Intersubunit physical couplings fostered by the left flipper domain facilitate channel opening of P2X4 receptors

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P2X receptors are ATP-gated trimeric channels with important roles in diverse pathophysiological functions. A detailed understanding of the mechanism underlying the gating process of these receptors is thus fundamentally important and may open new therapeutic avenues. The left flipper (LF) domain of the P2X receptors is a flexible loop structure, and its coordinated motions together with the dorsal fin (DF) domain are crucial for the channel gating of the P2X receptors. However, the mechanism underlying the crucial role of the LF domain in the channel gating remains obscure. Here, we propose that the ATP-induced allosteric changes of the LF domain enable it to foster intersubunit physical couplings among the DF and two lower body domains, which are pivotal for the channel gating of P2X4 receptors. Metadynamics analysis indicated that these newly established intersubunit couplings correlate well with the ATP-bound open state of the receptors. Moreover, weakening or strengthening these physical interactions with engineered intersubunit metal bridges remarkably decreased or increased the open probability of the receptors, respectively. Further disulfide cross-linking and covalent modification confirmed that the intersubunit physical couplings among the DF and two lower body domains fostered by the LF domain at the open state act as an integrated structural element that is stringently required for the channel gating of P2X4 receptors. Our observations provide new mechanistic insights into P2X receptor activation and will stimulate development of new allosteric modulators of P2X receptors.

P2X receptors are trimeric membrane ion channels (1, 2) activated by extracellular ATP (3). So far, seven P2X subtypes (P2X1–P2X7) have been identified, which are expressed virtually in almost all mammalian tissues, including nervous, immune, and cardiovascular systems (3–5). P2X receptors possess a molecular architecture distinct from other ion channel protein families (4, 6, 7) and are implicated in a wide range of physiological and pathological processes (2, 4, 5, 8), such as neuroinflammation, synaptic transmission, primary afferent signaling, chronic pain, central control of respiration, vascular remodeling, and the regulation of blood pressure. Accordingly, P2X receptors hold great interest as new therapeutic targets for inflammation and cardiovascular and neurological diseases (8–14). For this purpose, it is essential to fully understand the detailed gating mechanism of P2X receptors at the atomic level (9, 15, 16).

High resolution X-ray structures at apo/closed and ATP-bound open states are available for zebrafish P2X4 (zfP2X4) (7, 9), human P2X3 (hP2X3) (17), Amblyommia maculatum P2X (AmP2X) (18), and panda P2X7 (19) receptors, which greatly aid our understanding of the working principles of those unique receptors, such as the ligand recognition, pore architecture, and conformational changes associated with the channel activation. Based on these structures, optical control of P2X receptors independent of natural stimulus has been achieved via powerful optogating approaches (20–22). The structural comparison of the closed and open structures suggests a possible gating mechanism of P2X receptors (Fig. 1, A and B) (9, 16, 23, 24). First, at the ATP-binding site, ATP promotes the jaw closure between the head and dorsal fin (DF)3 domains, making the DF domain move upward to the head domain to accommodate ATP. Meanwhile, the bound ATP pushes the left flipper (LF) domain out of the ATP-binding site. Second, because both the LF and DF domains are structurally coupled with the lower body domain, the movements of those two domains lead to a concomitant outward flexing of lower body domains in the open state, which markedly expands the central vestibule (Fig. 1B). Finally, lower body domains are directly coupled with two transmembrane (TM) domains TM1 and TM2, and therefore their outward flexing can directly promote the opening of ion

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3 The abbreviations used are: DF, dorsal fin; LF, left flipper; TM, transmembrane; PDB, Protein Data Bank; IVM, ivermectin; pF, picofarad; NPM, N-phenylmaleimide; MTSEA, 2-aminoethyl methanethiosulfonate; MTSES, 2-sulfonatoethyl methanethiosulfonate; DTNB, 5′-dithiobis(2-nitrobenzoic acid); ANOVA, analysis of variance; MD, molecular dynamics; CV, collective variable; pS, picosiemens; β-ME, β-mercaptoethanol; CHX, cycloheximide.
Physical couplings fostered by left flipper domain

Figure 1. Bound ATP-evoked allosteric changes associated with channel opening of P2X4 receptors. A, allosteric changes essential for the channel activation of P2X4 receptors. The white dotted lines denote the outward flexing of two lower body domains and the concomitant expansion of the central vestibule of P2X4 receptors. The gray and red arrows indicate the conformational changes after ATP binding and the cation-permeating pathway, respectively. B, superposition of resting (blue) and open (red) conformations of P2X4 receptor and zoom-in view of the expansion of central vestibule. The gray arrows indicate the movements of the DF, LF, and lower body domains associated with the expansion of central vestibule.

The proposed gating mechanism based on the open and closed X-ray structures highlights that the repelling action of ATP on the LF domain would favor the outward flexing of lower body domains and concomitant pore dilation (9). The reason why the motion of the LF domain favors this outward flexing of the lower body domains is yet unknown. Recently, it has been demonstrated that ATP binding-induced alteration in interdomain hydrophobic interactions and the concomitant relative motions between the LF and DF domains are indispensable allosteric events for channel activation of P2X4 receptors (32), although the underlying mechanism of this process remains undetermined. One possibility is that the hydrophobic interactions between the LF and DF domains at the resting state build up an energy barrier that prevents the activation of P2X receptors (32). The expelling of the LF domain from the ATP-binding pocket might help to overcome this energy barrier and favor the channel activation of P2X4. If so, bound ATP would finally soothe this loop structure to reduce the energy barrier during the channel opening. However, more inter- and intrasubunit contacts among the LF, DF, and lower domains were established after bound ATP pushed the LF domain out of the ATP-binding pocket (details see below and Ref. 9), implying a less flexible LF domain at the open state was developed after the repulsion. Thus, previous studies and structural models (9, 32) only partially elucidate the function of the LF domain during channel gating. What exactly is the reason behind the bound ATP-induced repulsion of this flexible loop out of the ATP-binding pocket? Is it a passive allosteric change for only adapting the LF domain to accommodate the allostery of other domains or a more important allostery making a major contribution to couple the ATP binding to the final pore opening? Getting a clear understating of this allostery will provide new mechanical insights into the gating process of P2X4 receptors.

Using multidisciplinary approaches, we proposed that ATP-bound induced conformational changes of the LF domain enable it to establish inter-subunit physical couplings among the DF and two lower body domains, which are essential for the channel opening of P2X4 receptors. This observation will enrich our understandings of the role of the LF domain in channel gating and provide new mechanistic insights into the channel activation of P2X receptors.

Results

Inter-subunit physical contacts among the lower body and DF domains established by the LF domain are essential for the channel function of P2X4 receptors

Revealed by the homology models of rat P2X4 (rP2X4) receptors (32, 37) built from zfP2X4 X-ray structures of the apo and open states (9), the LF domain is a loop structure surrounded by the head, DF, right flipper, and lower body domains (Fig. 2A). Its N and C termini are covalently coupled with the β12 and β13 sheets of the lower body domain (Fig. 2B and C), respectively. Alanine-scanning mutagenesis of all residues of the LF domain (Fig. 24, D and E), ranging from Arg-277 to Tyr-292 of rP2X4, indicated that the residues in the N and C termini rather than
those in the middle region, including Arg-278, Leu-279, Asp-280, Arg-282, and Pro-290 (Fig. 2, D and E), are essential for the channel function of P2X4 receptors. Mutations on identical residues in zfP2X4 (Arg-281, Asp-283, and Lys-285, Fig. 3A) significantly reduced ATP (1 mM)-induced currents (Fig. 3, B and C), indicating a crucial role of these residues in both rP2X4 and zfP2X4.

A comparison of the LF domain at the open and closed states based on the homology models of rP2X4 revealed that all mutants impairing the maximal current amplitude virtually contribute to the newly established intersubunit physical couplings among the two lower body and DF domains after the LF domain was repelled out of the ATP-binding site (Fig. 2, B and C). Arg-278 and Asp-280 foster an intrasubunit salt bridge in both the closed and open structures of P2X4, whereas an additional hydrogen bond (H-bond) was formed between the side chain of Arg-282 and the oxygen atom of the main chain of Arg-278 at the open state (Fig. 2B, lower panel). ATP binding also contributes to the formation of the H-bond between the side chain of Asn-192 (located in the lower body domain of another subunit) and the main chain atom of Arg-282 (Fig. 2B, lower panel). N192A mutant partially reduced the maximal current amplitude of P2X4 (Fig. 2, D and E). Additionally, the atoms of the main chain of Val-288, Ser-289, and Pro-290 developed new contacts with the side chain of Arg-203 (in the lower body domain of another subunit) (Fig. 2C, lower panel).

Alanine substitution of Arg-203 significantly impaired channel activation of rP2X4 receptors (Fig. 2, D and E). Mutations on the identical residue (Arg-206) in zfP2X4 significantly reduced ATP (1 mM)-induced currents (Fig. 3B and C), suggesting this newly established contact is also crucial for the channel function of zfP2X4 receptors. The intersubunit hydrophobic contacts among Val-288, Leu-214, and Ile-205 were significantly changed after ATP binding (Fig. 2C) (32). Mutations on Val-288, Leu-214, Ile-205, and the identical residues of zfP2X4 significantly reduced maximal current densities of both rP2X4 and zfP2X4 receptors (32). In contrast, although Glu-245 forms intersubunit contact with Arg-282 via H-bonds to tighten the LF domain at the resting state (Fig. 2B, upper panel), E245A and E245R had no effect on the maximal current amplitude and the EC50 of ATP (the concentration of ATP yielding current that is half of the maximum) of rP2X4 (see below), indicating that this contact is redundant for the channel function. Thus, ATP binding-induced conformational changes of the LF domain and the following intersubunit physical couplings among the DF and two lower body domains fostered by the deformed LF domain at the open state are pivotal for the channel function of both rP2X4 and zfP2X4 receptors.

Because of the low potency of ATP on the zfP2X4 receptor expressing in mammalian cells (7, 32, 37), the following mutagenesis, protein-expression measurements, and electrophysiological recordings were carried out on rP2X4 receptors.
Additionally, although the essential role of the residues involved in forming intersubunit physical couplings after ATP binding was conserved between rP2X4 and zfP2X4 receptors (Fig. 3, A and D) (32), some residues of the middle region of the LF domain are not exactly the same (Fig. 3D). Thus, to get a more appropriate prediction about the functional studies of the rP2X4 receptors, the following free-energy profile reconstructions and MD simulations were also based on the homology models of rP2X4 rather than on the crystal structures of the zP2X4 receptor.

**Impaired intersubunit physical couplings significantly influence channel gating rather than channel assembly, protein stability, and ATP-EC50 of P2X4 receptors**

Multiple factors can influence the maximal current densities of P2X receptors. The fact that the surface expression levels of these loss-of-function mutants exhibited no pronounced changes when compared with wild-type (WT) rP2X4 (Fig. 4, A and B) suggests that the decreased ATP currents were not acting on channel expression and trafficking, except for R278A and D280A. The complete abolishment of the ATP-induced currents in rP2X4R278A and rP2X4D280A (Fig. 2, D and E) may partially attribute to a decreased surface expression of those two mutants. Another possibility is that the LF domain and the established intersubunit physical couplings may structurally support the trimeric assembly or prevent the neighboring subunit from clashing with each other upon ATP binding. We introduced additional mutations, R203A and R282A, into mutant V288C/T211C, which could form trimeric receptors in non-reduced SDS-PAGE, due to the formation of intersubunit disulfide bonds as we previously demonstrated (32). Trimeric bands of mutants, V288C/T211C/R203A and V288C/T211C/R282A, were observed in the non-reducing Western blotting (Fig. 4C), indicating that the impaired intersubunit physical couplings did not render a significant deficiency in the channel assembly of P2X4 receptors. Additionally, we incubated the transfected HEK-293 cells with cycloheximide (CHX, 20 μg/ml), an inhibitor of protein biosynthesis (41), in time-scale experiments. In contrast to the profound reduction in maximum currents, after incubation with CHX for 10 h, the protein level of the mutants made no significant changes (Fig. 4, D and E), indicating that alterations in those positions did not render channel instability of P2X4 receptors. Moreover, in contrast to the profound reduction in maximum currents (Fig. 4, F and G), little to no change was observed in the surface expression (Fig. 4, H and I) and EC50 values for the functional mutants, namely R203K (EC50 = 1.43 ± 1.1 μM) and R282W (1.44 ± 1.2 μM), compared with WT channels (EC50 = 1.96 ± 1.2 μM) (Fig. 4J). Thus, the decreased maximal current densities in those mutants were not related to surface protein expression, channel assembly, protein stability, or EC50 of ATP.

To gain more structural information about the role of those newly established intersubunit physical couplings in the channel function of P2X4 receptors, the free-energy profiles for those interactions were reconstructed by metadynamics (42–44), “a powerful algorithm that can be used for both reconstructing the free energy and accelerating rare events in systems described by complex Hamiltonians” (45). The N-O distances of Arg-282…Glu-245 and Arg-203…Val-288 are distinct due to the high energy barrier existing in the passing path (Fig. 5A). After ATP binding, Arg-282 moved away from the Glu-245 (N-O distances of 4.6 and 14.9 Å, at the resting and open states, respectively), whereas Arg-203 moved closer to Val-288 (N-O distances of 11.4 and 3.1 Å, at the resting and open states, respectively), indicating new intersubunit contacts among these residues were established. These two distance measurements were defined as collective variables (CVs) of metadynamics (Fig. 5A). The lower free-energy paths (gray line) plotted onto free energy profiles passing those two CVs from open to resting states were carried out under the conditions with (Fig. 5B) and without (Fig. 5C) bound ATP to figure out the correlation between these allosteric changes and the ATP-bound open state. The bound ATP made this passing difficult due to the high energy barrier existing in the passing path (Fig. 5B), indicating that the existence of both the bound-ATP and new intersubunit contacts had “locked” the P2X4 receptor at the open state. In contrast, when the bound ATP was taken away from the ATP-binding site (Fig. 5C), this passing became spontaneous because free-energy profiles revealed a more sta-
ble CV similar to that of the resting state (CVR) than that of the open state (CVO), indicating that the LF domain-mediated establishment of intersubunit physical couplings is an allosteric change correlated well with the ATP-bound open state. Because these mutations had little to no changes in the EC50 of ATP, the protein expression and channel assembly, despite profound reduction in maximum currents (Fig. 4), they may impair the channel gating of P2X4 receptors.

To test this hypothesis, we performed single channel recordings in the outside-out configuration. The control experiment showed that the main conductance state of channels opened by saturated ATP (100 μM) in patches excised from HEK-293 cells expressing WT rP2X4 (Fig. 5D) was similar to that shown in previous reports (46–48). The channel with a unitary conductance of ~10 pS at 120 mV was observed in the most of the patches. Channels opened and closed frequently and were highly flickering, and thus the precise determination of the open and shut time could not be made. Additionally, unitary currents could only be observed at the first 5 s and disappeared after ~10 s (Fig. 5D). By contrast, there were no or only few channel openings resembling unitary rP2X4 current in rP2X4R203A (Fig. 5E) and rP2X4R282A (Fig. 5F) in response to saturated ATP (100 μM). When ATP (100 μM) was co-applied with ivermectin (IVM) (3 μM), a widely used P2X4 enhancer (48–50), unitary

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**Figure 4. Effects of mutants on the channel functions of rP2X4 receptors.** A and B, representative Western blotting (A) and mean values (B) of the membrane expression of P2X4 with alanine replacements transfected in HEK-293 cells. At least three experiments were performed for each mutant: *, p < 0.05; **, p < 0.01 versus WT, one-way ANOVA with Bonferroni post hoc test. C, protein samples extracted from transfected HEK-293 cells separated by non-reducing Western blotting. Monomeric, dimeric, and trimeric receptors are indicated by triangle arrows on the left. Molecular mass markers are shown on the right. Similar results were observed in at least three independent experiments. D and E, representative Western blotting (D) and mean values (E) of rP2X4 protein for WT, R203A, R278A, D280A, and R282A. Cells were treated with 20 μg/ml CHX in a time-course experiment as indicated. The results were observed in at least three independent experiments for statistical analysis. F and G, representative traces (F) and mean values (G) of the responses of WT rP2X4 and various mutants to ATP (100 μM, mean ± S.E., n = 3–7). *, p < 0.05; **, p < 0.01 versus WT (dashed line), one-way ANOVA with Bonferroni post hoc test. H and I, representative Western blotting (H) and mean values (I) of the membrane protein expression for WT, R282W, and R203K. At least three experiments were performed for each mutant: *, p < 0.05; **p < 0.01 versus WT, one-way ANOVA with Bonferroni post hoc test. J, effects of mutations on the ATP-EC50 of rP2X4. The solid line is a fit of the Hill equation to the ATP-dependent activation. Each point represents the mean ± S.E. of four measurements.
currents could be observed both in rP2X4R203A and rP2X4R282A (Fig. 5G). Similar to a previous finding (48), IVM exhibited a small effect on the unitary current amplitude (Fig. 5G, H) of rP2X4R203A, rP2X4R282A, and WT and significantly prolonged the open time of channels (Fig. 5G). Perhaps this is because IVM directly acts on the interface between transmembrane domains TM1 and TM2 (50), the only entrance of various ions. The generation of unitary currents of rP2X4R203A and rP2X4R282A in the patches exposed to IVM and ATP further supported the idea that the mutated channels with impaired intersubunit physical couplings behave with normal surface expression, channel assembly, and the EC50 of ATP. It has been well established that IVM is able to significantly increase the channel opening probability (Po) of P2X4 receptors (48). Thus, the absence of unitary P2X4 currents in mutated channels exposed to saturated ATP and the significant channel activity observed in the presence of both ATP and IVM suggested that the impairment in intersubunit physical couplings significantly decreased the open probability of P2X4 receptors.

Engineered intersubunit metal bridges that change the intersubunit physical couplings remarkably influence the channel gating of P2X4 receptors

To further examine this idea, we applied different metal bridges to weaken or strengthen the physical couplings among the DF and two lower body domains fostered by the LF domain at the open state. We have recently shown that an intradomain
Zn\(^{2+}\) bridge in the LF domain produces an unexpected inhibition (32) on the current of mutant channel rP2X4\(^{His-286/V288H}\) without identifying the underlying mechanism. Here, the structural model of rP2X4\(^{His-286/V288H}\) in the open state suggested that Zn\(^{2+}\) might form an intersubunit rather than an intrasubunit Zn\(^{2+}\) bridge with the introduced histidine residue V288H and natural histidine residue His-286 of one subunit, as well as with the main chain oxygen atoms of Pro-207 and Ile-209 of another subunit (Fig. 6A). Indeed, post-application of Zn\(^{2+}\) after ATP markedly reduced the remaining currents of rP2X4\(^{His-286/V288H}\) (ratio = 0.26 ± 0.06, n = 4, green arrow, Fig. 6, B and C). This blockage was specific to the presence of histidine at both 288 and 286 positions because Zn\(^{2+}\) application only caused mild inhibition on the single histidine mutant H286A/V288H as well as WT P2X4 (His-286/Val-288) receptor (Fig. 6C). Introduction of the additional mutation into His-286/V288H (His-286/V288H/P207A and His-286/V288H/I209A) affected both the inhibition (Fig. 6C) and the dose-response curve of Zn\(^{2+}\) (Fig. 6D) of rP2X4 (IC\(_{50}\) the half-inhibition of Zn\(^{2+}\) = 68.4 ± 10, 39.4 ± 2, and 129.8 ± 17 \(\mu\)M, for rP2X4\(^{His-286/V288H}\), rP2X4\(^{His-286/V288H/P207A}\), and rP2X4\(^{His-286/V288H/I209A}\), respectively), indicating that Pro-207 and Ile-209 may contribute to the intersubunit binding of Zn\(^{2+}\) at the open state.

The structural model of rP2X4\(^{V288H/His-286}\) at the open state revealed that the Zn\(^{2+}\) bridge between His-286, V288H, and the oxygen atoms of the main chain of Pro-207 and Ile-209 is capable of trapping the C-terminal of the LF domain into the flexible loop \(\beta9\)-a3 (Fig. 6A, right panel). This idea is further supported by the measurement of the angle between three C atoms of residues His-288, Arg-278, and Ile-205 of P2X4\(^{His-286/V288H}\) but increased in mutant His-286/V288H/I209A when compared with that of WT P2X4 receptor. F, zoom-in view of the constructed zinc-bridge model of P2X4\(^{His-286/V288H}\) based on the open structure showing details of the distances (light-blue dotted line) between Zn\(^{2+}\) and the coordinating NE2, sulfur, and oxygen atoms of His-286, His-288, and Cys-209.
Physical couplings fostered by left flipper domain

Figure 7. Effect of Zn$^{2+}$ on the unitary rP2X4$^{\text{His-286/V288H/I209C}}$ currents. A and B, representative current recordings from excised outside-out membrane expressing single channel (A) or multiple channels (B) exposed to ATP and the following ATP-$Zn^{2+}$ co-application for the mutant His-286/V288H/I209C. Full opening (O) and closing (C) are indicated by black and yellow lines, respectively. Y axis denotes the ratio of the number of events to the number of bins (the bin number is set to 320). Similar results were obtained in at least three other independent recordings.

ranged from 23–25° (Fig. 6E) during MD simulations, a value smaller than that of WT rP2X4 (25–28°) (Fig. 6E), indicating that the C terminus moved downwards and became closer to the flexible loop $\beta 9-\alpha 3$ than WT rP2X4 (Fig. 6A). Because the N terminus of the LF domain was located in the rigid lower body domain (β12) of another subunit (Fig. 6A), the C-terminal movement toward the flexible loop $\beta 9-\alpha 3$ significantly weakened intersubunit physical couplings between the DF and two lower body domains fostered by the deformed LF domain after ATP binding. To prevent this movement, we introduced an additional substitution in loop $\beta 9-\alpha 3$ (His-286/V288H/I209C) to push the C terminus away from the flexible loop $\beta 9-\alpha 3$ through an additional coordination bond between Zn$^{2+}$ and the free thiol group of I209C (Fig. 6F), and to regain contacts between the C terminus and rigid the $\alpha 3$ helix of the DF domain (Fig. 6F). This additional replacement on Ile-209 by cysteine significantly increased the angle formed by Ca atoms of residues His-288, Arg-278, and Ile-205 of rP2X4$^{\text{His-286/V288H/I209C}}$ during MD simulations (Fig. 6, E and F), indicating that the LF domain had escaped from the flexible loop $\beta 9-\alpha 3$ and produced more contacts with the rigid $\alpha 3$ helix of the DF domain (Fig. 6F, right lower panel).

Indeed, the additional application of Zn$^{2+}$ after ATP led to a remarkable potentiation (ratio = 3.55 ± 0.36, n = 12) rather than an inhibition on the remaining current of His-286/V288H/I209C (right trace of Fig. 6, B, green arrow, and C, green column), revealing the pivotal role of intersubunit physical couplings between the DF and two lower body domains established by the LF domain in the open state. This potentiation requires the presence of both histidine residues His-288 and His-286 in the LF domain of one subunit, and the Cys-209 located in the interface between the DF and lower body domains of another subunit, because Zn$^{2+}$ application only slightly inhibited rather than potentiated the currents of H286A/V288H/I209C, His-286/Val-288/I209C, and H286A/Val-288/I209C (Fig. 6C).

The effect of Zn$^{2+}$ on the unitary rP2X4$^{\text{His-286/V288H/I209C}}$ currents was also measured in the outside-out configuration of patch clamp. There were no channel openings resembling unitary rP2X4 currents in the presence of saturated ATP (Fig. 7A), although currents with a unitary conductance of ~10 pS at −120 mV were evoked when ATP and Zn$^{2+}$ were co-applied (Fig. 7A). In additional macroscopic recordings, a few unitary P2X4 currents (~10 pS) were observed when only 100 μM ATP was applied (Fig. 7B); however, a following co-application of ATP and Zn$^{2+}$ evoked a large current (~30 channels simultaneously opening) that rapidly declined to a steady-state level, where individual openings and closings can be measured, indicating that the engineered metal bridge rendered a significant increase in the open probability of rP2X4 receptors but had no remarkable effects on the rP2X4 desensitization. The unitary current conductance was similar before (~10 pS) and after Zn$^{2+}$ treatment (~10 pS, Fig. 7, A and the lower panel of B), suggesting these physical couplings had no effect on the unitary current conductance of rP2X4 receptors. Thus, physical couplings among the DF and two lower body domains fostered by the LF domain increase the open probability rather than change the current unitary conductance and channel desensitization of rP2X4 receptors.

Restraining the LF domain from fostering physical couplings via intersubunit disulfide cross-linking impairs channel activation of P2X4 receptors

To further examine the contribution of the intersubunit physical couplings fostered by the deformed LF domain during the channel gating of rP2X4 receptors, we immobilized the LF domain at the resting state by introducing cysteine residues that form the intersubunit disulfide bridge (Fig. 8A). The $C_{\alpha}-C_{\beta}$ distances of Ser-201–Asp-283 (14.2 Å versus 7.1 Å, resting versus open states) and Ser-201–Leu-284 (19.4 Å versus 7.9 Å, resting versus open states) (Fig. 8, B and C) were longer than that of a disulfide pair (~5 Å) (51). Therefore, the interdomain disulfide bond at those positions could immobilize the LF domain at the resting state and restrain the LF domain from fostering intersubunit physical coupling at the open state (Fig.
The WT rP2X4 and single mutants S201C, D283C, and L284C migrated on SDS-polyacrylamide gels predominantly at a position expected for monomeric form (57 kDa; Fig. 8D). In contrast, no obvious monomeric form was observed for the subunits containing cysteine at both positions (S201C/D283C and S201C/L284C). The observed higher molecular weights presumably represent disulfide bond trimer, because -mercaptoethanol (−ME, 1%) reduced those to a monomeric size (Fig. 8D). Although −ME caused a modest shift of WT and single cysteine replacement mutants as we previously reported because of its effects on the large number of native cysteine residues (32), it should still be reasonable to conclude that interdomain/intersubunit disulfide bonds are actually formed between the LF and lower body domains in S201C/D283C and S201C/L284C.

As a result, after expression in HEK-293 cells, the mutated channels with double cysteine substitutions (S201C/D283C, 4.06 ± 1.7 pA/pF, n = 7; and S201C/L284C, 6.39 ± 1.3 pA/pF, n = 8) produced much smaller responses to ATP when compared with WT (249 ± 25 pA/pF, n = 20) and mutants with single cysteine replacements S201C (91.7 ± 37 pA/pF, n = 6), D283C (145 ± 37 pA/pF, n = 7), and L284C (137 ± 53 pA/pF, n = 7). Applications of dithiothreitol (DTT) increased the ATP-induced currents of S201C/D283C and S201C/L284C for...
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Figure 9. Formation of intersubunit disulfide perturbs the conformation of the middle region of the LF domain at the open state. A, zoom-in view of the constructed zinc-bridge model of P2X4\textsubscript{S201C/D283C} based on the open structure exhibits the details of the C\textsubscript{\textbeta}–C\textsubscript{\textbeta} distances (green dotted line) of Cys-201 and Cys-283 and the distances measured between Zn\textsuperscript{2+} and the coordinating sulfur atoms from Cys-201 and Cys-283 (yellow dotted line). B and C, sample traces (B) and summarized (C, mean ± S.E., n = 5–9) effects of extracellular Zn\textsuperscript{2+} treatment on ATP (100 \textmu M, saturated)-evoked remaining currents of WT and S201C/D283C. **, p < 0.01 after versus Zn\textsuperscript{2+} application, paired Student’s t test. D, state-dependent cross-linking of rP2X4\textsubscript{S201C/D283C}. DTT (+/−) indicate with (+) or without (−) the treatment of DTT (10 \textmu M for 10 min) after the cell surface biotinylation; ATP (+/−) indicate with (+) and without (−) a treatment of ATP (100 \textmu M for 1 min) before the cell lysis; β-ME (+/−) indicate the presence (+) and absence (−) of β-ME (1%, 10 min) in the loading buffer.

~4–5- and ~2–3-fold (ratio = 4.49 ± 0.67, n = 3, p = 0.003 and 2.19 ± 0.39, n = 4, p = 0.006), respectively (Fig. 8, E, G, and H), which were reversed by applications of H\textsubscript{2}O\textsubscript{2} (Fig. 8E), suggesting that immobilization of the LF domain led to impaired channel activation of P2X4. In contrast, DTT slightly reduced ATP-evoked currents of the WT rP2X4 receptors, which was reversed by H\textsubscript{2}O\textsubscript{2} (Fig. 8F). All the results suggested that the breaking of disulfide bonds is responsible for DTT-induced increases in current amplitudes of S201C/D283C and S201C/L284C. Additionally, increasing ATP concentration (1 \textmu M) had no effect on DTT-induced potentiation efficacy on rP2X4\textsubscript{S201C/D283C} and rP2X4\textsubscript{S201C/L284C} currents (Fig. 8, G–J) (S201C/D283C: 4.5 ± 0.7- and 4.4 ± 0.8-fold for 10 \textmu M and 1 \textmu M ATP-induced currents, n = 3 and 3, respectively, p = 0.93, t test; S201C/L284C: 2.2 ± 0.4- and 1.9 ± 0.3-fold potentiation for 10 \textmu M and 1 \textmu M ATP-induced currents, n = 4 and 3, respectively, p = 0.59, t test). Thus, the impaired channel gating of rP2X4\textsubscript{S201C/D283C} and rP2X4\textsubscript{S201C/L284C} by disulfide cross-linking is not related to alterations in ATP sensing.

However, disulfide cross-linking may interrupt both conformations of the LF domain at resting and open states. To provide direct evidence that restraining the LF domain from fostering intersubunit physical couplings at the open state can influence channel activation of P2X4 receptors, we introduced an intersubunit Zn\textsuperscript{2+} bridge between the LF and lower body domains to slightly perturb the conformation of the middle region of the LF domain at the open state (Fig. 9A). This Zn\textsuperscript{2+} bridge at the open state was established via DTT treatments (10 \textmu M for 10 min) at the resting state of rP2X4\textsubscript{S201C/D283C}, which enables the free cysteine residues Cys-201 and Cys-283 to chelate Zn\textsuperscript{2+} at the open state. The C\textsubscript{\textbeta}–C\textsubscript{\textbeta} distance of the two free cysteine residues in the natural Zn\textsuperscript{2+} bridge of crystal structures ranges from 4.5 to 5.0 Å (52), a value relatively smaller than C\textsubscript{\textbeta}–C\textsubscript{\textbeta} distance of Ser-201 and Asp-283 (7.1 Å, Fig. 8C) at the open state. Therefore, a rebuilt Zn\textsuperscript{2+} bridge (Cys-201…Zn\textsuperscript{2+}…Cys-283, Fig. 9A; C\textsubscript{\textbeta}–C\textsubscript{\textbeta} distance of Cys-201 and Cys-283 = 5.2 Å) after DTT application on P2X4\textsubscript{S201C/D283C} could perturb the conformation of the middle region of the LF domain. Indeed, post-administration of Zn\textsuperscript{2+} after ATP inhibited 51.7 ± 2.3% of the remaining ATP currents of P2X4\textsubscript{S201C/D283C} but not WT P2X4 (n = 9, p = 0.0006, Fig. 9, B and C) under the condition that the disulfide bond has been previously interrupted by DTT at the resting state. This point was further tested by a measurement of state-dependent cross-linking of rP2X4\textsubscript{S201C/D283C}. After DTT breaking, the cross-linking between S201C and D283C rebuilt more quickly after rP2X4\textsubscript{S201C/D283C} was treated by ATP, when it is compared with channels without ATP treatment (Fig. 9D). Alkaline-scanning mutations on the LF domain have demonstrated that the residues of the middle region (Figs. 2, D and E, and 10A) were not as crucial as the residues in the N- and C-terminal regions in the channel activation of P2X4 receptors. However, an immobilization of the LF domain using disulfide cross-linking or slightly shortening the pair-residue distance (from 7.1 to 5.2 Å) between the middle region of the LF and lower body domains using a metal bridge rendered a significantly impaired channel activation of rP2X4 receptors. Therefore, intersubunit physical couplings among the DF and two lower body domains fostered by the LF domain at the open state act as a whole structural element that is stringently required by
the channel opening of P2X4 receptors, and any impairment in its integrity will lead to an impaired channel activation.

Moreover, deletions of a single amino acid (Δ283, Δ284, Δ285, Δ286, and Δ287), two amino acids (Δ283–284, Δ284–285, Δ285–286, and Δ286–287), and even three amino acids (Δ283–285, Δ284–286, and Δ285–287) in the middle region produced little change on the maximal current of P2X4 receptors (Fig. 10, B and C). However, truncating four amino acids (Δ283–286) fully abolished the channel activation of P2X4 (n = 8, Fig. 10, B and C), without changing the channel expression and trafficking of P2X4 receptors (Fig. 10, D and E). Thus, a proper length of the middle region (at least two residues remained among those of five amino acids) is prerequisite for the LF domain being functional, further confirming that the middle region is a structural element required by the P2X4 receptors at the open state (see “Discussion”).

**Weakening intersubunit physical couplings via covalent modifications impairs the channel activation of P2X4 receptors**

Finally, we interrupted the conformation of the N terminus of the LF domain through covalent modifications to partially weaken these intersubunit physical couplings at the open state (Fig. 11A). Upon channel activation, the intrasubunit salt bridge between Arg-278 and Asp-280 as well as the two H-bonds (Arg-282…Arg-278 and Arg-282…Asn-192) formed an “interaction network” that was crucial for the deformation of the LF domain at the open state (Fig. 2B, lower panel). Trp-194 is located adjacent to those four amino acids (Fig. 11A and the lower panel of Fig. 2B). We substituted Trp-194 with cysteine with the intention to prevent the interactions between Arg-278, Asp-280, and Arg-282 via covalent modifications (Fig. 11A).

Using a cysteine modification technique such as methanethiosulfonate (33), Ellman’s (53), and alkylating reagents (54) (Fig. 11B), we introduced charged groups (the aminoethyl group of 2-aminoethyl methanethiosulfonate (MTSEA) and sulfonatoethyl group of 2-sulfonatoethylmethanethiosulfonate (MTSES)), a bulky group (1-phenylpyrrolidine-2,5-dione group of N-phenylmaleimide (NPM)), and both charged and bulky groups (5-thio-2-nitrobenzoic acid group of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)) into the position (Cys-194) around Arg-278, Asp-280, and Arg-282 via covalent modifications (Fig. 11A). Applications of MTSEA (1 mM), MTSES (1 mM), DTNB (1 mM), and NPM (1 mM) markedly reduced the current amplitude of P2X4W194C receptors.
(Fig. 11, C and D), which were partially or fully reversed by DTT treatments (Fig. 11C), except NPM treatments, because the alkylating reaction is irreversible. In contrast, DTNB, MTSEA, MTSES, and NPM had no effects on the current amplitude of P2X4S201C (Fig. 11, C and D), a mutant with a cysteine at 201 that stays slightly away from Trp-194, Arg-278, Asp-280, and Arg-282 (Figs. 11A and the lower panel of 2B). In addition, MTSEA, MTSES, DTNB, and NPM did not change the current amplitude of WT P2X4 (Fig. 11, C and D). Thus, specific covalent modifications at W194C are responsible for their inhibitory effects on the P2X4W194C.

Similarly, covalent modification may also interrupt both conformations of the LF domain at resting and open states. To provide direct evidence that covalent modification could prevent the LF domain from fostering intersubunit physical couplings at the open state, MTSEA (1 mM) treatment on ATP (100 μM, saturated)-evoked currents of P2X4WT, P2X4S201C, or P2X4W194C, which were rescued following application of DTT except for NPM treatments. **, p < 0.01 after versus before covalent modifications. Cells were voltage-clamped (amphotericin-perforated patch clamp) at −60 mV, and currents were evoked by ATP (100 μM, 15 s) at 8-min intervals. E and F, sample traces (E) and summarized (F, mean ± S.E., n = 3–5) effects of post MTSEA (1 mM) treatment on ATP (100 μM, saturated)-evoked remaining currents of P2X4WT, P2X4S201C, or P2X4W194C. * , p < 0.05; **, p < 0.01 after versus before MTSEA application, paired Student’s t test. G and H, sample traces (G) and summarized effects (H) (mean ± S.E., n = 4–20) of mutations on the maximal amplitude of currents caused by saturated ATP (100 μM). *, p < 0.05 versus WT (dashed line), one-way ANOVA with Bonferroni post hoc test.

**Discussion**

Here, we propose that the LF domain, a flexible loop structure, underwent allosteric changes after ATP binding, which promotes the formation of intersubunit physical couplings among the two lower body domains and the DF domain that facilitates opening of P2X4 receptors (Fig. 12). At the open state, bound ATP repels the LF domain out of the ATP-binding site, which coordinates the LF, DF, and two lower body domains into an integrated structural element. The integration of the LF domain in the cleft among those domains was achieved through the salt-bridge Arg-278…Asp-280, the newly formed hydrogen-bonding contacts Arg-282…Arg-278, Asn-192…Arg-282, Val-288…Arg-203, and Ser-289…Arg-203, and new hydrophobic interactions among Val-288, Ile-205, and Leu-214 after ATP binding (Fig. 12). The following evidence demonstrated that this new integrated structural element is stringently required by...
the channel gating of the P2X4 receptors. First, mutations of the residues involved in the establishment of physical couplings significantly reduced ATP currents of P2X4 receptors. The gel analysis has excluded the possibility that this newly integrated structural element made contributions to the channel stability and channel assembly. The absence of unitary current in R203A and R282A in response to saturated ATP and the “gain-of-function” of channel activity in these two mutants when IVM was co-applied with ATP confirmed the crucial role of intersubunit interactions among the DF, LF, and lower body domains (Fig. 1, A and B). All of those features made the LF domain fit for coordinating both outward flexing of two domains and upward motion of the DF domain (Fig. 1, A and B). Because the DF domain is structurally coupled to the lower body domain through loop β9–α3 (Figs. 1, A and B, and 2, A–C), its upward motion evoked by ATP binding will cause the outward flexing of the lower body domains.

Three lower body domains form the big central vestibule (9) of P2X receptors (Fig. 1, A and B), and the outward flexing of lower body domains leads to the expansion (9) of this big central vestibule (Fig. 1, A and B). It is worth noting that the expansion of the central vestibule is crucial for the channel gating of trimeric ion channels (1, 2, 38), such as P2X receptors and ASIC channels. Small molecules (53, 55), toxins (56), and covalent modifications (53) acting on the residues of this region can directly affect the channel activation of ASIC channels. The outward flexing of the lower body domains and the expansion of the central vestibule might facilitate the channel activation of P2X receptors by the following reason. The central rigid lower body domains are structurally coupled with TM domains and the pore region (Fig. 1B). Therefore, the deflection of the LF domain evoked by bound ATP directly causes the motions of TM region through those rigid lower body domains, which may facilitate the gating transition from the resting state to the open state (Fig. 1A). Thus, the established physical couplings between the DF and two lower body domains by the deformed LF domain are pivotal to the outward flexing of lower body domains and the concomitant pore dilation of P2X4 receptors.

Our data also showed that the flexible middle region of the LF domain, consisting of seemingly negligible residues, affected channel gating of P2X4 receptors. We have recently suggested that hydrophobic interactions between Val-288, Ile-205, Leu-214, and the aliphatic chain of Lys-190 in P2X4 receptors may develop an energy barrier for the channel gating (32). As revealed by changes in both apparent affinity and maximal cur-

**Physical couplings fostered by left flipper domain**

Figures 12. Illustration of intersubunit physical couplings established by the LF domain at the open state. The established physical couplings integrate the DF, lower body, and LF domains into a structural element stringently required by the channel gating of rP2X4 receptors. The movements of extracellular domain and TM domain were referred by light-blue arrows. The black dotted lines connecting pink dots denote H-bonds or hydrophobic interactions between the key residues. Only two of three subunits, where subunit A and B are colored in green and purple, respectively, are shown for the clarity.
Physical couplings fostered by left flipper domain

rent before and after DTT application on disulfide cross-linking in mutant P2X4V288C/T211C, bound ATP-induced repelling action on Val-288 from the ATP-binding site may behave with two main functions. One is to reduce the energy barrier for channel gating, and the other is to accommodate ATP molecules. The N terminus of the LF domain is relatively rigid because of its structural coupling with the lower body domain and the existence of a salt bridge between Arg-278 and Asp-280 in this region; thus, only a flexible middle region can buffer the repelling action of ATP on Val-288. Following the expulsion of Val-288 from the ATP-binding site and structural rearrangements of Val-288, Ile-205, Leu-214, and Lys-190, a lot of new intra- and intersubunit contacts were established, including Arg-282. At this stage, the middle region located on the interface between two “interaction clusters,” Asp-280...Arg-278...Arg-282 and Val-288...Arg-203...Ser-289, may act as a linker to stabilize those two “clusters” and maintains the intersubunit physical couplings between lower body domains and the DF domain at the open state. Thus, the middle region may contribute to the flexibility at the resting state to buffer the repelling action of ATP on Val-288 and provide the proper length between two termini (Fig. 10) that facilitate the LF domain to foster proper intersubunit physical couplings among the two lower body and DF domains at the open state.

Finally, despite the indispensable role of the LF domain in channel activation of P2X4, we cannot neglect the fact that the sequence of the LF domain is not conserved among various subtypes of P2X receptors (Fig. 3D). At the open state, Arg-203 contacts with the main chain atoms of Val-288 and Ser-289, contributing to the establishment of intersubunit physical couplings. However, the arginine is replaced by glycine at the identical position of P2X2 and P2X3 subtypes (Fig. 3D). Similarly, the salt bridge formed by Arg-278 and Asp-280 in P2X4 is also absent in the P2X1 subtype (Fig. 3D). The identical residue of Arg-282 in P2X4 is absent in the P2X1 subtype (Fig. 3D). The sequence variation is much greater in the middle region of the LF domain throughout the P2X receptor family, which is completely absent in P2X6. However, this absence is not the only reason why no one could record ATP current in cells expressing P2X6 (57). Because of those non-conserved sequences, the three-dimensional (3-D) architecture of the LF domain varies in different subtypes. Further studies are required to determine how those distinct sequences make up the functional LF domain in various subtypes. Nevertheless, it provides a foundation for developing subtype-specific blockers of P2X4 receptors by targeting on this non-conserved region of the LF domain throughout the P2X receptor family.

In summary, ATP binding-induced repelling action on the LF domain promotes the formation of physical couplings among two lower body domains and the DF domain and facilitates the outward flexing of lower body domains (Fig. 12), leading to the expansion of the central vestibule and the concomitant pore dilation. This study provides new mechanistic insights into the channel gating of P2X4 receptors and may contribute to develop new strategies for subtype-specific blockers of P2X receptors.

Experimental procedures

Drugs, cell culture, mutagenesis, and receptor expression

ATP, ZnCl2, and most of the other drugs were purchased from Sigma. The plasmid rP2X4 and zfP2X4.1 are the gifts from Drs. Lin-Hua Jiang, Alan North, and Eric Gouaux. Each mutant was constructed by the QuikChange mutagenesis kit and was verified by DNA sequencing. All constructs were expressed in cultured HEK-293 cells in DMEM at 37 °C in a humidified atmosphere of 5% CO2 and 95% air. Transfections of plasmids were performed using Hilityx (Dojindo Laboratories, Kumamoto, Japan). Electrophysiological measurements were performed on HEK-293 cells 24–48 h after transfection.

Electrophysiology

As in our previous descriptions (32, 53), conventional whole-cell configuration under the voltage clamp at room temperature (23 ± 2 °C) was used for electrophysiological recordings. 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 reference electrode in bath solution ranged from 3 to 5 megohms. 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 Digidata 1440 interface using Clampex and 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 7.35–7.40. Patch electrodes were filled with standard internal solution containing 30 mM NaCl, 120 mM KCl, 1 mM MgCl2, 0.5 mM CaCl2, and 5 mM EGTA at the conditional neutral pH 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 experiment. As we described previously (32), ATP solutions were prepared for 2 h in the batch buffer and applied using a fast pressure-driven computer-controlled microperfusion system OctaFlow08P (ALA Scientific Instrument). ATP currents were normalized to cell membrane capacitance. Dose-response curves data were collected from the recording of a range of ATP concentrations; the corresponding currents were normalized to the maximal current amplitude; ATP-gated currents were recorded after regular 3–5-s ATP application every 2–8 min. Pulses were spaced up to 8–20 min to avoid receptor desensitization at higher ATP concentration (10–100 μM) applications. The amphoterin-in-perforated patch-clamp technology (58) was also used for recordings of dose-dependent responses and covalent modifications of WT P2X4, P2X4W194C, and P2X4S201C. During this procedure, ATP-gated currents were recorded after regular 15–20-s ATP applications every 8–10 min to avoid receptor desensitization. Single-channel recordings using outside-out configuration were carried out in HEK-293 cells at room temperature (23 ± 2 °C) 24–48 h after transfection. Recording pipettes were pulled from borosilicate glass (World Precision Instruments, Inc.) and fire-polished to yield resistance of 5–10 megohms. The holding potential was −120 mV. The external solution and internal solutions are the same.
as those of whole-cell recordings. Single channel recordings were sampled at 50 kHz with a 2-kHz filter and a low-pass filtered at 200 Hz, using an Axopatch 200B amplifier in conjunction with pClamp 10 software (Axon Instruments). Occasional large brief noise spikes were visually identified and removed from current traces.

**Cell-surface biotinylation and Western blotting analysis**

Cell-surface biotinylation and Western blotting were performed according to our previous descriptions (32, 59). Briefly, HEK-293 cells expressing rP2X4 or its mutants were washed in chilled PBS+/− and then were incubated with sulfo-NHS-LC-biotin. The reaction was then terminated by treating the cells with glycine in PBS. Then the cells were collected and lysed with RIPA buffer. Using agarose resin linked to NeutrAvidin, the biotinylated proteins were then separated from the intracellular protein fraction. The resins were washed, and bound proteins were eluted with the boiling SDS sample buffer, whereas 10% of the volume of the supernatant was diluted and used as the total protein fraction. The samples were separated by SDS-PAGE and transferred to the polyvinylidene difluoride (PVDF) membrane and then were incubated overnight at 4 °C with anti-EE tag (1:1000, Abcam, catalogue number ab40767) or anti-GAPDH (1:1000, SungeBiotech, catalogue number KM9002) antibodies. Appropriate HRP-conjugated secondary antibodies for EE tag (25 °C, 1 h, 1:1000, goat-rabbit IgG(HL)-HRP; SungeBiotech, catalogue number: LK2001) or GAPDH (25 °C, 1 h, 1:3000, goat-mouse IgG(HL)-HRP; SungeBiotech, catalogue number: LK2003) were further incubated and finally visualized by exposure with the ImageQuant RT ECL solution (Thermo Fisher Scientific). All Western blottings and gels are accompanied by the location of molecular weight markers (Thermo PageRuler Prestained Protein Ladder 10−170 kDa, catalogue number 26617). Protein expression analysis of each mutant and WT receptors was repeated at least by three independent experiments.

**Homology modeling**

For homology modeling of rP2X4 and its mutants using program MODELLER (60), the structures of the closed (PDB code 4DW0) and open (PDB code 4DW1) zfP2X4 receptors were taken as the templates. Zinc bridge models were also constructed according to our previous procedure (32) using MODELLER. Briefly, for zinc-binding site reconstructions, a coordinate atom of histidine or free cysteine residues. Then, distance constraint (2.0–2.6 Å) was added between zinc and the coordination atoms of histidine or free cysteine residues. Then, these models applied by OPLS_2005 force field (61) were further minimized by DESMOND (62). The resulting models were further optimized by 1.2-ns MD simulations using program DESMOND with OPLS_2005 force field. After such a time scale of MD simulations, various parameters of zinc bridges, including distances between atoms, bond angles, and dihedral angles, were very close to those obtained by analysis of crystals (52).

**MD simulations**

As we described previously (32, 63), all MD simulations were performed using the program DESMOND (62) with a constant number of particles, pressure, and temperature and periodic boundary conditions, which use a particular “neutral territory” method called the midpoint method (62) to efficiently exploit a high degree of computational parallelism. A default OPLS_2005 force field (61), following the functional form of the OPLS-AA family of force fields with additional stretch, bend, and torsional parameters for better coverage of ligand functional groups, was employed for the protein, ions, and ligand molecules. The energy-minimized homology models of rP2X4 and its mutants at the resting or open states were used as the starting structures for MD simulations. The large dimeric phosphatidylcholine bilayers in various simulation systems were constructed to generate a suitable membrane system where the TM region of the WT P2X4 and its mutants could be embedded. The protein/dimeric phosphatidylcholine system was then solvated in a bath of simple point charge water molecules. Counter ions were subsequently added to compensate for the net negative charge of the system. NaCl (150 mM) was added into the simulation box that represents background salt at physiological conditions. To maintain the system at a constant temperature of 300 K and constant pressure, Berendsen thermostat and barostat algorithms were applied to couple protein and other molecules. All of the bond lengths, including hydrogen atoms, were constrained by the Linear Constraint Solver algorithm. Electrostatic interactions between charged groups at a distance of less than 12 Å were calculated explicitly; long range electrostatic interactions were calculated using the smoothed particle mesh Ewald method. All of the MD simulations were run on the DAWNING TC2600 (AMD Opteron™ 8374HE CPUs). Preparation, analysis, and visualization were performed on a 12-CPU CORE DELL T7500 graphic working station. The MD trajectory analysis were performed using Simulation Even Analysis and Simulation Interactions Diagram tools of DESMOND.

**Metadynamics**

Metadynamics (43–45) is a technique where the potential for one or more chosen variables (“collective variables”) is modified by periodically adding a repulsive potential of Gaussian shape at the location given by particular values of the variables. All metadynamics analysis were conducted by the program DESMOND (62) under NPT and periodic boundary conditions using the default parameters at constant temperature (320 K) and pressure (1 bar) by using the Berendsen method. All simulations used the all-atom OPLS_2005 force field for proteins, ions, lipids, and the simple point charge waters. The parameters for height, width of the Gaussian, and the interval were set to 0.12 kcal/mol, 0.05 Å, and 0.09 ps, respectively. The sum of the Gaussians and the free-energy surface were generated by Meta-dynamics Analysis Tools of DESMOND.

**Data analysis**

The results are expressed as the means ± S.E. Statistical comparisons were made using one-way ANOVA and Student’s t test, where p < 0.05 (*) or p < 0.01 (**) was considered significant. Concentration-response relationships for ATP activation of WT or mutated channels were obtained by measuring currents in response to different concentrations of ATP, and all of
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the results used to generate a concentration-response relationship were from the same group. The data were fit to Hill Equation 1,

\[ I/I_{\text{max}} = \frac{1}{1 + (\text{EC}_{50}/[\text{ATP}])^n} \]  

(Eq. 1)

where \( I \) is the normalized current at a given concentration of ATP; \( I_{\text{max}} \) is the maximum normalized current; \( \text{EC}_{50} \) is the concentration of ATP yielding a current that is half of the maximum, and \( n \) is the Hill coefficient.

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