X4 receptors display pore dilation in the absence of Ca2+. With Ca2+, the P2X4 receptors exhibited only a disengaged trimer and no dye uptake was observed. Thus, our data provide a new insight into ATP-induced structural changes in P2X4 receptors that correlate with pore dynamics.

Introduction

P2X receptors (P2XRs) are cell-surface ATP-gated cation channels, and seven subtypes (P2X1–7) are known [1]. One functional P2XR channel is composed of three subunits. Each P2XR subunit is predicted to have a large extracellular domain (ECD), two transmembrane-spanning domains (TMD), and N and C termini intracellular domains (ICD) [1]. It has been suggested that the second half of the ECD (residues 170–330) has sequence and secondary structure similarities to the catalytic site of class II aminoacyl-tRNA synthetase [2]. A six-stranded antiparallel β-pleated sheet structure is believed to exist in the ECD of P2XRs. 3-D homology modeling in P2X4Rs suggests that this region coordinates ATP binding and the allosteric coupling of the conformational changes in the ATP binding domain with corresponding changes at the transmembrane channel gate through a linker region (the α-helix between the β6 strand and TM2 region) [3]. In addition to the allosteric coupling of the ATP-binding sites at ECDs and the channel gate at TMD, P2XRs have different permeability states that were originally discovered by Cockcroft and Gomperts [4]. With P2XRs, extracellular Ca2+ levels greatly affect the permeability dynamics [5]. In the presence of Ca2+, P2X,R only opens a small cation-permeable channel pore but in the absence of extracellular Ca2+, it forms a larger pore that allows larger molecules including N-methyl-D-glucamine (NMDG)+, propidium iodide, and ethidium bromide (EtBr) to pass. Although there is a functional relationship between ECD and TMD, the ATP-induced structural changes in ECD are poorly understood. Recent extensive studies by Khakh’s group have clearly demonstrated the allosteric coupling of ICDs and the ion channel permeability of P2XRs [6,7]. These results strongly support the hypothesis of the allosteric coupling of channel pores in TMD and other domains including ECDs.

In recent structural studies of P2XRs two approaches have been used: electron microscopy (EM) and atomic force microscopy (AFM). In EM, single particle averaging analysis and the Ni-NTA gold labeling of human P2X4Rs have clearly demonstrated the distance between the C-terminal tails, the molecular volume, and the 3-D structure [8]. In AFM research, an antibody tagging study has revealed the trimer structure of P2XRs [9,10]. AFM has the important advantage of allowing proteins to be observed under liquid conditions, and this makes it possible to activate P2XRs by ATP during AFM studies. In an AFM study combined with ATP treatment, P2X4Rs exhibited a pore-like structure [11]. In addition to drug treatment, AFM can be used for imaging both lipid bilayers [12] and proteins inserted in lipid membranes [13]. Extensive AFM studies by Engel and Müller’s groups have obtained high-resolution topographs of many proteins including aquaporin [14], connexin [15], F-ATP synthase [16], and tubulin [17]. A recent study by Cisneros clearly demonstrated the topography of orientation regulated and...
Author Summary

ATP is not only a source of intracellular energy but can act as an intercellular signal by binding membrane receptors. Purinergic receptors, which bind with nucleotides including ATP are known as P2 receptors and are divided into two types: ion channel-type P2X receptors and metabotropic-type P2Y receptors. P2X receptors are thought to undergo conformational changes in response to ATP binding, leading to the opening of transmembrane channels, through which cations enter the cells. A growing body of evidence shows that P2X receptors control various physiological and pathophysiological cellular responses. However, the receptor structure and the conformational changes it experiences upon stimulation remained to be clarified. Here, we employed an atomic force microscope (AFM) to observe P2X receptor behavior at the single channel level. We chose to analyze the P2X4 receptor, because it is known to increase the transmembrane pore size (i.e., pore dilation) in the absence of extracellular calcium. Activated P2X4 receptor exhibited a trimeric topology with a pore-like structure in the center. When calcium was present the receptor exhibited a trimer without a pore structure at its center. These structural changes corresponded well with the changes of ion permeability of P2X4 receptor.

covalently assembled homotrimer OmpF proteins [18]. In their report, the authors employed the single particle correlation averaging method to obtain 3-fold symmetrized images of OmpF trimer that are identical to the topographs of 2-D crystals of OmpF. Because many P2XR channels are also homotrimers, this approach can be used for the high-resolution imaging of P2XRs. Although the use of AFM provides significant advantages the imaging speed is usually very slow (several tens of seconds). Many ion channel reactions occur in less than a second, so fast scanning is essential for observing the P2XR reaction with AFM. To address this issue, we employed a recently developed fast-scanning AFM [19] that allows us to observe biological molecules including nucleic acids [20], lipids [12], and proteins [21,22] at high temporal resolution. Fast-scanning AFM in combination with single particle averaging is considered a powerful tool for analyzing single P2X4Rs channels with high spatial and temporal resolution.

Results

Expression, Purification, and AFM Observation of P2X4Rs on Poly-D-Lysine-Coated Mica

The expression of rat P2X4R protein in human 1321N1 astrocytoma cells was estimated by western blotting. P2X4R was detected only in P2X4R gene-overexpressed cells (Figure 1A). In silver-stained native-PAGE, one band corresponding to a trimer (about 150 kDa) (Figure 1B) was observed. The same protein analyzed by SDS-PAGE and silver-staining exhibited a band corresponding to a monomer (about 50 kDa). For the AFM analysis of P2X4Rs, we used freshly cleaved mica as a substrate because it has an atomically flat surface and is usually used for protein observation with AFM. All the AFM images were presented as gray-scale height images. In many cases, the P2X4R particles were only loosely attached to the uncoated mica and so they moved during the AFM observation. To obtain a stronger attachment for electrostatic interactions, we coated the mica with positively charged poly-D-lysine (PDL) (1 mg/ml, 30 min at room temperature [RT]) and set the pH of the imaging buffer (AFM imaging buffer A) at 8.0 because the isoelectric point of P2X4R is pH 7.41. All the P2X4Rs on the PDL-coated mica were observed in AFM imaging buffer A. Under this condition, the P2X4Rs were attached stably to the substrate (Figure 2A). The P2X4R control particles were relatively homogenous and nearly all circular, ellipsoidal, or triangular with obtuse angles (Figure 2B, upper panels). PDL-polymer were also observed (Figure 2B,[ii], arrows). In this study, we defined the dimensions of the P2X4Rs as their diameter and height on the basis of our criteria (see also Materials and Methods and Figure S1). The nonstimulated P2X4Rs had a diameter of 12.6 ± 0.2 nm (mean ± standard error of the mean [SEM]) (n = 200) and a height of 2.3 ± 0.1 nm. To observe activated P2X4Rs, we added ATP before the AFM observation. ATP did not induce any significant changes at 100 µM (unpublished data), but the P2X4Rs changed greatly at 1 mM (Figure 2B, lower panels). Under this condition, at least several minutes of ATP treatment was required before the P2X4Rs underwent structural changes. After the structural changes caused by 1 mM ATP, most of the P2X4Rs

Figure 1. Expression and Purification of P2X4R

(A) Overexpression of P2X4R in human 1321N1 astrocytoma cells. In Western blotting, P2X4R protein was only detected in cells transfected with the P2X4R gene.

(B) Purification of P2X4R protein. The molecular weight of the purified P2X4R protein band was detected at about 150 kDa in native-PAGE (left) and 50 kDa in SDS-PAGE (right). After electrophoresis, the gels were stained with silver staining.

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P2X4 receptor.
Figure 2. AFM Observations of P2X4Rs on PDL-Coated Mica

(A) P2X4Rs attach stably to PDL-coated mica. P2X4Rs on PDL-coated mica exhibited stable attachment and the majority did not shift position during AFM observation. Scale bar, 10 nm.

(B) AFM images of P2X4Rs at (i) low resolution, (ii) high resolution, and (iii) single particle level. (i) At low resolution, the P2X4Rs were relatively homogenous. Slight differences were observed after ATP treatment (1 mM, 30 min), but they are not very clear at this resolution. Scale bar, 20 nm. (ii, iii) At high resolution and at a single particle level, there were significant structural differences between the control P2X4Rs and the P2X4Rs after ATP addition. Each single particle was selected based on our criteria (Materials and Methods, Figure S1C). In the control, the P2X4Rs were nearly circular, ellipsoidal, or triangular with obtuse angles. After ATP addition, the P2X4Rs had a tripartite morphology. PDL-polymers were also observed (arrows). Scale bar, 10 nm.

(C) Percentage of trimeric P2X4Rs was significantly increased after ATP (1 mM, 30 min). ***, p < 0.001.

(D) Averaged images of P2X4Rs. (i) Nonsymmetrized averaging of P2X4Rs in the control (left) and after ATP addition (right). (ii) Symmetrized averaging of P2X4Rs in the control (left) and after ATP addition (right). 3-fold symmetrized images were obtained after symmetrized averaging. Scale bar, 5 nm.

(E) Three lobes are individual subunits in one P2X4R trimer. The distance between lobes was significantly less than that between trimers. ***, p < 0.001.

(F) P2X4R trimer shifts position as one unit. (i) When a P2X4R trimer moves during AFM, the three lobes were not dissociated but moved as a trimer. Scale bar, 20 nm. (ii) Enlarged images of single P2X4R trimer in a rectangle at 5 s, nonsymmetrized and symmetrized averaging images of ten particles in the same scan area. Scale bar, 10 nm. AFM observation was performed in AFM imaging buffer A.

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appeared to be trimers (84.9 ± 5.0%, n = 393) (Figure 2C). The ATP-treated P2X4Rs had a diameter of 14.2 ± 0.2 nm (n = 205) and a height of 3.0 ± 0.1 nm. The diameter of one lobe in a P2X4R trimer was 5.9 ± 0.2 nm (n = 40). To obtain clear topographs of P2X4Rs, we averaged single P2X4R images by using the same approach employed by Cisneros et al. [18] and on the basis of our criteria (Figure S1). The nonsymmetrized averaging of ATP-treated P2X4Rs revealed a tripartite morphology (Figure 2D[i], right) that was enhanced by 3-fold rotational symmetrization (Figure 2D[ii], right). Nonstimulated P2X4Rs were circular or triangular with obtuse angles after averaging (Figure 2D, left panels). For averaging, we used the particles shown in Figure 2B(iii) (n = 60). Then, we checked whether these trimers were one unit of P2X4R trimers or simply three adjacent particles. If each lobe was an individual P2X4R trimer that was incidentally assembled into a trimer, the distance between lobes would not be significantly different from the distance between trimers. The distance between the lobes in a P2X4R trimer and the distance between two adjacent trimers were 8.7 ± 0.1 nm (n = 100, between lobes) and 35.5 ± 2.7 nm (n = 115, between trimers), respectively (Figure 2E). Sometimes, P2X4R particles on PDL-coated mica shifted position within the same scan area. In this situation, single lobes in a P2X4R trimer (15 min after 1 mM ATP treatment) did not move individually but moved along with the other two lobes (Figure 2F[i]). Enlarged images of single P2X4R trimer in a rectangle at 5 s are shown on the left in Figure 2F(ii). The nonsymmetrized and symmetrized averaging of ten particles in the same scan area at 0 s is shown in the center and on the right, respectively, in Figure 2F(ii).

Time-Lapse Imaging of ATP-Induced Structural Changes in Single P2X4R

To observe the ATP-induced continuous structural changes in P2X4Rs, we performed imaging using fast-scanning AFM with a scan rate of two frames per second. P2X4Rs were observed in AFM imaging buffer B. Under our conditions, faster scan rates than this degraded the signals and increased noise so that we were unable to obtain sufficient resolution. It is known that a mica surface is negatively charged [23], and so we used uncoated mica rinsed with a high concentration of KCl (1 M, 30 min at RT) to reduce electrostatic interactions between the mica surface and the ATP or P2X4Rs. Under this condition, many P2X4Rs shifted position during AFM imaging. To obtain a clear topology of P2X4R, ten P2X4R particles were averaged at the same time point. The resulting 3-fold symmetrized images of P2X4Rs clearly exhibited the structures at each time point. Before the uncaging (~2.5 to ~0.0 s) of caged ATP (200 μM), P2X4R exhibited a circular structure (Figure 3, see also Video S1). At 0.5 s after uncaging, the P2X4R structure changed greatly and a clear trimeric structure was observed. After this change, the distances between individual lobes gradually increased (~5 s). The conformational change in the nonsymmetrized P2X4Rs is also shown in Figure S2. The same reaction was reproduced in three independent experiments. Another result of the ATP-induced structural changes in P2X4Rs is shown in Figure S3. Some P2X4Rs were stable at one location during AFM imaging. Several examples of ATP-induced structural changes in a single P2X4R are shown in Figure S4. At a single particle level, although the P2X4R topologies were relatively blurry, individual subunits became visible after uncaging and appeared to move away from each other. When the ATP was washed off, the pore dilation-like structure returned to a circular structure (unpublished data).

AFM Observation and Functional Analysis of Membrane-Inserted P2X4Rs

To estimate the orientation of observed structures, P2X4Rs were reconstituted in a lipid bilayer. Figure 4A(i) is a diagram showing the predicted structure of a P2X4R subunit. A six-stranded anti-parallel β-plated sheet structure is reported to exist in the second half of the ECD in P2X4R subunits [2,3]. The entire structure of trimeric P2X4R is predicted on the basis of this homology modeling data, as shown in Figure 4A(ii). In AFM, this β-plated sheet structure should be observed as one large domain. Figure 4B shows our working hypothesis, which is that when P2X4Rs are reconstituted in a lipid bilayer and if they are inserted in an upward orientation, they should respond to ATP thus resulting in structural changes and increased Ca2+-permeability. When P2X4Rs were inserted in the lipid bilayer that formed on mica, the AFM images of P2X4Rs in membranes were similar to the P2X4Rs that were dissociated from the membrane. The P2X4Rs had circular structures in the control and trimeric structures after binding with ATP (200 μM, 1 min) (Figure 4C and 4D). P2X4Rs reconstituted in a lipid bilayer did not require as high a concentration of ATP as those on PDL-coated mica. Under this condition, the structures of most of the P2X4Rs (83.3 ± 5.4$, n = 70) changed into a tripartite form. The P2X4Rs in the membranes were 11.4 ± 0.3 nm in diameter and 5.8 ± 0.1 nm high (including the height of the membrane) in the control (n = 50) and 13.3 ± 0.3 nm in

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Figure 3. Fast-Scanning AFM Observations of the ATP-Induced Structural Changes in the P2X4Rs

Time-lapse imaging of ATP-induced structural changes of P2X4Rs. Before activation, P2X4Rs were in circular shape and exhibited some fluctuation (∼2.5 s to ∼0.0 s). Caged ATP (200 μM) was uncaged at 0 s. After uncaging, the P2X4R structure changed to a trimer structure within 0.5 s. Then, P2X4R exhibited a further structural change and adopted a pore dilation-like conformation. Ten P2X4R particles were averaged for each frame. Scale bar, 10 nm. AFM observation was performed in AFM imaging buffer B.

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Pore Dilation-Like Structural Changes and Dye Uptake of P2X4Rs

In the time-lapse imaging of ATP-induced structural changes in P2X4Rs, we observed a characteristic pore dilation-like structure (Figure 3, ∼5.0 s). This pore dilation-like structure was also observed in membrane-reconstituted P2X4Rs (Figure 4D). Before the appearance of this structure, the P2X4Rs on the uncoated mica exhibited nondilated trimer structures (Figure 5A, center). We observed two P2X4R structures similar to these two different forms on PDL-coated mica (Figure 5B). 15 min after ATP (1 mM) addition, the P2X4Rs exhibited a nondilated trimer structure (Figure 5B, center) but they exhibited a pore dilation-like structure 30 min after ATP addition (Figure 5B, right). Then we estimated the dye uptake function of P2X4Rs using the same Ca2+ imaging system. EtBr-dye uptake measurement was performed at the same time as the Ca2+ imaging. Here, no increase was observed in red fluorescence after ATP addition (Fig. 4E[iii]). The Ca2+ imaging was performed in Ca2+ imaging buffer.

Discussion

Our main findings in this study are that (i) it is possible to achieve time-lapse imaging of the dynamic structural changes
of P2X₄Rs evoked by ATP; (ii) the three subunits are close to each other and it is impossible to observe individual subunits in the control but they disengage and move away from each other after ATP binding; and (iii) the two types of structural changes observed in AFM appear to correspond to two functional states, namely the Ca²⁺-permeable state and the dye permeable state.

Recent structural studies with direct imaging methods including EM and AFM or with other methods including fluorescence resonance energy transfer (FRET)-based analysis have provided strong motivation for structural studies of P2XRs. These reports clearly demonstrated trimeric stoichiometry using antibody-tagging [9,10] or Ni-NTA gold labeling on the His-tag of the C termini in P2XRs [8], and the shape, architecture, and size of P2X₄Rs in a nonstimulated state and the distance between the C termini of P2X₄Rs [8]. We needed to determine the way in which P2X₄Rs change their entire structure in response to ATP binding. To address this issue we analyzed homotrimeric P2X₄Rs. To this end, we overexpressed P2X₄R gene in human 1321N1 astrocytoma cells. Because this cell does not express P2X₄Rs [24], purified P2X₄Rs from the membrane fraction of this cell are considered to form homotrimers. In fact, the purified P2X₄R presented as a single band corresponding to a trimer (about 150 kDa) in native-PAGE but as a monomer (about 50 kDa) in SDS-PAGE, and purified P2X₄R was functional as estimated in terms of Ca²⁺ permeability and EtBr uptake of P2X₄R. Under 2 mM Ca²⁺ conditions, the green fluorescence intensity immediately increased after ATP addition (see also Video S2). There was no significant increase in red fluorescence (DNA/EtBr) intensity. Each trace is the mean ± SEM of five independent experiments. Scale bar, 100 μm.

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perhaps causes the reduced response of P2X₄R to ATP. Under our conditions, the strong attachment of P2X₄R may change the structural flexibility and/or the intersubunit interaction that reduces the responsivity to ATP. The reduced attachment of P2X₄Rs to mica without PDL dramatically increased the velocity of the ATP response, and thus supported our hypothesis.

Despite the low ATP reactivity of P2X₄Rs on PDL, we observed clear structural differences between the control and the ATP-treated condition. We believe that the three lobes observed after ATP addition were three individual subunits of one P2X₄R trimer. First, the distance between the lobes was significantly smaller than that between trimers. If each lobe was an individual P2X₄R trimer that was incidentally assembled into a trimer, the distance between lobes would not be significantly different from the distance between trimers. Second, during the AFM observation, some P2X₄R trimers occasionally shifted position, and these trimers moved as trimers (i.e., the three lobes did not dissociate). Third, in time-lapse analysis, the circular structure changed into a trimer after ATP treatment both in the averaged particle images and in single particles. This result also suggests that the trimeric stoichiometry exists even in P2X₄Rs before ATP binding. From this observation, we considered circular particles without individual subunits before ATP binding to be trimeric P2X₄Rs because those subunits were closer together than the spatial resolution of our AFM system. If this is the case, the diameter of the P2X₄R in the control should be double that of one lobe. In fact, the diameter of the P2X₄R in the control (about 12.6 nm) was approximately double that of one lobe (5.9 × 2 nm). These three lobes were also observed when P2X₄Rs were inserted into a lipid bilayer, suggesting that these lobes are the predicted six-stranded antiparallel β-pleated sheet structures in the ECDs of P2X₄Rs. EM analysis of P2X₄Rs revealed propeller-like domains in the ECDs [8] that were similar to the six-stranded antiparallel β-pleated sheet-like structure that we observed in the ECDs. In their report, the authors clearly demonstrated that the EM-based distance between the C termini of the P2X₄Rs was 6.1 nm and the FRET-based distance between the C termini was 5.6 nm. The three propeller-like domains at the opposite end of the P2X₄R to the gold-labeled C termini means the distances between these domains would be similar. As described above, when three lobes are assembled close together in the control, the distance between the centers of two lobes is twice the lobe radius (2.95 × 2 nm), which agrees well with the distance between P2X₄R C termini estimated by FRET and EM [8]. As mentioned above,
the AFM images of P2X₄Rs in a lipid bilayer and on mica were comparable; this result strongly suggests the upward direction of the P2X₄Rs on mica. However, the height of P2X₄R on mica was less than the height of a lipid bilayer composed of phospholipids (about 4 nm) [12]. In nonsymmetrized averaging, one of the three lobes in the P2X₄R trimer on mica was lower than the other two. The height of the P2X₄Rs on mica was only slightly greater than that from the surface of a lipid bilayer to the top of the inserted P2X₄Rs, indicating the possibility that the P2X₄Rs do not stand vertically and TMD and/or ICD are bent during the AFM observation. From these observations, we concluded that P2X₄Rs lean to one side on mica and TMD or ICD may be bent and concealed behind the ECDs. Similarly, the simple adsorption of P2X₄Rs [11] on mica also results in these molecules having a top view-like structure in AFM images, thus supporting our conclusions.

In the time-lapse imaging, we observed two different structural changes: (i) from one circular structure to a trimeric structure (0.0 → 0.5 s after uncaging) and (ii) the subsequent moving away of each lobe (0.5 → 5.0 s). The second structural change reminds us of an important function of the P2X family, namely pore dilation. In an early study, Khakh et al. clearly demonstrated that P2X₄R exhibits NMDG⁺ permeable pore dilation in the absence of extracellular Ca²⁺ [5]. In their work, the P2X₄Rs exhibited sustained activity for several minutes, indicating that our pore dilation-like structure is not a desensitized P2X₄R state. Our work represents direct evidence of the functional and structural relationship of pore dilation in P2X₄R. Under a 0 Ca²⁺ condition, we observed both pore dilation-like structural changes in ECDs and EtBr uptake. This pore dilation-like change was reproducible under various conditions including on mica, on PDL-coated mica and in a lipid bilayer, strongly suggesting that this structural change is a fundamental reaction of P2X₄R. At 2 mM Ca²⁺, we observed no EtBr uptake but there was a Ca²⁺ flow via P2X₄R that also corresponded to the previous report [5]. Under this condition, the pore dilation-like structure of P2X₄R was not observed but P2X₄R trimers similar to the structure seen immediately after ATP binding were evident. The averaged trace of the green fluorescence intensity exhibited a near-plateau state after an initial increase. This result may indicate that the number of desensitized P2X₄Rs increase during a long ATP exposure. From these observations, we considered that the structural changes in the ECDs of P2X₄Rs are related to permeability dynamics. Recent reports on P2X₇Rs suggested the possibility that their EtBr uptake is mediated by accessory Pannexin-1 (Panx1) channels [26]. In their report, the authors demonstrated that human 1321N1 cells express Panx1, so it is possible that there is functional coupling between overexpressed P2X₄Rs and Panx1 in this cell. We concluded that EtBr can pass through P2X₄Rs independent of Panx1 (at least in our study) for the following reasons. First, we used purified P2X₄Rs and only a single band was observed in the native-PAGE/silver staining. As Panx1 (about 50 kDa) forms a hexameric channel [27], Panx1 contamination would be detected as another band (about 300 kDa). Second, Panx1 and connexins are known to have structural similarities [28] and connexins are observed as hexameric structures [15] in AFM. We observed no hexameric structures in our AFM study. Third, the issue of Panx1 coupling with P2X₇R remains to be clearly settled because another group has demonstrated

![Figure 5. Pore Dilation-Like Structural Changes Are Related to the Dye-Uptake of P2X₄R](image-url)
that P2X₇R exhibits pore dilation independent of Panx1 [29].
P2X₂R also exhibits the pore dilation independent of Panx1 [7].
These results indicate that Panx1 may not be a fundamental component of the pore dilation state of the P2XR family. Fourth, in contrast to connexin hemichannels, Panx1 is active at physiological extracellular Ca²⁺ concentrations [28]. In our simultaneous Ca²⁺/dye uptake measurement, EtBr uptake was not observed at 2 mM Ca²⁺. However, our data and these reports do not rule out the possibility of functional coupling between P2X₄Rs and Panx1 in cells.

Thus, our present study provides direct evidence of structural changes in the ECDs of P2X₄Rs that are involved in permeability dynamics. We have achieved the first direct, time-lapse imaging, to our knowledge, of ATP-induced...

Figure 6. Model of Structural Changes of ECDs in P2X₄R and Corresponding Pore States Based on AFM and Functional Analysis Data

In the control, the ECDs are close to each other and so individual ECDs (or subunits) cannot be observed with AFM. Immediately after ATP binding, three ECDs are disengaged and the TMD pore is Ca²⁺ permeable.

(A) In the absence of Ca²⁺, the distances between the ECDs are further increased and the TMD pore becomes EtBr permeable (right).

(B) With 2 mM Ca²⁺, P2X₄R does not exhibit any further structural changes after the disengagement of the ECDs and exhibits no permeability changes.

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structural changes of P2X4R using a new technique, namely fast-scanning AFM. Our approach provides new insights into the structure of P2XRs, and an extension of this approach to other P2X subtypes will help us to understand the structural and functional relationships of the P2X family.

Materials and Methods

Reagents. Reagents were obtained from the following sources. DMEM, EDTA, and FBS were purchased from Gibco. Aprotinin, bestatin, deoxycholate, ethidium bromide, fluorescein, glycine, leupeptin, NaCl, EGTA, penicillin, pepstatin A, PDL, protein A sepharose, puromycin, sodium orthovanadate, Trition X-100, HEPE, 3-[3-Cholamidopropyl]dimethylammonio]-1-propanesulfonate, 4–2(aminomethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), and L-α-phosphatidylcholine (PC) were obtained from Sigma-Aldrich. P2X4R was supplied by Invitrogen. The silver staining kit and MeOH were purchased from Wako Pure Chemicals. Sephadex G-25M, aprotinin, bestatin, leupeptin, NaCl, EGTA, penicillin, pepstatin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicillin, pepstatin A, leupeptin, NaCl, EGTA, penicill
Procedure for averaging P2X4Rs. We performed the averaging in accordance with an early study [18]. First, we selected individual P2X4R particles on the basis of our criteria and then averaged them using EMAN software (nonsymmetrized averaging). Under our conditions, most P2X4R particles were defined as the diameter of the circle used for determining the particle center. The diameter of a trimeric P2X4R was defined as the average value of the long and short axes. (i) The diameter of a trimeric P2X4R was defined as the diameter of a circle that circumscribed the three lobes. (ii) Criteria for P2X4R height. In the control, the particle height was simply defined as the height from the mica surface (left). The height of a trimeric P2X4R was defined as the average value of the heights of the three lobes (right).

Criteria for Determining the Center, Diameter, and Height of P2X4Rs

(i) The center of a triangular P2X4R was defined as the center of a triangular circumcircle (left). The center of circular P2X4R was defined as the center of a circle connecting the highest points of three lobes. (ii) Experimental examples of determining the particle center.

Supporting Information

Figure S1. Criteria for P2X4R size. The diameters of triangular and circular P2X4Rs were defined as the diameter of the circle used for determining the particle center. The diameter of a trimeric P2X4R was defined as the average value of the long and short axes. (i) The diameter of a trimeric P2X4R was defined as the diameter of a circle that circumscribed the three lobes. (ii) Criteria for P2X4R height. In the control, the particle height was simply defined as the height from the mica surface (left). The height of a trimeric P2X4R was defined as the average value of the heights of the three lobes (right).

Statistical analysis. Average results are expressed as the mean ± SEM. Data were analyzed with the Student’s t-test to determine the differences between groups. Significance was accepted when p < 0.05.
Direct Imaging of Single P2X<sub>4</sub> Receptors

Found at doi:10.1371/journal.pbio.1000103.sv002 (836 KB AVI).

Video S3.  EtBr Uptake of P2X<sub>4</sub>Rs under 0 mM Ca<sup>2+</sup> Conditions
Under 0 mM Ca<sup>2+</sup> conditions, the red fluorescent (DNA/EtBr) intensity gradually increased after ATP addition. Found at doi:10.1371/journal.pbio.1000103.sv003 (817 KB AVI).

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Author contributions. YS, KS, MT, SK, KI, and KT conceived and designed the experiments. YS and KS performed the experiments. YS, KS, MT, SK, KI, and KT analyzed the data. YS, MT, and SK contributed reagents/materials/analysis tools. YS wrote the paper.

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