A conserved residue in the P2X4 receptor has a nonconserved function in ATP recognition

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Highly conserved amino acids are generally anticipated to have similar functions across a protein superfamily, including that of the P2X ion channels, which are gated by extracellular ATP. However, whether and how these functions are conserved becomes less clear when neighboring amino acids are not conserved. Here, we investigate one such case, focused on the highly conserved residue from P2X4, E118 (rat P2X4 numbering, rP2X4), a P2X subtype associated with human neuropathic pain. When we compared the crystal structures of P2X4 with those of other P2X subtypes, including P2X3, P2X7, and Amp2X, we observed a slightly altered side-chain orientation of E118. We used protein chimeras, double-mutant cycle analysis, and molecular modeling to reveal that E118 forms specific contacts with amino acids in the “beak” region, which facilitates ATP binding to rP2X4. These contacts are not present in other subtypes because of sequence variance in the beak region, resulting in decoupling of this conserved residue from ATP recognition and/or channel gating of P2X receptors. Our study provides an example of a conserved residue with a specific role in functional proteins enabled by adjacent nonconserved residues. The unique role established by the E118-beak region contact provides a blueprint for the development of subtype-specific inhibitors of P2X4.

The side-chain orientation of a given residue is closely related to protein functions such as ligand recognition (1) and conformational transitions (2) of these proteins during allosteric processes. Fine interpretation of the relationship between residue function and side-chain orientation at atomic level will advance our understanding of protein function (3) and provide more accurate structural information for future drug design (4, 5). In general, the relationship between 3D structural information and function is more conserved in a protein superfamily than that of primary amino acid sequence (6, 7). And it has also been suggested that in a given functional protein superfamily, conserved amino acids have similar functions among different members (8, 9). However, if there are nonconserved residues located around the highly conserved residues, how do these surrounding residues affect the function of those conserved residues? Do the conserved and nonconserved residues encode different structural information together? Although there has been a great deal of studies aimed at understanding how do primary sequences determine the 3D structure and function of proteins, studying the relationship between sequence, structure, and function (RSSF) remains a daunting task. However, in recent years, a number of different crystal or cryo-EM structures have been identified (10–12), including structures from different subtypes and species, or at different states (apo or open states), such as the P2X receptors (13–21), providing invaluable information used to study the RSSF of a particular protein superfamily.

P2X receptors are ligand-gated ion channels activated by extracellular ATP, and seven different subtypes (P2X1–7) have been cloned in recent years (22). These different P2X receptors are implicated in different physiological processes, such as synaptic transmission (23), pain sensation (24–26), and immune and cardiovascular regulations (27, 28). Therefore, P2X receptors are considered as potential therapeutic targets for neuropathic pain, inflammation, rheumatoid arthritis, chronic cough, and others (29–31). Prior to the first determination of the crystal structure of P2X receptors, studies on the structure and function relationship of these receptors focused on various conserved residues and their role in ATP recognition, channel pore location, and others (32–35). In the past decade, crystal or cryo-EM structures of several P2X receptors were determined, including crystal structures of zebrafish (Danio rerio) P2X4 (zfP2X4) (13–15), gulf coast tick (Amblyomma maculatum) P2X (AmP2X) (16), chicken (Gallus gallus)
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domesticus) P2X7 (ckP2X7) (17), giant panda (Ailuropoda melanoleuca) P2X7 (pdpP2X7) (21), human (Homo sapiens) P2X3 (hP2X3) (18, 19), and cryo-EM structures of rat (Rattus norvegicus) P2X7 (rP2X7) (20) in the closed/open/desensitized/antagonist-bound states. Thus, the study of P2X RSSF has entered a poststructure era where we can more accurately re-evaluate the structure and function relationship of P2X receptors.

In this study, we compared the conformation/side-chain orientation of some conserved residues in several recently structurally identified P2X subtypes and found that a highly conserved residue E121 (equal to E118 in rP2X4) in the head domain of zfP2X4 has a different side-chain orientation from the other subtypes. Through a combination of molecular dynamics (MD) simulations, chimera construction, mutagenesis, and electrophysiological studies, we suggest that the unique side-chain orientation of E121 provides additional contacts between this residue and amino acids in the beak region, thus establishing a special role in determining the apparent affinity of ATP in rP2X4 but not in other P2X subtypes.

Results

E121 is a highly conserved residue in zfP2X4 with a different side-chain orientation compared with other P2X subtypes

To reassess the role of conserved residues in P2X channel function, we compared the side-chain orientations of all conserved residues in several P2X subtypes whose 3D structures have been determined by X-ray crystallography, including zfP2X4 (Protein Data Bank [PDB] IDs: 4DW0 and 4DW1), hP2X3 (PDB IDs: 5SVJ and 5SVK), AmP2X (PDB ID: 5F1C), ckP2X7 (PDB ID: 5XW6), and pdpP2X7 (PDB ID: 5U1L). The cryo-EM structures of rP2X7 were not included into this comprehensive comparison since we could not exclude possible influence of two different methods (single-particle cryo-EM and X-ray crystallography) in determining residue side-chain orientation. Through a comprehensive structural superimposition, we found that three highly conserved residues R298, K316, and E121 (zfP2X4 numbering) have different side-chain orientations in different P2X subtypes (Table 1). R298 and K316 produce direct contacts with the agonist ATP in the open structure of zfP2X4 (Fig. 1A), and it is therefore reasonable to infer that the different side-chain orientation of these two residues may contribute to distinct recognition patterns for free ATP$^4$ or MgATP$^2$- and/or distinct ATP affinities in various P2X subtypes (36). E121 is located far away from the ATP-binding site in the head domain of zfP2X4 (Fig. 1, A and B), and its role in the channel function of the P2X receptors remains unclear.

The dihedral angle $\chi_{Ca-CB-CY-CS}$ of E121 in zfP2X4 at the resting (PDB ID: 4DW0) and open (PDB ID: 4DW1) states was $166.1^\circ$ and $-174.4^\circ$, respectively (Fig. 1B), showing extended conformations. In other P2X isoforms, such as hP2X3 in the resting (PDB ID: 5SVJ) and open states (PDB ID: 5SVK), and ckP2X7 with an incorporation of competitive inhibitor 2',3'-O-(2,4,6-trinitrophenyl) adenosine 5'-triphosphate (PDB ID: 5XW6), the $\chi_{Ca-CB-CY-CS}$ of identical residues ranged from $60^\circ$ to $-80^\circ$ (Fig. 1, D–F) for a bent conformation (Fig. 1, D–F). The side chain of the equivalent residue of pdpP2X7 was shortened (D121; Fig. 1C and G), so we could not measure the dihedral angle of this residue. The recent determined cryo-EM structures of rP2X7 reveal the extended conformation of the same residue E121 at the resting state ($\chi_{Ca-CB-CY-CS} = 172.9^\circ$) and the bent conformation at the open state ($\chi_{Ca-CB-CY-CS} = -55.2^\circ$) (Fig. 1H).

To test whether the conformation of E121 in zfP2X4 and equivalent residues in other P2X subtypes are stable in a physiological condition, a series of 0.5-μs MD simulations were performed on aforementioned crystal or cryo-EM structures. These simulations showed that during simulations of zfP2X4, E121 always maintained an extended conformation ($\chi_{Ca-CB-CY-CS} = 180^\circ$ or $-180^\circ$) both in the resting and open states, whereas the same residues in other structures remained in the bent conformation ($\chi_{Ca-CB-CY-CS} = -60^\circ$–$-80^\circ$) (Fig. 2). The $\chi_{Ca-CB-CY-CS}$ of E121 also remained at ±180$^\circ$ and ±60 to 80$^\circ$ when MD simulations were performed based on cryo-EM structures of rP2X7 at the resting and open states, respectively, indicating that the MD simulation did not change the initial $\chi_{Ca-CB-CY-CS}$ of E121 of rP2X7 (Fig. 1H). Thus, the side-chain orientation of these equivalent residues is not a transient “snapshot” in the structure determination but is also relatively stable in the structure fluctuations.

Alanine substitutions of E121 in zfP2X4 and E118 in rP2X4 significantly alter the apparent affinity of ATP

Mutagenesis performed in these residues showed that zfP2X4E121A resulted in an approximately 15-fold increase

| Dihedral angles | zfP2X4 | hP2X3 | AmP2X | pdP2X | ckP2X7 |
|-----------------|--------|--------|--------|--------|--------|
| $\chi_{Ca-CB-CY-CS}$ | E121 | E109 | E129 | D121 | E109 |
| -166.1 | 75.2 | -76.6 | -73.2 | 63.3 |
| R298 | 171.7 | 178.6 | 172.5 | 174.3 | 179.3 |
| $\chi_{Ca-CB-Nx-Nx}$ | -87.8 | 99.1 | 101.9 | -174.4 | 61 |
| -86.5 | 113.1 | 107.0 | 87.6 | 108.3 | 105.8 |
| -166.4 | 173.8 | 163.1 | -172.9 | -169.7 | 162.2 |
| $\chi_{Ca-CB-CY-CS}$ | -177.6 | 175.8 | -173.9 | -176.7 | -52.4 |
| -177.6 | 175.8 | 177.4 | 160.9 | 160.9 |
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in the apparent affinity of ATP (concentration yielding half-maximal activation, $EC_{50} = 1426 \pm 733$ and $89.6 \pm 18.1 \mu M$ for zfP2X4WT and zfP2X4E121A, respectively, $p < 0.05$; Fig. 3A), and other subtypes of corresponding mutations did not or slightly change the apparent affinity of ATP ($EC_{50} = 0.45 \pm 0.07$ and $0.58 \pm 0.07 \mu M$ for hP2X3WT and hP2X3E109A, respectively, Fig. 3C; $43.5 \pm 4.7$ and $32.9 \pm 4.2 \mu M$ for AmP2XWT and AmP2XE129A, respectively, Fig. 3D; $4.77 \pm 0.69$ µM for rP2X7 WT; 7zP2X4, zebrafish (Danio rerio) P2X4.

Figure 1. Side-chain orientations of E121 in zfP2X4 and equivalent residues in other P2X receptors. A, residues E121, R298, and K313 highlighted in the open structure of zfP2X4 (Protein data Bank [PDB] ID: 4DW1). The different domains are named head, dorsal fin (DF), left flipper (LF), right flipper (RF), body and fluke, respectively. B, zoom-in view of the location of E121 (upper) and 2Fo–Fc omit map for E121 (lower) in zfP2X4 at the resting (PDB ID: 4DW0, left) and open (right) states. Red circles indicate the atoms chosen to define the dihedral angle $\chi_{C\alpha-C\beta-C\gamma-C\delta}$. C, the sequence alignment of the head domain in P2X family. Pink boxed regions highlight the highly conserved residue E121/E118 and nonconserved residue R143/H140 (zfP2X4/p2X4 numbering) in P2X receptors. Sand bar indicates residues in the beak region. D–G, side-chain orientations of E109 in the apo (PDB ID: 5SVJ) and open (PDB ID: 5SVK) crystal structures of hP2X3, E129 in the open structure of AmP2X (PDB ID: 5F1C) (E), E109 in the crystal structure of ckP2X7 in complex with inhibitor (PDB ID: 5XW6) (F) and D121 in the apo crystal structure of pdP2X7 (PDB ID: 5U1L) (G). H, the side-chain orientation of E121 in the cryo-EM structure of rP2X7 at the resting (PDB ID: 6U9V) and open (PDB ID: 6U9W) states. rP2X4, rat (Rattus norvegicus) P2X4; zfP2X4, zebrafish (Danio rerio) P2X4.

Figure 2. Molecular dynamics (MD) simulations used to test the stability of dihedral angle $\chi_{C\alpha-C\beta-C\gamma-C\delta}$ in various P2X structures. A–H, the fluctuation of dihedral angle $\chi_{C\alpha-C\beta-C\gamma-C\delta}$ during 0.5-µs MD simulations of zfP2X4 (A, resting; B, open), hP2X3 (C, resting; D, open), AmP2X (E, open), ckP2X7 (F, with bound inhibitor), and rP2X7 (G, resting; H, open). The trajectories were sampled every 200 ps. AmP2X, gulf coast tick (Amblyomma maculatum) P2X; ckP2X7, chicken (Gallus gallus domesticus) P2X7; hP2X3, human (Homo sapiens) P2X3; rP2X7, rat (Rattus norvegicus) P2X7; zfP2X4, zebrafish (Danio rerio) P2X4.
Figure 3. Altered apparent ATP affinities of ATP in P2X mutants. A–I, concentration–response curves of ATP in WT and equivalent alanine substitutions in zfP2X4 (A), rP2X4 (B), hP2X3 (C), AmP2X (D), ckP2X7 (E), pdpX7 (F), hP2X7 (G), hP2X1 (H), and hP2X2 (I). The solid lines are fitted by Hill equation (mean ± SD, n = 3–7; EC50 = 1426 ± 733 and 89.6 ± 18.1 μM, nH = 0.94 ± 0.16 and 1.02 ± 0.18 for zfP2X4WT and zfP2X4E121A; EC50 = 7.65 ± 1.56 and 45.92 ± 9.05 μM, nH = 1.20 ± 0.22 and 1.11 ± 0.15 for rP2X4WT and rP2X4E118A; EC50 = 0.45 ± 0.07 and 0.58 ± 0.07 μM, nH = 0.94 ± 0.16 and 1.02 ± 0.18 for hP2X3WT and hP2X3E109A; EC50 = 95.7 ± 17.6 and 96.6 ± 10.9 μM, nH = 1.14 ± 0.16 and 0.91 ± 0.16 for rP2X7WT and rP2X7E121A; EC50 = 97.4 ± 35.6 and 5.31 ± 1.26 μM, nH = 0.94 ± 0.16 and 1.46 ± 0.63 for hP2X1WT and hP2X1E119A; EC50 = 13.16 ± 1.97 and 12.92 ± 1.68 μM, nH = 1.15 ± 0.17 and 1.26 ± 0.15 for hP2X2WT and hP2X2E127A, respectively). The scatter of each maximum concentration of ATP (3 mM) used for zfP2X4E121A.

The underlying mechanism of the altered apparent ATP affinity in zfP2X4E121A should be further investigated; however, previous studies have shown that zfP2X4 is poorly expressed in mammalian cells and insensitive to extracellular ATP (EC50 > 1 mM) (13, 14), making it difficult to study the mechanism by using comprehensive mutagenesis together with electrophysiological recordings. Therefore, the following chimera constructions, double-mutant cycle analysis, and interaction–function correlation analysis were carried out in rP2X4 rather than zfP2X4 receptors. In addition, to obtain more accurate information for rP2X4 studies, we built homology models of rP2X4 and performed MD simulations of rP2X4 WT and its mutants.

Similarly, alanine substitution on the equivalent residue E118A of rP2X4 significantly changed the apparent ATP affinity of rP2X4 (7.65 ± 1.56 and 45.9 ± 9.1 μM for rP2X4WT and rP2X4E118A, p < 0.05, respectively; Fig. 3B). As controls, the corresponding mutants in hP2X1 and hP2X2 had no effect on the apparent affinity of ATP (EC50 = 0.86 ± 0.16 and 0.92 ± 0.17 μM for hP2X1WT and hP2X1E119A, p > 0.05; EC50 = 13.16 ± 1.97 and 12.92 ± 1.67 μM for hP2X2WT and hP2X2E127A, p > 0.05; Fig. 3, H and I). Only negligible ATP current could be detected in cells expressing homomeric mammalian P2X5 and P2X6 receptors, and therefore, effects of the same mutants on these two P2X subtypes were not measured. Thus, the apparent affinity of ATP was only affected by the substitution of alanine in E118 or E121 in P2X4, but not in all other P2X receptor subtypes tested, although this glutamate is highly conserved.

Additional mutants in E118 were constructed, and their effects on the affinity of rP2X4 were measured by a sequential ATP application protocol. The ratio (R) of currents (I1 max/I0 μM) induced by 8 μM (EC50) and 1 mM (saturated) ATP was used to simplify the effect of various mutants on the apparent affinity of rP2X4 (Fig. 4A). rP2X4 WT had an R value of 2.16 ± 0.22, with
Figure 4. Role of E118 in determining the apparent affinity of rP2X4. A and B, representative current traces (A) and pooled data (B) for WT and rP2X4 mutants. About 8 μM ATP was first applied (gray bars) and then switched to 1 mM ATP (green bars). R values are derived from the ratio of 1 mM ATP-induced current to 8 μM ATP-induced current. Open circles of each scatter represent each measurement through whole-cell recordings, n = 15, 7, 6, 5, 3, and 6, from left to right, respectively. *p < 0.05, **p < 0.01 versus WT, one-way ANOVA with Bonferroni post-test (Fig. 6, 42) = 11.54, p < 0.0001), rP2X4, rat (Rattus norvegicus) P2X4.

higher R values indicating higher EC50 values for the mutants (Fig. 4, A and B). Any small change in the polarity or size at position 118 significantly impaired the apparent ATP sensitivity of rP2X4, for example, by replacing the Glu by Asp (EC50 = 232.3 ± 50.3 μM) did not change compared with rP2X4zf-beak (232.3 ± 50.3 μM) (p > 0.05; Fig. 5D). In addition, double-mutant cycle analysis indicated that E118 correlates with the introduced new beak region sequences (ΔΔG = −1.25 kcal/mol for rP2X4E118Atzf-beak, rP2X4E118Atzf-beak, and rP2X4E118A/ztz-beak, and rP2X4E118A/ztz-beak, indicating strong interaction between the identical residue and introduced new beak regions in chimera channels (>0.35 kcal/mol) (37). Although the rP2X4E118A/ztz-beak-based E118A mutant has no influence on the EC50 of ATP, it is still reasonable to infer that sequences in the beak region are implicated in E118/E121-mediated ATP recognition of P2X4 receptors.

The role of E118 in the apparent affinity of rP2X4

We further studied how E118, together with the adjacent beak region, determines the apparent ATP affinity of rP2X4. During MD simulations of apo P2X receptors, the head domain behaves with a tendency to spontaneously move downward and a closure of ATP-binding site jaw, at least for P2X2, P2X3, and P2X4 (38–43). Here, rP2X4E118A also exhibits similar head domain movement during MD simulations (Fig. 6A), indicating that E118A does not compromise the inherent dynamics of the head domain.

We then explored the possibility that polar contacts between E118 and adjacent residues may contribute the role of E118 in ATP recognition of P2X4 receptors. At the resting state, the polar residues within 5 Å of E118 are S124 and D138 (Fig. 6B). The alanine substitution of these two residues had no significantly increased the EC50 of ATP from 0.12 to over 1 mM (Fig. 5C). However, the EC50 of rP2X4E118A/ztz-beak (161.7 ± 26.5 μM) did not change compared with rP2X4zf-beak (232.3 ± 50.3 μM) (p > 0.05; Fig. 5D).
significant effect on R value of rP2X4 (Fig. 6F). At the open state, polar residues within 5 Å are S124 and S141 (Fig. 6C). In 0.5-μs MD simulations, we could observe stable hydrogen bond (H-bond) contacts between E118 and S141/S142 (Fig. 6D). The fact that S141A/S142A, as well as E118A/S141A/S142A, led to significantly changed R values of rP2X4 (I1m M/I8 μM = 9.10 ± 5.13 and 7.93 ± 2.03 for S141A/S142A and E118A/S141A/S142A, respectively; Fig. 6F) suggested that the E118 ... S141/S142 interaction observed at the open state is essential for the apparent affinity of ATP in rP2X4.

Further double-mutant cycle analysis was performed to quantify these interactions. S141A/S142A, E118A, and E118A/S141A/S142A resulted in right shifts of dose-dependent response curves of ATP (EC50 = 7.65 ± 1.56, 51.50 ± 16.14, 45.92 ± 9.05, and 59.61 ± 13.22 μM for WT, S141A/S142A, E118A, and E118A/S141A/S142A, respectively; Fig. 6F). Accordingly, the free energy alterations were calculated to be 1.04, 1.11, and 1.20 kcal/mol for E118A, S141A/S142A, and E118A/S141A/S142A, respectively (Fig. 6H). The coupling energy ΔΔG of E118 and S141/S142 was −0.96 kcal/mol, which confirms the strong interaction between E118 and S141/S142 cluster (Fig. 6H).

In addition, we introduced tryptophan into the V161, a residue located near the E118 and S141/S142. Because V161 is located in the rigid β-sheet (B7) of the head domain, V161W will certainly block the S141/S142 ... E118 interactions during channel opening of rP2X4 (Fig. 6E). Indeed, the R value of V161W increased to 13.7 ± 2.4 (Fig. 6F), indicating a significant decrease in the apparent affinity of ATP in rP2X4.

H140 mediates the correlation of E118 with ATP recognition of rP2X4

H140 is located in the lower beak region and may be involved in ATP recognition and channel gating of rP2X4 (Fig. 7A; right) (14, 15). During 0.5-μs MD simulations of rP2X4 WT with bound ATP, a tightening of the cleft between the dorsal fin and the rostral/head region and a shortening of the distance between ATP and H140 can be observed (Fig. 7B). In contrast, A118 swung upward with H140 together and away from the bound ATP during MD simulations of rP2X4E118A (Fig. 7, C–E). This altered allostery will unwind the interaction between ATP and H140 and the subsequent coordinated movement of the beak and dorsal fin domains, ultimately affecting the ATP recognition and channel gating of P2X4.

Double-mutant cycle analysis was also applied to confirm the coupling between E118 and H140. Single mutations E118A and H140A and double-mutant E118A/H140A resulted in increased EC50 values of ATP to 45.9 ± 9.1, 53.3 ± 9.1, and 55.1 ± 14.4 μM, respectively (Fig. 7F). The coupling free energy...
ΔΔG of E118 and H140 was calculated to be $-1.02$ kcal/mol (Fig. 7), indicating that H140 and E118 are interdependent in contributing to ATP recognition and channel gating of P2X4.

In other P2X subtypes, there is no correlation between the highly conserved residue and ATP recognition

Finally, we explored why this conserved residue has no effect on the apparent ATP affinity of other P2X subtypes (Fig. 8) by using MD simulations. In 0.5-μs MD simulations of hP2X3WT or hP2X3E109A at the open state, P128, the residue corresponding to H140 of rP2X4, remained away from ATP ranging from 8.4 to 14 Å (the distance between the Cα of P128 and N9 atom of ATP; Fig. 8, A–C). Although the P128 moved closer to ATP in hP2X3E109A, it is still impossible to establish a direct contact between the beak region and ATP at this distance (Fig. 8, A and C). Thus, sequence differences in the beak region and/or the structure of this region in hP2X3 might compromise the role of E109 in the apparent ATP affinity of hP2X3.

In order to understand the role of H140 in the apparent ATP affinity of P2X4, we used MD simulations to explore why this residue has no effect on ATP recognition of other P2X subtypes. In MD simulations of hP2X3 WT or hP2X3 E109A at the open state, P128, the residue corresponding to H140 of rP2X4, remained away from ATP ranging from 8.4 to 14 Å (the distance between the Cα of P128 and N9 atom of ATP; Fig. 8, A–C). Although the P128 moved closer to ATP in hP2X3 E109A, it is still impossible to establish a direct contact between the beak region and ATP at this distance (Fig. 8, A and C). Thus, sequence differences in the beak region and/or the structure of this region in hP2X3 might compromise the role of E109 in the apparent ATP affinity of hP2X3.

Similarly, MD simulations of AmP2X and AmP2X E129A reveal ~8 Å distance between the beak region and ATP (Fig. 8, D–F). Therefore, it is possible that sequence differences of the beak regions in other P2X subtypes interrupt the coupling of this conserved residue, like E118 in rP2X4, to the final ATP recognition of the P2X receptor.

**Discussion**

In RSSF studies, highly conserved residues often thought to have a conserved role in protein architecture and function. In our study, E121 (zfP2X4 numbering) is a highly conserved residue, but its side-chain orientation differs from that of other P2X subtypes. Accordingly, the zfP2X4E121A mutant significantly altered the apparent affinity of ATP rather than the corresponding mutants in other P2X subtypes. The specific role of this unique side-chain orientation–determined conserved residue in ATP recognition is due to the coupling established by this conserved residue and adjacent non-conserved residues to the ATP recognition of P2X4. Our findings suggest that a highly conserved residue in the primary amino acid sequence could “own” the nonconserved role in proteins, and that the conserved residue is able to establish a special function with adjacent nonconserved residues.

The ability of rP2X4 E118 in determining the ATP recognition and/or channel gating is possibly through the “E118 ... S141/S142 ... H 140 axis,” especially for the H-bond contact between E118 and S141/S142 complexes. The following evidence can support this idea. First, several E118 mutants suggest that the strong polarity and proper side-chain size of this residue are important (Fig. 4), and stable H-bond contacts between E118 and S141/S142 could be detected during MD simulations.
simulations (Fig. 6D). Second, the carboxyl group of E118 is oriented toward S141/S142 at the open state, which would facilitate the H-bond contacts among these residues (Fig. 6C). Third, the R values of S141A or S142A are slightly higher than that of rP2X4 WT, but the R value of double-mutant S141A/S142A increases to a level equal to that of E118A (Fig. 6F). This idea is further confirmed by the double-mutant analysis of E118 and S141/S142 (Fig. 6H). Fourth, during MD simulations of rP2X4E118A, loss of E118...S141/S142 contact was associated with upward movement of the beak region, which resulted in the displacement of H140 from the ATP recognition site (Fig. 7). H140 mediates the coupling of E118 to the ATP recognition of rP2X4. A, superimposed apo and open structures of rP2X4 to show the downward motions of the head domain and beak region, as well as the H140 moving closing to the bound ATP. Arrows highlight bound ATP-induced allosteric change. The yellow-dashed line indicates the distance between the Cα of H140 and N9 atom of ATP. B and C, superimposition of the initial open structure and averaged conformations of rP2X4WT (B) and rP2X4E118A (C) after 0.5-μs molecular dynamics (MD) simulations. Red arrows indicate the conformational changes during MD simulations. D and E, fluctuations of the dihedral angle of H140 (D) and distance between ATP and H140 (E) during 0.5-μs MD simulations of rP2X4WT and rP2X4E118A. The trajectories were sampled every 200 ps. F, the ATP concentration–response curves of rP2X4WT, rP2X4E118A, rP2X4H140A, and rP2X4E118A/H140A (mean ± SEM, n = 3–5, EC50 = 53.3 ± 9.1 and 55.1 ± 14.4 μM, nH = 1.24 ± 0.16 and 0.99 ± 0.15 for H140A and E118A/H140A, respectively). The data on rP2X4WT and rP2X4E118A are the same as those displayed for Figure 4B. G, double-mutant cycle analysis between E118 and H140A. rP2X4, rat (Rattus norvegicus) P2X4.

Figure 8. Molecular dynamics (MD) simulations of hP2X3WT, AmP2XWT, and their equivalent mutants. A, distance measurements between ATP and P128 during 0.5-μs MD simulations of hP2X3WT and hP2X3E109A. B and C, superimpositions of initial open structure and conformations after MD simulations of hP2X3WT and hP2X3E109A. The dashed lines indicate the distance between the Cα of P128 and N9 atoms of ATP. D, distance measurements between ATP and I115 during 0.5-μs MD simulations of AmP2XWT and AmP2XE129A. E and F, superimpositions of initial open structure and snapshots after MD simulations of AmP2XWT and AmP2XE129A. The trajectories were sampled every 200 ps. AmP2X, gulf coast tick (Amblyomma maculatum) P2X; hP2X3, human (Homo sapiens) P2X3.
Double-mutant cycle analysis of E118 and H140 (ΔΔG = −1.02 kcal/mol) also demonstrated the functional coupling of these two residues (Fig. 7G). Thus, the established “E118 ... S141/S142 ... H140 axis” is the mechanism for the special role of E118 in determining the ATP recognition.

Our findings also support the idea that the nonconserved residue is able to significantly alter the conformation and function of highly conserved residues, especially when these conserved residues are located in a loop region. Based on the sequence alignment, we could find that although zP2X4E121 is highly conserved but the other residues in the beak region (a loop structure; Fig. 1C) are nonconserved throughout the P2X family. Accordingly, in the crystal structure of zP2X4, the side chain of E121 points outside the head domain, showing an extended conformation with its carboxyl group facing to the lower beak region. However, in other P2X subtypes, the side chain of the equivalent residue bends inward to the head domain, making no contact with the lower beak region. Interestingly, differences in the beak region sequences of zP2X4 and rP2X4, from the same subtype but different species, persist, making no contact with the lower beak region. Inter-chain of the equivalent residue bends inward to the head domain, forming no contact with the lower beak region. However, in other P2X subtypes, the side chain of the equivalent residue bends inward to the head domain, making no contact with the lower beak region. Inter-chain of the equivalent residue bends inward to the head domain, showing an extended conformation with its carboxyl group facing to the lower beak region. However, in other P2X subtypes, the side chain of the equivalent residue bends inward to the head domain, making no contact with the lower beak region.

Metal ions have been shown to stimulate this site (45) and the beak region, is an allosteric site, and several compounds/P2X receptors. The upper lumen, formed by the head domains and the beak region, is an allosteric site, and several compounds/metal ions have been shown to stimulate this site (45–47). Understanding the relationship between the side-chain orientation of E118 within this site and the ATP-recognition P2X receptors will facilitate the design and optimization of potent and highly selective new analogs based on the known compounds. Furthermore, there is no compound that has been shown to act directly on the P2X4 receptor at this site, and we can screen for new P2X4-selective lead compounds.

Electrophysiological recordings

Whole-cell patch clamp recordings were performed at room temperature (25 ± 2°C), using an Axopatch 200B amplifier (Molecular Devices). Current signals were sampled at 10 kHz and filtered at 2 kHz and analyzed by pClamp 10 (Molecular Devices). HEK293 cells were superfused with standard extracellular solution containing (in millimolar) 150 NaCl, 5 KCl, 10 glucose, 10 Hepes, 2 CaCl2, and 1 MgCl2 (pH 7.4). The pipettes were pulled from glass capillaries through two-stage puller (Narishige PC-10) and filled in a general intracellular solution containing (in millimolar) 120 KCl, 30 NaCl, 0.5 CaCl2, 1 MgCl2, 10 Hepes, 5 EGTA (pH 7.2), and a resistance varied between 2 and 4 MΩ. During gap-free recordings, the membrane potential was held at −60 mV, and the ATP solution was applied with the Y-tube. As we previously described (19, 48), nystatin-perforated patch clamp was carried out to reduce the dialysis of intracellular constituents and current rundown. Nystatin-perforated intracellular solution contains (in millimolar) 75 K2SO4, 55 KCl, 5 MgSO4, and 10 Hepes (pH 7.2). Nystatin was purchased from Sangon Biotech, and all other drugs were purchased from Sigma–Aldrich. Maximal currents or R ratios were obtained by whole-cell patching, whereas concentration–response curve recordings were performed using perforated patch clamp to avoid channel desensitization.

Homology modeling and MD simulations

The homology models of rP2X4WT and rP2X4E118A were constructed based on the crystal structures of zP2X4 (PDB IDs: 4DW0 and 4DW1) by using Modeller as we described previously (42, 49). The alignment of the target sequences was manually adjusted to match published alignments (13); the obtained model was checked and validated by ProCheck (50); the energy-minimized structures of P2X were used as the initial structures for MD simulations. A large 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine bilayer, available in System Builder of DESMOND (Schör dinger) (51), was used to generate a suitable membrane system in which the transmembrane domain of the P2X could be embedded. The P2X/
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1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine system was dissolved in simple point charge water molecules. Counter ions were then 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 condition. The default relaxation protocol of Desmond for each system was applied before the simulations were run: (1) 100 ps simulations in the canonical (constant moles, volume and temperature) ensemble with Brownian dynamics using a temperature of 10 K with solute heavy atoms restrained; (2) 12 ps simulations in the canonical (constant moles, volume and temperature) ensemble using Berendsen thermostat at 10 K with small-time step and solute heavy atoms restrained; (3) 12 ps simulations in the isothermal-isobaric (constant moles, pressure and temperature) ensemble using the Berendsen thermostat and barostat at 10 K and 1 atm with solute heavy atoms restrained; (4) 12 ps simulations in the isothermal-isobaric (constant moles, pressure and temperature) ensemble using the Berendsen thermostat and barostat at 300 K and 1 atm with solute heavy atoms restrained; (5) 24 ps simulations in the isothermal-isobaric (constant moles, pressure and temperature) ensemble using the Berendsen thermostat and barostat at 300 K and 1 atm with no restraints. After equilibration, the MD simulations were performed for 500 ns. Long-range electrostatic interactions were computed using the smooth particle mesh Ewald method. The integration time step used was 1 fs, and the coordinate trajectories were saved every 200 ps. All simulations were performed by using Desmond with a constant number of particles, pressure (1 bar), and temperature (300 K) and periodic boundary conditions by using Nose–Hoover chain thermostat. Proteins, ions, lipids, and the simple point charge waters were assigned to all-atom OPLS_2005 force field (52, 53). Simulations were run on DELL T7910 graphic working station (with NVIDIA Tesla K40C-GPU). Preparation, analysis, and visualization were performed on a 12-CPU CORE DELL T7500 graphic working station.

Data analysis

Bonferroni’s multiple comparisons test (ANOVA) was used when there were more than two groups. *p < 0.05 or **p < 0.01 was considered significant. Concentration–response curves were fitted to the equation as \( I/I_{\text{max}} = 1/(1 + (EC_{50}/[ATP])^n) \), where \( I \) is the normalized current at a given concentration of ATP, \( I_{\text{max}} \) is the maximum normalized current, \( EC_{50} \) is ATP dose for half-maximal effect, and \( n \) is the Hill coefficient. Statistical comparison between \( EC_{50} \) values was performed using extra sum-of-squares F tests. The change in Gibbs free energy upon each mutant was described by the following equation: \( 
\Delta G = -RT \ln \left( \frac{EC_{50} \text{ of WT}}{EC_{50} \text{ of mut}} \right) \), where the constants \( R \) and \( T \) are 1.987 cal/mol/K and 293 K, respectively (54). The interaction free energy in the double-mutant cycles was calculated from \( \Delta \Delta G_{\text{interaction}} = \Delta G_{\text{mut1} + \text{mut2}} - (\Delta G_{\text{mut1}} + \Delta G_{\text{mut2}}) \). If the change in the free energy of the double mutant (\( \Delta G_{\text{mut1} + \text{mut2}} \)) equals the sum of the free energy changes of the corresponding single mutations (\( \Delta G_{\text{mut1}} \) and \( \Delta G_{\text{mut2}} \)), which means that the value of \( \Delta \Delta G_{\text{interaction}} \) is close to zero, the two residues or clusters will not interact. In contrast, if the two residues interact, the change in the free energy of the double mutant will differ from the sum of the changes of the corresponding single mutations, and \( \Delta \Delta G_{\text{interaction}} \) should be larger than ±0.35 kcal/mol (37) that has been reported for noninteracting residues.

Data availability

All data are contained within the article.

Author contributions—Y. Y. initiated the project. Y. Y. and J. W. designed research. P.-F. C., X.-F. M., L.-F. S., Y. T., Y.-Z. F., P. L., Z. X., and J. W. performed researches. P.-F. C., M. X. Z., C.-R. G., J. W., and Y. Y. analyzed data. P.-F. C., J. W., M. X. Z., and Y. Y. wrote the article. All authors discussed the results and commented on the article.

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Abbreviations—The abbreviations used are: AmP2X, gulf coast tick (Amblyomma maculatum) P2X; cP2X7, chicken (Gallus gallus domesticus) P2X7; HEK293, human embryonic kidney 293; HP2X3, human (Homo sapiens) P2X3; MD, molecular dynamics; PDB, Protein Data Bank; pdP2X7, giant panda (Ailuropoda melanoleuca) P2X7; rP2X7, rat (Rattus norvegicus) P2X7; RSSE, relationship between sequence, structure, and function; zfp2X4, zebrafish (Danio rerio) P2X4.

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