Structural insights into the competitive inhibition of the ATP-gated P2X receptor channel

Go Kasuya, Toshiaki Yamaura, Xiao-Bo Ma, Ryoki Nakamura, Mizuki Takemoto, Hiromitsu Nagumo, Eiichi Tanaka, Naoshi Dohmae, Takanori Nakane, Ye Yu, Ryuichiro Ishitani, Osamu Matsuzaki, Motoyuki Hattori & Osamu Nureki

P2X receptors are non-selective cation channels gated by extracellular ATP, and the P2X7 receptor subtype plays a crucial role in the immune and nervous systems. Altered expression and dysfunctions of P2X7 receptors caused by genetic deletions, mutations, and polymorphic variations have been linked to various diseases, such as rheumatoid arthritis and hypertension. Despite the availability of crystal structures of P2X receptors, the mechanism of competitive antagonist action for P2X receptors remains controversial. Here, we determine the crystal structure of the chicken P2X7 receptor in complex with the competitive P2X antagonist, TNP-ATP. The structure reveals an expanded, incompletely activated conformation of the channel, and identified the unique recognition manner of TNP-ATP, which is distinct from that observed in the previously determined human P2X3 receptor structure. A structure-based computational analysis furnishes mechanistic insights into the TNP-ATP-dependent inhibition. Our work provides structural insights into the functional mechanism of the P2X competitive antagonist.
ATP is the main cellular energy source and also serves as an extracellular signal transmitter. P2X receptors are non-selective cation channels gated by extracellular ATP. The vertebrate P2X receptors include seven subtypes (P2X1–P2X7) that form homo- or hetero-trimers and are involved in diverse physiological processes, such as muscle contraction, pain sensation, inflammation, and perception. Each subunit of the P2X receptor is composed of the large extracellular domain that contains the ATP and other ligand binding sites, the two transmembrane helices that form a non-selective cation pore, and the intracellular N- and C-termini that modulate channel gating.

Among the P2X receptor subtypes, the P2X7 receptors have a unique, long intracellular C-terminus with protein binding, phosphorylation, and lipid recognition sites. The activation of P2X7 receptors stimulates the release of inflammatory cytokines, such as interleukins and tumor necrosis factor-alpha (TNF-α). Therefore, P2X7 receptors play a crucial role in inflammation, immunity, neurological function, and apoptosis. Accordingly, P2X7 receptors are potential therapeutic candidates for rheumatoid arthritis, hypertension, and atherosclerosis, and clinical trials of chemical compounds targeting P2X7 have been conducted for P2X7-associated diseases. In addition, the human P2X7R gene is located at chromosome position 12q24, and is highly polymorphic. To date, more than 600 single-nucleotide polymorphisms (SNPs) have been detected, and some of them cause amino-acid substitutions. These substitutions induce losses or gains of functions in P2X7 receptors, and are associated with tuberculosis infection, ischemic stroke, and mood disorders.

The previously determined structures of the zebrafish P2X4 (zP2X4; the "zf" refers to zebrafish) receptor, the Gulf Coast tick P2X (ampP2X; the "am" refers to the tick’s scientific name Amblyomma maculatum) receptor and the human P2X3 (hP2X3; the "h" refers to human) receptor provided structural insights into the recognition of agonists and antagonists, as well as the channel activation and desensitization. Quite recently, the crystal structures of the panda P2X7 (pdP2X7; the "pd" refers to panda)…

**Fig. 1** Functional properties of ckP2X7 and crystallization construct ckP2X7 \(_\text{cryst}\). a FSEC profiles on a Superdex 200 10/300 GL column (GE Healthcare) for the EGFP-fused ckP2X7 WT (black), the EGFP-fused ckP2X7 \(_\text{cryst}\) (red), and the EGFP-fused amP2X \(_\text{cryst}\) (cyan), expressed in HEK293S GnTI’ cells. The arrows indicate the estimated elution positions of the void volume, the EGFP-fused P2X (trimer), and the free EGFP. b Representative traces of ATP-evoked currents of ckP2X7 WT and ckP2X7 \(_\text{cryst}\). c Summarized effect (mean ± s.e.m., n = 3–15) of the mutation on the ATP current of ckP2X7 \(_\text{cryst}\). **p < 0.05 vs. ckP2X7 WT. d** Representative traces of the effects of TNP-ATP on the 50 µM ATP-evoked current of ckP2X7 WT. ATP solution was prepared under Ca\(^{2+}\) free conditions. e The dose-response curve of TNP-ATP with ckP2X7 WT was fitted to the Hill equation (solid line, IC\(_{50}\) = 3.55 ± 1.13 µM). Data points are means ± s.e.m. for n = 3. f Measurement of the TNP-ATP binding ability of the ckP2X7 \(_\text{cryst}\), monitored by the excitation spectrum change of TNP-ATP upon protein binding. Error bars indicate ± s.e.m. for n = 5. The calculated K\(_{D}\) for TNP-ATP binding is 12.26 ± 2.64 µM.
Table 1 Data collection and refinement statistics

| TNP-ATP-bound ckP2X7 |
|-----------------------|
| **PDB code** | 5XW6 |
| **Data collection** | |
| Beamline | X06SA-PXI |
| Wavelength (Å) | 1.000 |
| Space group | P4₁,2,2 |
| **Cell dimensions** | |
| a, b, c (Å) | 113.0, 113.0, 333.5 |
| α, β, γ (°) | 90.0, 90.0, 90.0 |
| Resolution (Å)* | 3.1 (3.23 – 3.10) |
| R_{sym} | 0.097 (0.578) |
| I/σ* | 5.2 (1.6) |
| Completeness (%)* | 99.8% (100.0) |
| Multiplicity* | 6.0 (6.1) |
| CC1/2 (%)* | 98.6 (64.9) |

| **Refinement** | |
| Resolution (Å) | 3.1 |
| No. reflections | 40,046 |
| R_{work}/R_{free} | 0.226/0.251 |
| No. of atoms | |
| Total | 7932 |
| Protein | 7751 |
| Ligand/other | 180 |
| Average B-factors (Å²) | |
| Protein | 75.5 |
| Ligand/ion | 74.4 |
| Water | 100.5 |
| R.m.s. deviations | |
| Bond lengths (Å) | 0.007 |
| Bond angles (°) | 0.990 |
| Ramachandran | |
| Favored (%) | 96.1 |
| Allowed (%) | 3.9 |
| Outlier (%) | 0.0 |

*Highest resolution shell is shown in parentheses

Overall structure. The overall assembly and subunit folding of ckP2X7_cryp are consistent with those of the previously determined P2X structures20–24, 30, sharing the chalice-like trimeric architecture with a large hydrophilic extracellular domain, two transmembrane helices, and intracellular termini, resembling the shape of a dolphin (Supplementary Fig. 1c)20. In the extracellular domain, the electron density for the TNP-ATP molecule was detected at the inter-subunit ATP binding pocket (Fig. 2a, b; Supplementary Fig. 5). While TNP-ATP acts as an antagonist of P2X7 receptors, the extracellular domain structure of ckP2X7_cryp is similar to those of the ATP-bound, activated hp2X3 and zlp2X4 structures (Supplementary Fig. 8a, d), rather than those of the apo, closed hp2X3, zlp2X4, and pdp2X7 structures or the antagonist bound, closed hp2X3 and pdp2X7 structures (Supplementary Fig. 8b, c, e, f, h), consistent with the previous NMR analysis of the zlp2X4 receptor27. The transmembrane pore is formed by the TM2 helices with the residues from Asp319 to Leu344 lining the pore, consistent with the previous electrophysiological analysis (Fig. 2c–f)31, 32. In the most constricted region, the pore radius is smaller than 0.3 Å, which is too narrow for ion conduction. The constricted region is formed by the Met340 and Cys341 residues near the cytoplasmic side (Fig. 2d–f). In contrast, in the zlp2X4 and hp2X3 structures in the apo, closed state, the most constricted region is located near the extracellular side, rather than the cytoplasmic side (Supplementary Fig. 3)20, 21, 23. In those structures, the residues corresponding to Thr322 and Ser326 in ckP2X7 define the constricted channel gate (Supplementary Figs. 2 and 3). This partial pore opening on the extracellular side in the ckP2X7_cryp structure is apparently due to the extracellular domain architecture, which is similar to those of the ATP-bound, activated P2X structures.
Supplementary Fig. 8a, d). Notably, the transmembrane domain architectures of the previously determined ATP-bound structures of zfP2X4 and amP2X, including the large inter-subunit gaps, are partially distorted from those in the native lipid environment, potentially due to detergents or truncations utilized for crystallization 21, 22, 33. However, such abnormal inter-subunit gaps are not observed in the transmembrane region of the ckP2X7 cryst structure (Fig. 2c, d). Overall, this structure represents a TNP-bound, inactivated state of the P2X7 receptor.

Mapping of functionally important mutations. Numerous SNPs of P2X7 have been identified, and some are associated with amino-acid substitutions implicated in several diseases, such as cancer, chronic pain, osteoporosis, and depression 16–18. We mapped the amino-acid residues involved in the pore constriction region of the ckP2X7 structure, viewed from the intracellular side. Amino-acid residues involved in the pore constriction region are depicted by stick models. e The pore radius for the TNP-ATP-bound ckP2X7 structure along the pore center axis. The pore size was calculated with the program HOLE. f Pore-lining residues of the TNP-ATP-bound ckP2X7 structure are shown in stick representations.

Fig. 2 The architectures of the TNP-ATP-bound ckP2X7 structure. a The TNP-ATP-bound ckP2X7 structure, viewed parallel to the cell membrane. The blue, red, and yellow colors correspond to each subunit. The omit Fc –Fo density map contoured at 2.5 σ is presented for the TNP-ATP molecular density. b The recognition manner of TNP-ATP. The molecule is colored according to the dolphin-like model 20. c The surface model with a cartoon representation of the TNP-ATP-bound ckP2X7 structure. d The surface model with a cartoon representation of the transmembrane domain in the TNP-ATP-bound ckP2X7 structure, viewed from the intracellular side. Amino-acid residues involved in the pore constriction region are depicted by stick models. e The pore radius for the TNP-ATP-bound ckP2X7 structure along the pore center axis. The pore size was calculated with the program HOLE. f Pore-lining residues of the TNP-ATP-bound ckP2X7 structure are shown in stick representations.
respectively). These results indicate that the amino-acid substitutions observed in the extracellular domain are not involved in the direct trimer formation and ATP binding by the hP2X7 receptor, but they affect the conformational change of the extracellular domain required for channel activation. Intriguingly, in our inactivated ckP2X7 crystal structure, all of the loss-of-function substitutions are located in the secondary structure (α-helix and β-sheet) regions, suggesting that the loss-of-function substitutions may affect the structural integrity in the inactivated state. In the transmembrane domain, two substitutions, ckVal335 (hAla348Thr) and ckLeu344 (hThr357Ser), which are associated with toxoplasmosis and bipolar disorder16, are observed close to

**Fig. 3** Comparison of TNP-ATP binding sites in the TNP-ATP-bound structures of ckP2X7 and hP2X3. **a–d** Overall (a) and close-up (b–d) views of the TNP-ATP binding sites in the TNP-ATP-bound ckP2X7 structure. **e–h** Overall (e) and close-up (f–h) views of the TNP-ATP binding sites in the TNP-ATP-bound hP2X3 structure (PDB ID: 5SVQ) (b). The molecules are colored according to the dolphin-like model20. The TNP-ATP and amino-acid residues are depicted by stick models. The Mg²⁺ ion is depicted by a green sphere. Dotted black lines indicate hydrogen bonds (<3.3 Å).
Next, in the TNP-ATP-bound ckP2X7 structure, the phosphate groups of TNP-ATP adopt a totally extended conformation, whereas the phosphate groups of ATP in the previous structures form a bent, “U-shaped” conformation (Fig. 3a–d; Supplementary Fig. 6). This extended conformation of the phosphate groups in the TNP-ATP-bound ckP2X7 structure enables the formation of two hydrogen bonding interactions with the side chains of 
ckLys236 from the right flipper domain and 
ckLys298 from the upper body domain. The interactions between the right flipper domain and the phosphate groups are not visible in the previously determined ATP-bound P2X structures (Supplementary Fig. 6a–c). In contrast, the phosphate groups of TNP-ATP in the TNP-ATP-bound hP2X3 structure are partially bent in a “Y-shaped” conformation (Fig. 3e–h; Supplementary Fig. 6).

Finally, the ribose group of TNP-ATP in the TNP-ATP-bound ckP2X7 structure adopts a similar orientation to that in the ATP-bound structures, with the C2’ and C3’ atoms of the ribose group facing toward the head domain. This orientation of the ribose group and the following uracil is stabilized by weak interactions with the left flipper domain at the 
ckTyr274 residue, in a similar manner to that observed in the ATP-bound hP2X3 structure (Fig. 3a–d; Supplementary Fig. 6c, d). However, the ribose group of TNP-ATP in the TNP-ATP-bound hP2X3 structure is rotated by ~180°, and thus the C2’ and C3’ atoms of the ribose group face toward the left flipper region (Fig. 3e–h; Supplementary Fig. 6). The different orientation of the ribose group also changes that of the trinitrophenyl group: the trinitrophenyl group in the TNP-ATP-bound ckP2X7 structure contacts the head and dorsal fin domains, forming three hydrogen bonds with the side chains of 
ckLys66, 
ckThr112, and 
ckThr202 (Fig. 3a–d). Consistently, previous electrophysiological analyses of the P2X1 and P2X4 receptors indicated that the head and dorsal fin domains are associated with the TNP-ATP binding 
34, 35. In contrast, the trinitrophenyl group in the TNP-ATP-bound hP2X3 structure is buried in the hydrophobic region between the lower body domain and the mostly disordered left flipper region, and thus there is no hydrophilic interaction between the receptor and the trinitrophenyl group, despite its high hydrophilicity (Fig. 3e–h; Supplementary Fig. 6).

**TNP-ATP recognition.** In the TNP-ATP-bound ckP2X7 structure, the TNP-ATP molecule is located in the ATP binding pocket, and thus interacts with the head, upper body, right flipper, and left flipper domains from one subunit, and the lower body and dorsal fin domains from the neighboring subunit (Fig. 2b). Unexpectedly, the structure revealed that the binding mode of TNP-ATP is quite different from that in the recently reported TNP-ATP-bound hP2X3 structure (Fig. 3) 
23. First, the adenine ring of TNP-ATP adopts a similar orientation to that observed in the previously reported ATP-bound P2X structures 
21–23, and thus forms hydrogen bonds with the side chains of the highly conserved 
ckThr177 and the main chain carbonyl groups of 
ckThr64 and 
ckThr177 (Fig. 3a–d; Supplementary Figs. 2, 6). Additional hydrogen bonds are formed with the side chain of 
ckThr64 (Fig. 3a–d; Supplementary Figs. 2, 6). In contrast, in the TNP-ATP-bound hP2X3 structure, the adenine ring of TNP-ATP adopts a distinct orientation from those observed in the ATP-bound structures (Fig. 3e–h; Supplementary Figs. 2, 6). It interacts with the side chain of 
ckThr177 (ckThr177) and the main chain carbonyl group of 
ckLys63 (ckThr64), but not with the main chain carbonyl group of 
ckThr172 (ckThr177) (Fig. 3e–h; Supplementary Figs. 2, 6), due to the different orientation of the adenine ring.

**Putative antagonistic mechanism.** To gain insights into how TNP-ATP works at the receptor, we first compared the TNP-ATP-bound ckP2X7 structure with the apo, closed, and ATP-bound, open states of previously solved P2X receptor structures (Fig. 4; Supplementary Fig. 7). The structural comparisons of the P2X1 and P2X4 receptors indicated that the head and dorsal fin domains are associated with the TNP-ATP binding 
34, 35. In contrast, the trinitrophenyl group in the TNP-ATP-bound hP2X3 structure is buried in the hydrophobic region between the lower body domain and the mostly disordered left flipper region, and thus there is no hydrophilic interaction between the receptor and the trinitrophenyl group, despite its high hydrophilicity (Fig. 3e–h; Supplementary Fig. 6).
Effects of the trinitrophenyl group of TNP-ATP upon binding to P2X receptors. a, b Representative P2X7 structures during the 200 ns MD simulations in the presence of ATP (a) and in the absence of ligand (b) at the ATP binding pockets. The pink arrows are vector representations of the motions of the extracellular domains in the principal component analysis (PCA) of the ckP2X7cryt.

Discussion

In this work, we report the crystal structure of the chicken P2X7 receptor (ckP2X7) in complex with TNP-ATP, providing clarification of the TNP-ATP recognition by the P2X7 receptor, as well as precise structural interpretations of P2X7 mutations and SNPs (Figs. 2 and 3; Supplementary Fig. 4). The comparison with the previously determined P2X structures revealed that the ckP2X7 structure represents the “incompletely activated” conformation. By removing the trinitrophenyl group of TNP-ATP and all-atoms of TNP-ATP, we created the “TNP-ATP-bound form” and “Apo form” ckP2X7 structures, respectively. These two structures were embedded into the lipid bilayer. After performing 200 ns MD simulations, in the ATP-bound form ckP2X7 structure, the head domain underwent a significant downward movement toward the ATP binding pocket, while the other domains, including the dorsal fin domain involved in the adenine ring and ribose ring recognitions through hydrophobic interactions upon ATP binding, did not undergo any movements (Fig. 5a; Supplementary Fig. 6a–c). In contrast, in the apo form ckP2X7 structure, no specific structural rearrangement was observed (Fig. 5b). These results indicate that the trinitrophenyl group prevents the proper cleft closure motion for channel opening, especially by inhibiting the proper downward movement of the head domain. Consistently, the previous electrophysiological analyses showed that ATP binding and the following downward movement of the head domain toward the ATP binding pocket are crucial for the channel activation. Taking together, we propose that TNP-ATP binding may induce structural changes that are similar to those associated with ATP binding, and that the trinitrophenyl group of TNP-ATP may act as a wedge to inhibit the channel activation, by preventing the downward movement of the head domain upon the cleft closure motion of the ATP binding pocket (Fig. 6).
receptors, in which the head domain is involved in the TNP-ATP recognition, and the NMR analysis of the P2X4 receptor, in which TNP-ATP binding induces the expansion of the extracellular domain, in a similar manner to that observed with ATP-dependent activation. In contrast, in the structure of hP2X3 soaked with TNP-ATP, the overall conformation is essentially identical to the apo, closed state of hP2X3 (Fig. 3e–h; Supplementary Fig. 6c)23, and the trinitrophenyl group of diseases. Furthermore, the P2X7 receptor by the competitive antagonist, but also will differences. Investigations will be required to fully understand these structural differences. Intriguingly, although the left flipper domain in hP2X3 faces toward the trinitrophenyl group of TNP-ATP and is close enough to interact with it, this domain is mostly disordered in the TNP-ATP-bound structure of hP2X3. Overall, these striking structural differences between the P2X7 and P2X3 receptors might arise from the differences in the subtypes or potentially from the ligand soaking employed for the determination of the TNP-ATP-bound hP2X3 structure, whereas we employed a co-crystallization method for the structure determination of the TNP-ATP-bound ckP2X7. Further functional and structural investigations will be required to fully understand these structural differences.

In summary, our TNP-ATP-bound ckP2X7 structure not only provides insights into the antagonistic mechanism of the P2X7 receptor by the competitive antagonist, but also will facilitate structure-based drug design targeting this important ion channel family associated with various immune system diseases.

**Methods**

**Protein expression and purification.** The chicken P2X7 crystallization construct (ckP2X7cryst) was prepared by using the synthesized ckP2X7 WT (NCBI Accession Number: XP_001235163) sequence (GenScript) and subcloned into the pEG BacMam vector, with an N-terminal GFP-His8 tag, a tobacco etch virus (TEV) cleavage site and following the GS9S linker (Supplementary Table 1), and was expressed in HEK293S GnTI+ (N-acetylgalactosaminyl-transferease 1 Negative) cells (ATCC, cat. no. CRL-3022)38. Cells were collected by centrifugation (5000 × g, 10 min, 4 °C) and broken by sonication in buffer (50 mM Tris, pH 8.0, 150 mM NaCl) supplemented with 5.2 μg/ml 1 aprotinin, 2 μg/ml leupeptin, and 1.4 μg/ml-1 pepstatin A (all from Calbiochem). Cell debris was removed by centrifugation (10,000 × g, 10 min, 4 °C). Membrane was collected by ultracentrifugation (138,000 × g, 1 h, 4 °C). The membrane fraction was solubilized for 1 h at 4 °C in buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 2 mM CaCl2, 10% glycerol, 2% n-dodecyl-β-maltoside (DDM) (Calbiochem), and 0.2 Unit/l Aspiane (New Zealand Biolabs)). Insoluble materials were removed by ultracentrifugation (138,000 × g, 1 h, 4 °C), and the supernatant was incubated with Talon metal affinity resin (Clontech), washed with 20 mM imidazole and eluted with 250 mM imidazole. After TEV protease digestion and Endo H treatment, the fraction was purified by size-exclusion chromatography on a Superdex 200 Increase 10/300 GL column (GE Healthcare), equilibrated with SEC buffer (20 mM HEPES, pH 7.0, 100 mM NaCl, 10% glycerol, 0.05% DDM). The peak fractions of the protein were collected and concentrated to 3 mg/ml-1 using a centrifugal filter unit (Merck Millipore, 30 kDa molecular weight cutoff).

**Crystallization and data collection.** Before crystallization, 0.1 mM TNP-ATP (TOCRIS) was added to the protein solutions. The crystals were grown at 4 °C by sitting drop vapor diffusion method in a 96-well plate. The crystals of ckP2X7cryst appeared within 3 days under the following conditions (0.5 M sodium sulfate, 0.05 M lithium chloride, 0.05 M Tris, pH 8.0, 32% PEG400). After cryoprotection in reservoir solution supplemented with 25% glycerol, and flash-freezing in liquid nitrogen, the crystals were used for X-ray diffraction experiments. The X-ray diffraction data were collected at the Swiss Light Source (SLS) beamline X06SA-PXI, processed using DIALS39, and scaled using AIMLESS40.

**Structure determination.** The TNP-ATP-bound ckP2X7cryst structure was obtained by molecular replacement with MOLREP41, using one subunit of the ATP-bound ampP2X2 structure from the Gulf Coast tick (PDB: 5FIC) as the template. The structure thus obtained was further refined by using REFMACS42, PHENIX43, and COOT44, with three-fold non-crystallographic symmetry and secondary structure restraints. Data collection and refinement statistics are summarized in Table 1.

**Electrophysiology.** ATP and TNP-ATP were purchased from Sigma (St. Louis, MO, USA). The ckP2X7 WT and ckP2X7cryst constructs were subcloned into the BacMam vector, with a N-terminal GFP-His8 tag, and transfected into an insect cell line (Sf9 Express, Hilymax (Dojindo Laboratories, Kumamoto, Japan)). Both constructs were expressed in HEK-293 cells, cultured in DMEM medium at 37 °C in a humidified atmosphere of 5% CO2 and 95% air. Electrophysiological measurements were performed on HEK-293 cells, 24–48 h after transfection. The conventional whole-cell configuration under the voltage clamp at room temperature (23 ± 2 °C) was used to obtain the electrophysiological recordings.45, 46. Patch pipettes were pulled from glass capillaries, using the two-stage puller PP-830 (Narishige Co., Ltd.), and the resistance between the recording electrode filled with pipette solution and the recording electrode in bath solution ranged from 3 to 5 MΩ. Membrane currents were filtered at 2 kHz using a low-pass Bessel filter, and measured with an Axon 200B patch clamp amplifier (Molecular Devices). All currents were sampled and analyzed in the Digidata 1440 interface, using Clampex and the Clampfit 10.0 software (Molecular Devices). Cells were incubated in bath solution, containing 150 mM NaCl, 5 mM KCl, 10 mM glucose, 10 mM HEPES, 2 mM CaCl2, and 1 mM MgCl2, at the conditional neutral pH of 7.35–7.40. Patch electrodes were filled with a standard internal solution, containing 30 mM NaCl, 120 mM KCl, 1 mM MgCl2, 0.1 mM CaCl2, and 5 mM EGTA, at the conditional neutral pH of 7.35–7.40. During electrophysiological recordings, 80–90% of the series resistance was compensated and the membrane potential was held at ~60 mV throughout the entire ATP solutions were Microsurgically harvested, homogenized in 100 mM NaCl, pH 32, and the corresponding currents were normalized to the maximum amplitude of ATP (20 μM in Ca2+-free solution containing 5 mM EGTA), and ATP-gated currents were recorded after the regular 40–50 s ATP application every 8 min.

**Fluorescence measurement.** The fluorescence measurement using TNP-ATP as an indicator was performed based on the previous experiment. The purified ckP2X7cryst protein and the SEC buffer mixed with each concentration of TNP-ATP were incubated at 4 °C for over 1 h, respectively. The mixed solutions were dispensed in a 96-well flat-bottom plate, in a 6 μl sample volume. Fluorescence measurement was performed using a 2030 ARVO X3 Multilabel Reader (PerkinElmer), equipped with 405 nm emission and 535 nm excitation filters.

**Molecular dynamics simulations.** The simulation system was comprised of the ckP2X7cryst trimer, 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), ATP, water molecules, and 150 mM NaCl. The missing atoms, such as hydrogens in the protein, were added with the programs GROMACS 5.1.447 and VMD 1.9.348. The disordered side chains in the protein were modeled by COOT44. The periodic boundary conditions, including the explicit solvent and the 20 Å lipid bilayer, was prepared. The protein was embedded into the POPC lipid bilayer using the MemProMID pipeline49. The final size of the simulation box was 119 × 119 × 144 Å. The net charge of the system was neutralized by adding chloride and sodium ions. The topologies and force field parameters from GROMACS50 were used. MD simulations were performed using the program GROMACS 5.1.47. The system was first minimized using the steepest descent with a cutoff of 100 kJ mol−1 nm−1, with 1000 kJ mol−1 nm−2 harmonic position restraints for non-hydrogen atoms of the protein and ATP. Next, we performed an equilibration for 100 ps in the NVT ensemble (310 K, 1 atm), followed by an equilibration for 6 ns in the NPT ensemble (310 K, 1 atm), with the same restraints. Finally, we performed the production runs in the NPT ensemble for 200 ns, with the following bond length restraints between the atoms of ATP and the protein side chains: (1) ATP N1 and Thr177 OGI, (2) ATP N6 and Thr177 OGI, (3) ATP N6 and Thr64 O, and (4) ATP N7 and Thr64 O. These restrained bond configurations under the voltage clamp at room temperature (23 ± 2 °C) were used for bond constraint51. Principle component analysis was conducted by in-house scripts using Python 2.7.9 and MDAnalysis 0.15.052.

**Data availability.** Atomic coordinates and structure factors for the TNP-ATP-bound ckP2X7cryst structure have been deposited in the Protein Data Bank (http://www.pdb.org), under the accession code 5XW6. Other supporting data are available from the corresponding author on request.}

Received: 12 December 2016 Accepted: 2 August 2017

Published online: 12 October 2017
References

1. Burnstock, G. Purinergic nerves. Pharmacol. Rev. 3, 509–581 (1972).
2. Brune, K., Wagenbach, M. & Julius, D. New structural motif for ligand-gated ion channels defined by an ionotropic ATP receptor. Nature 371, 519–523 (1994).
3. Valera, S. et al. A new class of ligand-gated ion channel defined by P2X receptor for extracellular ATP. Nature 371, 516–519 (1994).
4. Chen, C., Akopian, A. & Sivilotti, L. A P2X purinoceptor expressed by a subset of sensory neurons. Nature 377, 428–431 (1995).
5. North, R. A. Molecular physiology of P2X receptors. Physiol. Rev. 82, 1013–1067 (2002).
6. Surprenant, A. & North, R. A. Signaling at purinergic P2X receptors. Annu. Rev. Physiol. 71, 333–359 (2009).
7. Kawate, T., Michel, J. C., Birdsong, W. T. & Gouaux, E. Crystal structure of the P2X7 receptor. Science 272, 735–738 (1996).
8. McQuillin, A. et al. Case-control studies show that a non-conservative amino-acid change from a glutamine to arginine in the P2RX7 purinergic receptor associates with clinical parameters. patients with papillary thyroid cancer: correlation with histological variants and disease. Recent Pat. DNA Gene Seq. 94, 695–698 (2009).
9. North, R. A. Molecular physiology of P2X receptors. Pharmacol. Rev. 66, 638–685 (2014).
10. Sluyter, S. D., Debetto, P. & Giusti, P. P2X7 receptors in neurological disorders and cardiovascular disorders. Cardiovasc. Psychiatry Neurol. 2009, 861324 (2009).
11. Burnstock, G., Nistri, A., Khakh, B. S. & Giniatullin, R. ATP-gated P2X receptors. J. Biol. Chem. 272, 372–346 (1997).
12. Chen, C., Akopian, A. & Sivilotti, L. A P2X purinoceptor expressed by a subset of sensory neurons. Nature 377, 428–431 (1995).
13. Skaper, S. D., Debetto, P. & Giusti, P. P2X7 receptors in neurological disorders and cardiovascular disorders. Cardiovasc. Psychiatry Neurol. 2009, 861324 (2009).
14. Skaper, S. D., Debetto, P. & Giusti, P. P2X7 receptors in neurological disorders and cardiovascular disorders. Cardiovasc. Psychiatry Neurol. 2009, 861324 (2009).
15. Bartlett, R., Stokes, L. & Sluyter, R. The P2X7 receptor channel: recent developments and the use of P2X7 antagonists in models of disease. Pharmacol. Rev. 53, 474–681 (2006).
16. Sluyter, S. D. & Stokes, L. Significance of P2X7 receptor variants to human health and disease. Recent Pat. DNA Gene Seq. 5, 41–54 (2011).
17. Mehta, N. et al. Purinergic receptor P2X7: a novel target for anti-inflammatory therapy. Bioorg. Med. Chem. 22, 54–88 (2014).
18. Skaper, S. D., Debetto, P. & Giusti, P. P2X7 receptors in neurological disorders and cardiovascular disorders. Cardiovasc. Psychiatry Neurol. 2009, 861324 (2009).
19. Roger, S. et al. Single nucleotide polymorphisms that were identified in affected mood disorders affect ATP-activated P2X7 receptor function. J. Psychiatr. Res. 44, 347–355 (2010).
20. Kawate, T., Michel, J. C., Birdsong, W. T. & Gouaux, E. Crystal structure of the ATP-gated P2X(4) ion channel in the closed state. Nature 460, 592–598 (2009).
21. Hattori, M. & Gouaux, E. Molecular mechanism of ATP binding and ion channel activation in P2X receptors. Nature 485, 207–212 (2012).
22. Kasuya, G. et al. Structural insights into divalent cation modulations of ATP-Gated P2X receptor channels. Cell Rep. 14, 932–944 (2016).
23. Manourou, S. E. et al. X-ray structures define human P2X3 receptor gating cycle and antagonist action. Nature 538, 66–71 (2016).
24. Karasawa, A. & Kawate, T. Structural basis for subtype-specific inhibition of the P2X7 receptor. Elife 5, e22153 (2016).
25. Virginio, C., Robertson, G., Surprenant, A. & North, R. A. Trinitrophenyl-substituted nucleotides are potent antagonists selective for P2X1, P2X3, and heteromeric P2X2/3 receptors. Mol. Pharmacol. 53, 969–973 (1998).
26. Lewis, C. J., Surprenant, A. & Evans, R. J. 2′,3′-O-(2,4,6- trinitrophenyl) adenosine 5′-triphosphate (TNP-ATP)-a nanomolar affinity antagonist at rat mesenteric artery P2X receptor ion channels. Br. J. Pharmacol. 124, 1463–1466 (1998).
27. Minato, Y. et al. Conductance of P2X4 purinergic receptor is determined by conformational equilibrium in the transmembrane region. Proc. Natl Acad. Sci. USA 113, 4741–4746 (2016).
28. Kawate, T. & Gouaux, E. Fluorescence-detection size-exclusion chromatography for precrystallization screening of integral membrane proteins. Structure 14, 673–681 (2006).
29. Khakh, B. S. & North, R. A. Neurromodulation by extracellular ATP and P2X receptors in the CNS. Neuron 76, 51–69 (2012).
30. Kasuya, G. et al. Structural insights into the nucleotide base specificity of P2X7 receptors. Sci. Rep. 7, 45208 (2017).
31. Samways, D. S. K., Li, Z. & Egan, T. M. Principles and properties of ion flow in P2X receptors. Cell. Physiol. Biochem. 8, 6 (2014).
32. Jindrichova, M. et al. Functional characterization of mutants in the transmembrane domains of the rat P2X7 receptor that regulate pore conductivity and agonist sensitivity. J. Neurochem. 133, 815–827 (2015).
the protein, and determined the structure with assistance from T.N., R.I. and M.H. R.N., M.T. and R.I. performed the molecular dynamics simulation. G.K., M.H. and O.N. wrote the manuscript. O.M., M.H. and O.N. supervised all of the research.

Additional information
Supplementary Information accompanies this paper at doi:10.1038/s41467-017-00887-9.

Competing interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.