Altered allostery of the left flipper domain underlies the weak ATP response of rat P2X5 receptors

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Although the extracellular ATP-gated cation channel purinergic receptor P2X5 is widely expressed in heart, skeletal muscle, and immune and nervous systems in mammals, little is known about its functions and channel-gating activities. This lack of knowledge is due to P2X5’s weak ATP responses in several mammalian species, such as humans, rats, and mice. WT human P2X5 (hP2X54328–349) does not respond to ATP, whereas a full-length variant, hP2X5 (hP2X5-FL), containing exon 10 encoding the second hP2X5 transmembrane domain (TM2), does. However, although rat P2X5 (rP2X5) has a full-length TM2, ATP induces only weak currents in rP2X5, which prompted us to investigate the mechanism underlying this small ATP response. Here, we show that single replacements of specific rP2X5 residues with the corresponding residues in hP2X5 (S191F or F195H) significantly enhance the current amplitude of rP2X5. Using a combination of engineered disulfide cross-linking, single-channel recording, and molecular modeling, we interrogated the effects of S191F and F195H substitutions on the allostery of the left flipper (LF) domain. On the basis of our findings, we propose that the bound ATP-induced distinct allostery of the LF domain with that of other functional subtypes has caused the weak ATP response of rP2X5 receptors. The findings of our study provide the prerequisite for future transgenic studies on the physiological and pathological functions of P2X5 receptors.

As a class of trimeric extracellular ATP-gated ion channels permeable to cations, Na⁺, K⁺, and Ca²⁺, P2X receptors are implicated in a variety of physiological and pathological processes (1–5). Several small molecules targeting P2X receptors, like AF-219 (phase III), have entered into clinical trials (6, 7), signifying the potential of these channels as the new drug targets. So far, seven P2X genes, encoding P2X1–7 subtypes, have been cloned from both excitable (such as neurons and muscle cells) and nonexcitable (like platelets and endothelial cells) cells, where they form both homomeric and heteromeric P2X receptor complexes (8–11). The different P2X subtypes expressed in various tissues have been implicated in diverse physiological and pathological processes (3, 4).

Compared with other functional subtypes, current knowledge about P2X5 is relatively limited (4). First cloned from rat celiac ganglia, P2X5 was later found in mesencephalic nucleus, heart, and spinal cord (11). Although ATP and its analog 2-Me-S-ATP could activate P2X5, with the concentration of ATP evoking half-maximum currents through P2X receptors (EC₅₀) reaching ~10–50 μM, the current amplitudes of homomeric P2X5 from several mammalian species, including humans, mice, and rats, were strikingly small as compared with that generated by other P2X subtypes (11–14). This is true for currents recorded in both primary cells and cell lines with P2X5 overexpression. The fact that the P2X5 gene was not lost during evolution or did not become a pseudogene, like TRP2C (15), implies that this P2X subtype plays important roles in physiological and/or pathological processes. Indeed, P2X5 has been implicated in cell fate determination of myocutaneous cells (16) and sensitizing acid pain evoked by lactic acid during exercises (17). Also, the expression of P2X5 is up-regulated in polycystic kidney disease (18). Furthermore, P2X5 and P2X7 receptors have been shown to be colocized in squamous epithelial cell layers, participating in cell proliferation to differentiation (19). Additionally, heteromeric P2X1/5 (20–22) receptors are involved in neuropathic pain (23) and Ca²⁺ signaling in cortical

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astrocytes (24). Functional evidence for the existence of hetero-
meric P2X2/5 receptors was also obtained recently (25). There-
fore, homomeric or heteromeric P2X5 receptors may partici-
participate in a variety of physiological and pathological processes.
However, many questions still remain unanswered, including
1) whether or not the small sustained current induced by ATP is
responsible for the P2X5-mediated pathophysiological func-
tions mentioned above, 2) whether P2X5 functions mainly
through forming heterotrimers with other P2X subunits, and 3)
whether homomeric P2X5 can produce sizable currents under
certain conditions, such as specific developmental stages. Gene
sequencing at the different developmental stages and trans-
genic studies could provide some clues for clarifying these
questions. In the meantime, elucidating the mechanism under-
lying the weak ATP response of homomeric mammalian P2X5
receptors and identifying functional variants, especially in
model animals like rats and mice, would advance our under-
standing of P2X5 gating and contribute to future functional
studies of P2X5 using transgenic animals.

It has been demonstrated that polymorphism in exon 10 of
the human P2X gene is responsible for the loss-of-function of
hP2X5 receptors (13). Kotnis et al. (14) cloned a full-length
hP2X5 (hP2X5-FL), which is present only in a small population
of African Americans. hP2X5-FL contains an intact exon 10,
which encodes the second transmembrane (TM2)9 domain of
this receptor, and is able to develop a large current in response
to extracellular ATP. However, even with the presence of the
full-length TM2, rP2X5 still exhibited weak ATP response. In
the present study, we sought to uncover the reason for the
discrepancy between the nonfunctional rP2X5 and the functional
hP2X5-FL. We constructed a series of chimeras between the two
P2X5 receptors for functional evaluation and identified two resi-
dues in the left flipper (LF) domain as responsible for the lack of
function of rP2X5. As revealed by cysteine cross-linking, single-
channel recordings, histidine protonation and deprotonation, and
molecular dynamics (MD) simulations, the bound ATP cannot
induce allosteric changes in the LF domain like other functional
P2X subtypes, which leads to a significant decrease in the open
probability of rP2X5, and thus, the weak response to ATP.

Results

The chimera with the rP2X5 sequence Val171–Lys205 swapped
by that of hP2X5-FL enhances the ATP-evoked response

Because the common hP2X5 variant lacking exon 10
(hP2X5Δ328–349) and rP2X5 produce little or negligible sus-
tained ATP-evoked currents (11, 26), but the full-length hP2X5
(hP2X5-FL, created by inserting exon 10 into hP2X5Δ328–349)
becomes functional (Fig. 1A), we constructed 10 chimeras (Fig.
1, B–D), namely chimera 1 (CH1) to chimera 10 (CH10), with
the following rP2X5 sequences replaced by the corresponding

\[ \text{regions of hP2X5-FL: 1–30, 31–50, 51–114, 115–170, 171–205, 206–230, 231–279, 280–341, 342–362, and 363–455, respectively. Among all of these chimeras, only rP2X5C193S and rP2X5C195H acquired sizable responses to ATP (100 μM), with the maximal current densities reaching 72.4 ± 10.8 and 162.0 ± 20.6 pA/pF, respectively (Fig. 1, C and D), which are much larger than the ATP response of WT rP2X5 (2.02 ± 0.77 pA/pF). Between the two chimeras, only the current character of rP2X5C193S is comparable with that of hP2X5-FL (Fig. 1C).

To examine whether the lack or gain of the ATP responses of
the chimeras could result from altered protein surface expres-
sion, we assessed the membrane surface and total protein levels
by a cell-surface biotinylation assay and Western blotting.
Although reduced surface expressions were detected for several
chimeras, the reduction was not large enough to account for the
lack of ATP responses of some of these chimeras except for
CH4 (Fig. 1, E and F). The unchanged or slightly reduced sur-
face expressions of rP2X5C193S and rP2X5C195H further suggest
that trafficking deficit could not be the cause for the absence of
ATP responses of WT rP2X5 (Fig. 1, E and F) and that the
replaced amino acid sequences are responsible for increasing
the channel function of rP2X5C193S and rP2X5C195H.

Single substitutions (S191F and F195H) significantly increase
the ATP-evoked response of rP2X5

We focused on rP2X5C193S in subsequent experiments. This
chimera contains amino acids 171–205 of hP2X5, which differs
from Val171–Lys205 of rP2X5 by 10 residues (Fig. 2A). To iden-
tify the key residues involved in ATP activation in this region,
we made a series of rP2X5 constructs containing individual
substitutions of the 10 residues with the corresponding one
from hP2X5-FL. Whole-cell recordings revealed that two of the
single amino acid substitutions, S191F and F195H, markedly
enhanced the ATP-evoked currents of rP2X5, with the maximal
current densities reaching 99.3 ± 2.2 and 143.0 ± 19.6 pA/pF, respectively (Fig. 2, B and C), whereas other substitu-
tions displayed no or very small (L181F, 18.8 ± 5.0 pA/pF)
ATP-induced currents (Fig. 2, B and C).

Based on the crystal structures of zebrafish P2X4 (zfP2X4
receptors (27, 28), we created the homology models of rP2X5.
The homology model of rP2X5 at the open state revealed a
possible direct interaction between Ser191 and ATP (Fig. 2D).
Therefore, the Ser → Phe substitution at Ser191 may increase
the affinity to ATP and thereby restore the rP2X5 response to
ATP. Indeed, as revealed by the concentration-response curves,
the apparent affinity of rP2X5S191F to ATP was increased by >20-
fold when compared with that of rP2X5WT (EC50 = 26.8 ± 13.9
and 0.96 ± 0.31 μM, for WT and S191F, respectively; Fig. 2E).
However, even with 20 mM ATP, the current generated by
rP2X5WT was negligibly small (Fig. 2B), suggesting that the
increased apparent affinity to ATP was not the only reason for the
enhancement of ATP response in rP2X5S191F. On the other hand,
Phe195 is not located within the ATP-binding pocket of rP2X5 (Fig.
2D), but the Phe → His substitution (rP2X5F195H) still exhibited
an increased ATP response (Fig. 2, B and C). Interestingly,
rP2X5F195H had a slightly decreased, rather than increased, appar-
ent affinity to ATP (EC50 = 45.4 ± 2.0 μM; Fig. 2E) as compared
with rP2X5WT, further supporting our speculation. Furthermore,
there was no significant difference in the cell-surface expression of S191F and F195H as compared with WT rP2X5 (Fig. 2, G and I), and increasing the amount of rP2X5WT cDNA in the transfection (6 μg) failed to improve the ATP response (Fig. 2C). Therefore, we postulated that the S191F and F195H substitutions might have elevated the ATP response of rP2X5 by altering the channel gating, rather than increasing the protein expression.

To further evaluate the effects of these residues on channel gating of P2X5, we replaced Phe191 and His195 of hP2X5-FL with the corresponding residues of rP2X5, namely hP2X5-FLF191S and hP2X5-FLH195F. The substitution of F191S, but not of H195F, noticeably decreased the maximal ATP response of hP2X5-FL (\(i_{\text{max}} = 39.2 \pm 9.9\) pA/pF for hP2X5-FLF191S and 278.0 ± 52.8 pA/pF for hP2X5-FLH195F; Fig. 2F). Both mutants showed a slight decrease in cell-surface expression, but such a decrease is unlikely to account for the large reduction in the current density of hP2X5-FLF191S, especially given that despite the similar decrease in surface expression, hP2X5-FLH195F exhibited similar current density as hP2X5-FLWT (Fig. 2, H and J). Altogether, these results suggest that Ser191 is mainly responsible for the extremely small ATP-evoked current of rP2X5 receptors.

The gain-of-function of rP2X5S191F may result from an altered allostery of the LF domain

In the homology model of rP2X5, the residue at position 191 makes direct interactions with ATP (Fig. 3A). Structural
Dynamic comparisons between rP2X5<sup>WT</sup> and rP2X5<sup>S191F</sup> using MD simulations followed by ligand root mean square (r.m.s.) fluctuation analysis revealed that the triphosphate groups (atoms 1–10) and the purine ring (atoms 20–30) of ATP were more stable in rP2X5<sup>S191F</sup> than in rP2X5<sup>WT</sup> (Fig. 3, B and C), probably because the phenylalanine at this position makes more contacts with ATP (Fig. 3D, middle). By contrast, a serine in the same position dramatically reduced the interaction between ATP and rP2X5 (Fig. 3D, top). Further analysis showed that the introduction of phenylalanine significantly enhanced the hydrophobic interaction between rP2X5<sup>S191F</sup> and ATP (Fig. 3E, middle). As revealed by a saturation-mutation screening of Ser<sup>191</sup>, in a certain range, along with the increase in the steric hindrance of the side chain, the response to ATP approached closer and closer to that of hP2X5-FL<sup>WT</sup> (I<sub>max</sub> = 23.3 ± 1.6, 69.1 ± 15.1, 99.3 ± 2.24, and 369 ± 31 pA/pF, for S191L, S191M, S191F, and S191Y, respectively; Fig. 3, F and G). Nevertheless, an oversized steric hindrance may also be unfavorable, such as S191W (Fig. 3, F and G). As for S191P, the imino group in the proline side chain made it unable to form the β-fold and consequently resulted in a functional loss (Fig. 3G).

Other amino acid substitutions did not significantly increase the ATP response of rP2X5 (Fig. 3, F and G), probably due to the altered shape and hydrophobic/hydrophilic properties of the ATP-binding pocket (like S191E/D/K/R/H) or improper/insufficient contacts with ATP (such as S191G/C/T/N/Q/V/I).

Figure 2. Single amino acid replacement (S191F or F195H) markedly enhances the ATP response of rP2X5. A, sequence alignment of the segment 171–205 in rP2X5-WT and hP2X5-FL, with amino acid residues different between rP2X5<sup>WT</sup> and hP2X5-FL labeled in gray. B and C, representative current traces (B) and pooled data (C; mean ± S.D. (error bars), n = 3–4) of rP2X5<sup>S191F</sup>, rP2X5<sup>F195H</sup>, and rP2X5<sup>WT</sup> induced by 100 μM ATP or 20 mM ATP. *, p < 0.05; **, p < 0.01 versus WT, one-way ANOVA with Bonferroni post hoc test (F<sub>(10, 30)</sub> = 113.7, p < 0.0001). D, zoom-in view of the constructed model of rP2X5 based on the open structure of zfP2X4 to illuminate the location of sites Ser<sup>191</sup> and Phe<sup>195</sup>. E, concentration-response curves of rP2X5 and its mutants (mean ± S.E. (error bars), n = 3–5). Each solid line is a fit of the Hill equation to the ATP-dependent activation. F, typical recordings (top) and pooled data (bottom) of hP2X5-FL and its mutants induced by 100 μM ATP (mean ± S.D. (error bars), n = 4–6). *, p < 0.05; **, p < 0.01 versus WT, one-way ANOVA with Bonferroni post hoc test (F<sub>(2, 11)</sub> = 10.87, p = 0.0025). G–J, representative Western blotting (G and H) and mean values (I and J) of protein expressions of rP2X5<sup>WT</sup> and its mutants (G and I) and hP2X5-FL and its mutants (H and J) transfected in HEK-293 cells. At least three experiments were performed. *, p < 0.05; **, p < 0.01 versus WT, one-way ANOVA with Bonferroni post hoc test (pooled data in I, F<sub>(2, 6)</sub> = 0.2886 (p = 0.7591) and F<sub>(2, 6)</sub> = 1.052 (p = 0.4059) for total and surface expressions, respectively; data in J, F<sub>(2, 6)</sub> = 6.948 (p = 0.0274) and F<sub>(2, 6)</sub> = 1.063 (p = 0.4025) for total and surface expressions, respectively).

Altered allostery of the LF domain during rat P2X5 gating

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Because 20 mM ATP could not effectively activate rP2X5WT (Fig. 2B), the enhanced channel function of rP2X5S191F cannot be simply attributed to the increased ATP affinity. Based on the crystal structures of P2X4 and P2X3 receptors at both open and closed states (27–29), we have previously demonstrated that the relative motions of the LF and DF domains induced by ATP, namely the downward movement of the LF domain and the upward movement of the DF domain, play a vital role in the channel activation of P2X receptors (30, 31). In addition, the downward motion of the LF domain facilitates the intersubunit physical coupling, which transmits ATP binding to channel gating of P2X receptors (32). Therefore, we built homology models of rP2X5WT, rP2X5S191F, and rP2X5S191Y at both the apo/closed and open states and examined the effects of S191F and S191Y substitutions on the motions of LF and DF domains as well as channel gating. As revealed by these models, phenylalanine at site 191 makes more hydrophobic interactions than serine with the residues of the DF (Ile231 and Leu217), lower body (Ile289 and the aliphatic chain of Lys193), and LF domains (Val208), which may promote the final conformational changes in those domains (Fig. 4A). Expectedly, MD simulation analysis of rP2X5WT and rP2X5S191F showed that S191F evoked significant conformational changes of the LF and DF domains at the apo/closed state, especially for the LF domain (Fig. 4B). Comparing Cα–Cα distances among 191⋅Ile289⋅Lys214 (Fig. 4, B and C) and pair distributions of the distance of center of mass (COM) among the LF, DF, and β-sheet (in the lower body domain) (Fig. 4D) during MD simulations of rP2X5WT and rP2X5S191F, we found that Phe191 more effectively pushed the LF domain away from the ATP-binding pocket than Ser191 (Fig. 4, B–E). Meanwhile, the DF domain migrated slightly outward and eventually shortened its distance from the LF domain (Fig. 4, B–E). These Phe191-induced alterations in the conformation of rP2X5 are in line with the conformational changes required for channel gating after ATP binding (Fig. 4A), implying that Phe191 may facilitate the LF domain to prime channel gating of rP2X5S191F. We also performed additional MD simulations on rP2X5S191Y and found that the bulkier side chain of Tyr191 produced even more contacts with ATP than Phe191 (Fig. 3, D and E, bottom panels), including stronger hydrophobic and water-aided hydrogen-bond (H-bond) interactions. These interactions led to ~10-fold increase in the apparent affinity to ATP (Fig. 2E). The additional contacts also led to altered conformation of the LF and DF domains both at the resting (Fig. 4, D and E) and open states for rP2X5S191Y (Fig. 3, D and E, bottom panels), indicating that the increased ATP response of S191Y pos-

**Figure 3.** Phenylalanine or tyrosine introduced at position 191 produces additional contacts with the bound ATP. A, zoom-in view of the constructed model of rP2X5S191F based on the open structure of zfP2X4. B and C, r.m.s fluctuation analysis of each atom (C) and of ATP when it binds to rP2X5WT or rP2X5S191F (B). D, timeline representations of the P2X5 WT– or mutant–ATP interactions and contacts, including H-bonds and hydrophobic, ionic, and water bridge interactions. Some residues producing more than one specific contact with the ATP are represented by a darker shade of orange. E, P2X5–ATP interactions monitored throughout the simulations of rP2X5WT, rP2X5S191F, or rP2X5S191Y. F and G, representative current traces and pooled data (mean ± S.E. (error bars), n = 3–4) of rP2X5WT, and mutations with substitutions in site Ser191 for hydrophobic residues activated by 100 μM ATP (F). *, p < 0.05; **, p < 0.01 versus WT, one-way ANOVA with Bonferroni post hoc test (F(19, 53) = 31.77, p < 0.0001).
sibly resulted from some different allosteric changes of the LF and DF domains as compared with that induced by S191F.

We further tested the idea that S191F and S191Y resulted in conformational changes of the LF and DF domains by using an engineered disulfide cross-linking approach (33) (Fig. 5A). As revealed by the MD simulations, if the LF domain was pushed outward by Phe191 (Fig. 5B), the Cα-Cα distance between Ile289 (in the LF domain) and Lys214 (in the DF domain) would become much closer (from 11.2 to 6.2 Å; Fig. 4, C and E), making the formation of the intersubunit disulfide bond possible. The MD simulations of the rP2X5S191F/K214C/I289C cross-linking model revealed that the dihedral angle χCB-Sγ-Sγ-Cβ of this engineered disulfide bond was near 91º (Fig. 5A and B), a typical dihedral angle for a disulfide bond in proteins (33). These predictions suggest that, compared with rP2X5K214C/I289C, it would be easier to detect disulfide bond formation in rP2X5S191F/K214C/I289C. Indeed, the protein band representing the trimeric receptors, which requires the formation of intersubunit disulfide bonds, was significantly increased in immunoblots of lysates from cells that expressed rP2X5S191F/K214C/I289C (Fig. 5, C and D). This band was reduced to the monomeric size by the treatment of rP2X5S191F/K214C/I289C with 1% DTT, demonstrating the disulfide cross-linking between the subunits (Fig. 5C). Meanwhile, under reducing conditions, the total protein levels (reduced into monomers) of rP2X5K214C/I289C and rP2X5S191F/K214C/I289C were not significantly different (Fig. 5, C and E), indicating that the increase in the trimeric rP2X5S191F/K214C/I289C was not due to nonspecific aggregation, which could occur because of excessive overexpression. Similar results were also observed in the mutant rP2X5S191Y/K214C/I289C (Fig. 5, C–E).

Therefore, S191F or S191Y substitution significantly altered the conformations of LF and DF domains of rP2X5. Because the LF domain is a flexible loop, whereas the DF domain is a rigid α-helix (Figs. 3A and 4B), Phe191 might exert more influence on the LF domain, which primes it to the conformation required for channel gating at the apo-state. This point is further supported by MD simulations (Fig. 4, B–E). Moreover, Phe191 could make direct hydrophobic contacts with residues of the DF, LF, and lower-body domains (Fig. 4A), and the bound ATP could further promote the relative motions of the LF and DF domains through its direct interaction with Phe191 (Fig. 4A). We have previously suggested that the interdomain hydrophobic interactions among the LF, DF, and lower-body domains pose an energy barrier for channel gating (31), and allosteric events that alter the hydrophobic interactions, with concomitant coordinated motions of LF and DF domains, are essential for channel gating of P2X4 receptors (30). Accordingly, the weak ATP response of rP2X5 may be attributed to the high energy barrier (30) for the channel gating of WT rP2X5 because its LF domain is deeply buried into the ATP-binding pocket. Thus, the alterations in the conformations of LF and DF domains, especially for the LF domain, introduced by S191F possibly decreased the energy barrier of channel gating and thereby resulting in the larger ATP response.

The increased current amplitude of rP2X5F195H is attributed to additional interactions between His195 and the LF domain

Additionally, the functional restoration of rP2X5F195H could also be attributed to an altered conformation of the LF domain. Although phenylalanine and histidine possess similar-size side
chains (Fig. 6A), the introduction of histidine in rP2X5F195H brings two nitrogen atoms into the side chain, which would facilitate the formation of an H-bond with other residues (Fig. 6A). Given that His195 is surrounded by nonpolar residues Val67, Ala65, and Ala206 in the lower-body domain (Fig. 6B), we could exclude the possibility that His195 directly interacts with these residues through an H-bond. Therefore, the gain-of-function of rP2X5F195H most likely resulted from polar contacts between the side chain of His195 (imidazole ring) and the LF domain (Fig. 6C). We performed MD simulations of rP2X5 under the conditions with or without bound ATP (Fig. 6D). Further H-bond interaction analysis between His195 and residues of the LF domain in rP2X5F195H suggested that His195 could produce more H-bond contacts with residues of the LF domain when ATP binds to rP2X5 (Fig. 6D), indicating that His195 mainly contributes to the conformational change of the LF domain at the open state instead of the resting state.

To test this hypothesis, we performed alanine-scanning mutagenesis in the LF domain of rP2X5F195H to identify residues that may produce additional contacts with the imidazole ring of His195. Indeed, F195H/S291A and F195H/G292A exhibited a complete loss of the ATP current of F195H ($I_{\text{max}} = 7.03 \pm 1.15$ and $1.48 \pm 0.34$ pA/pF for F195H/S291A and F195H/G292A, respectively; Fig. 6E and F), whereas F195H/D282A ($I_{\text{max}} = 59.0 \pm 11.3$ pA/pF) and F195H/T286A ($I_{\text{max}} = 94.2 \pm 12.8$ pA/pF) had partially decreased ATP-induced currents compared with rP2X5F195H. Thus, the increased current amplitude of rP2X5F195H relied on the interactions between His195 and residues in the LF domain, including Asp282, Thr286, Ser291, and Gly292, especially Ser291 and Gly292 (Fig. 6C). Importantly, equivalent mutations added on rP2X5S191F (rP2X5S191F/S291A and rP2X5S191F/G292A) and hP2X5-FLWT (hP2X5-FLS291A and hP2X5-FLG292A) did not affect the maximal ATP responses of the respective receptors (Fig. 6G), indicating that the interactions are specific to His195 and Ser291/Gly292-mediated functional restoration of rP2X5F195H.

To further examine whether histidine interacts with the LF domain through its nitrogen atoms in the imidazole ring of rP2X5F195H, we changed the pH value of bath solutions. The maximal ATP-evoked current of rP2X5F195H was significantly reduced ($I_{\text{max}} / I_{\text{pH}} = 0.38 \pm 0.05$, $p < 0.01$, $n = 4$) in the alkaline environment (pH 9.0) but unaffected (pH 6.0) or slightly
increased (pH 4.0) in the acidic pH (Fig. 6, H and I), suggesting that His\(^{195}\) needs to be protonated to form H-bonds with the oxygen atoms of the main chain of Gly\(^{292}\) or Ser\(^{291}\). In addition, according to the homology model of rP2X5\(^{F195H}\) at the closed state, the distances between the nitrogen atom of His\(^{195}\) (p\(K_a\) 6.0; Fig. 6 A, right) and the oxygen atom of Gly\(^{292}\) or Ser\(^{291}\) were 7.4 and 9.8 Å, respectively (Fig. 6 C). During the gating process, these distances will be shortened to 3.0–3.5 Å when H-bonds His\(^{195}\)-N ... H ... O-Gly\(^{292}/\)Ser\(^{291}\) were formed (Fig. 6 C). The H-bond contact would therefore facilitate the downward motion of the LF domain. The fact that the ATP response of rP2X5\(^{F195H}\) could be increased to a level similar to that of hP2X5-FL also suggests a lack or much weaker allosteric change of the LF domain during the channel gating of rP2X5\(^{WT}\). Moreover, pH variations did not affect the activation of rP2X5\(^{S191F}\) (Fig. 6, G and H), indicating that the H-bond contact His\(^{195}\)-N ... H ... O-Gly\(^{292}/\)Ser\(^{291}\) may be a mechanism specific for the functional restoration of rP2X5\(^{F195H}\). The increase in the current amplitude of chimera CH3 relies on a residue substitution located under the LF domain.

To understand the molecular basis for the partial gain-of-function of the chimera CH3, the 10 discrepant residues in the sequence (residues 51–114) of rP2X5 were individually replaced by their corresponding residues in hP2X5-FL (Fig. 7, A and B). Among the 10 residue substitutions, only mutation rP2X5\(^{V67I}\) could increase the ATP response of rP2X5 (\(I_{\text{max}}\) 28.6 ± 4.35 pA; Fig. 7, E and F), indicating that the partially increased channel function was not due to the altered surface expression of rP2X5\(^{V67I}\). Interestingly, differing only by one methyl group, the substitu-

Figure 6. The gain-of-function of F195H is also attributed to altered allostery of the LF domain. A, two-dimensional structures of phenylalanine (left) and histidine (right). The p\(K_a\) value of the nitrogen atom, being much closer to the carbon atom of the main chain, in the imidazole ring is 6.0, and this atom can act as either donor or acceptor of H-bonding contacts in different pH values. B and C, zoom-in view of the homology models of rP2X5\(^{F195H}\). Introduced His\(^{195}\) is surrounded by a series of nonpolar amino acids (B). The distances from the oxygen atoms of Ser\(^{291}\) and Gly\(^{292}\) to the nitrogen of His\(^{195}\) are 9.8 and 7.4 Å, respectively, if ATP does not repel the LF domain out of the ATP-binding pocket. Those distances might be shortened significantly once the channel is opening after the H-bonding contacts are established between His\(^{195}\) and Ser\(^{291}\) or Gly\(^{292}\). D, total number of H-bonds between His\(^{195}\) and residues of the LF domain detected in rP2X5\(^{F195H}\) during MD simulations. E and F, representative current traces (E) and pooled data (F) (mean ± S.D. (error bars), \(n = 3–5\) of rP2X5\(^{F195H}\), rP2X5\(^{F195H/S191A}\) and rP2X5\(^{F195H/G292A}\) respectively). G and H, summarized effects of mutations S291A and G292A on maximal ATP-evoked currents of hP2X5-FL and rP2X5\(^{S191F}\) (mean ± S.D. (error bars), \(n = 4–6\) ). H-bond contactogram (H) and mean values (I) (mean ± S.D. (error bars), \(n = 3–7\) of rP2X5\(^{F195H}\) and rP2X5\(^{S191F}\) induced by 100 \(\mu\text{M}\) ATP in acidic (pH 6.0 or 4.0) and basic (pH 9.0) bath solutions (\(F(3, 15) = 35.50 (p < 0.0001)\) and \(F(2, 9) = 0.02126 (p = 0.9790)\) for F195H and S191F, respectively).
tion of Val67 for isoleucine, which is located under the LF domain (Fig. 7B), significantly increased the ATP-evoked current of rP2X5. This may be due to the slightly increased steric hindrance that facilitates the repulsion of the LF domain after ATP binding, like the effect induced by S191F. Moreover, given that Ile67 is adjacent to Gly292 and Ser291 (Fig. 7B, bottom), it may have a similar effect as His195, pushing Ser291–Gly292 away from the ATP-binding pocket after ATP binding. Despite the difference among the mechanisms underlying the increased channel functions of S191F, F195H, and V67I, one thing is clear, that all three mutations more or less modulate the conformation of the LF domain. These mutations either force the LF domain to stay away from the ATP-binding pocket at the resting state or facilitate the LF domain to move away from the pocket after ATP binding. In other words, the ATP binding to rP2X5WT could not effectively cause the LF domain to move downward, a motion essential for the conformational transition from the ATP-binding pocket to the channel pore; therefore, rP2X5 receptors exhibit little or no response to ATP.

The proper conformational changes of the LF domain in rP2X5S191F or rP2X5S191Y allow normal state transition from resting to open and finally desensitization

Finally, to examine the influence of the normal allostery of the LF domain on channel gating of rP2X5, we compared the single-channel currents between rP2X5S191F and rP2X5WT.
Currents from outside-out patches revealed that saturated ATP concentrations could induce the opening of rP2X5WT, with the majority of the open channels under the subconductance states (0.6 pA, 120 mV) (Fig. 8, A and B). In contrast, most rP2X5S191F channels were at a completely opened state in the presence of saturating ATP (1.0–1.2 pA, 120 mV; Fig. 8, C and D). Moreover, the single-channel conductance of rP2X5S191F is not different from that of that full open state of rP2X5WT (10–11 picosiemens), indicating that the conformational changes of the LF domain facilitates the transitions of the channel states, rather than affecting the unitary conductance, which may be attributable to the fact that the LF domain is located far away from the ion permeation pathway (34).

In addition, by macroscopic outside-out patch recordings of rP2X5WT (Fig. 8E), we found that although the number of open channels was low, this number was kept stable even after 1-min application of ATP (0.1 μM), indicating that there were no or only a small proportion of channels that underwent desensitization after a long exposure to ATP. By contrast, the macroscopic patch recordings of rP2X5S191F (Fig. 8F) revealed that as low as 1 μM ATP instantaneously activated most of the channels in the patch, but 90% of them desensitized at ~1.5 s after the ATP application, and all channels desensitized at ~3–5 s after the ATP application. Therefore, S191F-induced conformational changes of the LF domain can effectively increase the open possibility of rP2X5, by promoting the state transitions from rest to open and finally to desensitization states (Fig. 9). Any factor that impedes this allosteric process would reduce the probability of the channels becoming fully open, trapping them in various subconductance states, as in the case of rP2X5WT (Fig. 8A). Similar results were also observed in both micro- and macroscopic outside-out patches of rP2X5S191Y (Fig. 8, G and H).

**Discussion**

P2X receptors are trimeric channels activated by extracellular ATP. They are involved in various physiological and pathological processes. Despite the wide expression of P2X5 in several systems, little is known regarding its gating properties and functions, partially due to the extremely small responses of several cloned mammalian P2X5 receptors to ATP. Here, we generate a series of rP2X5 chimeras containing a certain region of the hP2X5-FL sequence and examined their ATP responses, aiming to uncover the mechanisms underlying the weak ATP-evoked currents of rP2X5. Our results revealed that two rP2X5 chimeras, CH3 and CH5, produced significant ATP-evoked currents. Subsequent single-residue substitution experiments identified two key residues (Ser191 and Phe195) that are responsible for the weak responses of rP2X5 to ATP. Both S191F and
F195H substitutions could push the LF domain downward away from the ATP-binding pocket, which consequently increases the channel function of rP2X5, probably by reducing the energy barrier of gating that traps the channel in the subconductance states (Figs. 8 and 9).

Recent advances in the structural determinations of P2X receptors (including P2X3, P2X4, and P2X7) from different species (human, zebrafish, chicken, rat, and panda) have greatly improved our understanding of the molecular mechanism of the P2X gating processes (27–29, 35–37). Previous studies have demonstrated that the relative motions between the LF and DF domains, evoked by bound ATP, are critical for channel gating of P2X4 receptors, whereas impeding this movement can impair or even abolish channel activations (30). Although P2X5 receptors are different from well-investigated P2X4 receptors in many aspects, such as the amino acid sequences, the ATP sensitivity, and current properties, etc. (4), the fact that rP2X5 mutants (rP2X5S191F, rP2X5F195H, and rP2X5V67I) attained enhanced channel function by facilitating the LF conformation switch during the activation suggests a similar role of the LF domain in rP2X5 activation. The saturation mutation study at Ser191 showed that only amino acids with the proper size of the side chain at this site could significantly boost channel function of the rP2X5 receptors. Interacting with ATP and further pushing the LF domain outward, Phe191 or Tyr191 triggers a conformation switch correlated with the channel gating of P2X receptors (30). On the contrary, deprotonation of the side chain of His195 abolished its interaction with residues Gly292 or Ser291 in the LF domain and significantly impaired the channel function restored by F195H. All of these results demonstrate the importance of the allostery of the LF domain in the gating processes of P2X receptors. Because the allostery of the LF domain is critical for the gating processes of both P2X5 and P2X4 receptors (32), it is reasonable to suggest that ATP binding–induced conformational changes of LF domain are also very important for other subtypes of P2X receptors, despite the fact that the sequence of this domain is not very conserved among different subtypes (38).

Among all of the P2X5 receptors reported so far, robust ATP responses were observed in frog P2X5 and chicken P2X5 (39, 40), whereas rat, mouse, and human P2X5 exhibited small ATP-evoked currents (11–14). It seems that the species that appeared later in animal evolution are more likely to exhibit small ATP responses of P2X5. At present, there is no well-accepted explanation for this phenomenon. It is possible that these genes were cloned from organisms at different development stages or that it is simply due to the difference in cloning protocols. Despite the small ATP response of rP2X5, one cannot rule out the possibility that the P2X5 receptor is required for normal physiological functions of these species. Notably, most of the functional P2X5 receptors were cloned from animals in the early developmental stages; for example, the frog P2X5 was cloned from larval amphibian skin (39), and the chicken P2X5 was cloned from embryonic muscle tissues (40). It is possible that functional P2X5 receptors may also be present in animals at early developmental stages; for example, the frog P2X5 was cloned from larval amphibian skin (39), and the chicken P2X5 was cloned from embryonic muscle tissues (40). In this study, we aimed to determine the mechanism underlying the weak ATP response of rP2X5. Enlightened by the finding that inserting exon 10 of hP2X5 restored the channel function (13, 14), we constructed a series of rP2X5 chimeras and mutants and identified functional rP2X5 variants like rP2X5 S191F, rP2X5F195H, and rP2X5V67I. Although the functional variants we reported here may not exist in nature, we cannot rule out the existence of other functional natural rP2X5 variants, which may exert distinct functions in certain populations or under pathological conditions, like the hP2X5-FL (13, 14). Future gene sequencing at the different development stages of those species may be able to clarify this issue.

It is known that ion channel receptors could express both functional and nonfunctional forms at the same or different development stages (41). For example, there are two isoforms of nicotinic acetylcholine receptors (nAChRs), namely embryonic and mature nAChRs. In certain situations, the conversion of embryonic nAChRs to the mature ones is reversible (42). For example, blocking the synaptic activities by cutting off axons or...
Altered allostery of the LF domain during rat P2X5 gating

blocking nAChRs can accelerate the synthesis of new nAChRs. In addition, electric stimulation of the denervated muscle fibers can directly inhibit the synthesis of embryonic nAChRs (43). AMPA receptors can also be classified into two isoforms distinguished by calcium permeability: calcium-permeable (GluR2-Q) and calcium-impermeable (GluR2-R), which differ from each other by a single amino acid (44). Although most reported AMPA receptors are calcium-impermeable, studies have shown that calcium-permeable AMPA receptors play important roles as well. The latter have been associated with the abnormal calcium activity in the early stages of Alzheimer’s disease and could be a potential drug target for this disease (45). Our finding that single-residue substitution could switch nonfunctional rP2X5 to functional variants (e.g. rP2X5S191F, rP2X5F195H, and rP2X5V67I) raises the possibility that P2X5 might exist in both functional and nonfunctional isoforms at the same or different development stages. However, future studies are needed to conclusively demonstrate this point.

Finally, it is possible that a functional P2X5 receptor with robust ATP response may be harmful to mature animals, and the rP2X5 with weak ATP response may be necessary for normal physiological functions. For example, the P2X5 with weak ATP response can assemble heterogeneously with other functional subtypes (20–25, 46), like P2X1/5 and P2X2/5. Also, the small, sustained ATP response may be involved in the regulation of other ion channels or proteins, such as the ischemic pain induced by P2X5-mediated sensitization of acid-sensing ion channel 3 (ASIC3) during anaerobic exercise (17). Such kinds of interactions have been observed in other P2X subtypes as well. For example, when P2X2 was co-expressed with nAChRs, simultaneous activation of both types of channels produces much smaller currents when compared with currents evoked by activating a single channel type each time, suggesting a direct or indirect interaction between these two channel types (47). Very recently, it was demonstrated that ASIC3 and P2X3 form a pain-relevant cognate receptor (48). The two proteins do not appear to form a heteromeric channel, but they tightly associate with each other to form a protein complex, mediating unidirectional inhibition (48). Thus, P2X5 receptors might exert its role through assembling heterogeneously with other functional P2X subtypes or interacting with other proteins.

In summary, we demonstrate that single-residue substitution can increase the current amplitude of rP2X5 receptors (e.g. rP2X5S191F, rP2X5F195H, and rP2X5V67I). Expression of these functional mutants in model animals may provide important information about whether the rP2X5 receptors with weak ATP response are necessary for normal physiological functions and whether the functional variants with robust ATP response, like rP2X5S191F, might be harmful to mature animals.

Experimental procedures

Drugs and mutagenesis

Most drugs were purchased from Sigma. The cDNAs of rP2X5 and hP2X5 were kind gifts of Drs. Lin-Hua Jiang, Alan North, and Tian-Le Xu. They were then subcloned into the pEGFP-N1 and/or pCDNA3.0 (with EE-tag) vectors. All mutants were constructed by the QuikChange mutagenesis kit and were verified by DNA sequencing as we described previously (49, 50). The QuikChange mutagenesis kit was also used for inserting a short sequence into the expression construct by carefully designed primers. The exon 10 of hP2X5 is a little bit too long (~60 bp) to be inserted into the target construct through one PCR; therefore, we divided the whole exon 10 sequence into two parts and inserted them into the hP2X5 one by one to finally obtain full-length hP2X5 (hP2X5-FL). For the construction of various chimeras between the hP2X5-FL and rP2X5, ~20–60 bp of rP2X5 were replaced by the identical sequence of hP2X5-FL according to the procedure used in the insertion of exon 10 into hP2X5 above.

Cell culture and electrophysiology

As described previously (32, 51), human embryonic kidney 293 (HEK-293) cells were cultured in conditions containing 88% Dulbecco’s modified Eagle’s medium, 10% FBS, and antibiotics, and their suitable growth environment was 5% CO2 and 95% air in a humidified atmosphere at 37 °C. The transfection of plasmids was performed using Hilymax (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer’s protocol.

Whole-cell recordings under the voltage clamp were carried out 24–48 h after transient transfection using Axon 200B at room temperature (23 ± 2°C). The recording electrodes were filled with pipette solutions, and the resistance was about 3–5 mehmohms. Membrane currents were measured with a patch-clamp amplifier Axon 200B (Molecular Devices) and low-pass-filtered at 2 kHz. All currents were sampled and processed using a Digitata 1440 interface and a computer operating the Clampex and Clampfit version 14.0 software (Molecular Devices). The standard solution for cell incubating contained 150 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 1 0m M EGTA, and the pH was modulated to 7.2–7.4. The standard internal solution infused into patch electrodes included 30 mM NaCl, 120 mM KCl, 1 mM MgCl2, 0.5 mM CaCl2, and 5 mM EGTA, and the pH was modulated to 7.2 using Tris-base. During the process of electrophysiological recordings under voltage clamp conditions, the clamp value of membrane potential was set at ~60 mV. Drugs were dissolved in external solutions and applied by means of a fast pressure-driven computer-controlled microperfusion system OctaFlow08P (ALA Scientific Instruments). Dose-response curves were obtained from recording a variety of ATP concentrations, the corresponding currents were normalized to the maximum current amplitude, and currents were recorded every 20 min with ~10–20-s ATP application. Single-channel recordings using outside-out configuration were performed 24–48 h after transient transfection at room temperature (23 ± 2°C). Recording electrodes were drawn from borosilicate glass (World Precision Instruments, Inc.) and polished to produce resistance of about 5–10 megohms. The holding membrane potential was set at ~120 mV, and the external and internal solutions are the same as for the whole-cell recording. Single-channel currents were recorded at 50 kHz with a 2-kHz filter and low-pass-filtered at 300 Hz using the AxonPatch 200B amplifier in conjunction with pClamp 14 software (Molecular Devices).
Cell-surface biotinylation and Western blot analysis

Cell-surface biotinylation and Western blotting were carried out according to protocols used previously (30). rP2X5WT or mutants were expressed in HEK-293 cells. These cells were washed with chilled PBS and then bathed in solution containing sulfo-NHS-LC-biotin. This incubation reaction was ceased with glycine (20 mM) dissolved in PBS. Finally, the treated cells were gathered and lysed with radioimmune precipitation assay buffer. 10% of the volume of supernatant was separated and used as total protein fraction. The rest of the biotinylated proteins were attached to NeutrAvidin-linked agarose resin by 3–5-h incubation at 4°C and then rinsed several times with chilled PBS. These proteins were used as surface proteins. All proteins were boiled with SDS-loading buffer for 5 min at 95°C with 1% β-ME (for reducing experiments) or not (for nonreducing experiments). These proteins were analyzed by SDS-PAGE. With an 8–10% separation gel and a 4% stacking gel, these samples were separated and then transferred to a polyvinylidene difluoride membrane. The polyvinylidene difluoride membrane was blocked with 5% milk dissolved in PBS at room temperature for 1–2 h and then incubated overnight at 4°C with anti-EGF tag (1:1,000; Roche Applied Science), anti-EE tag (1:1,000; Cell Signaling Technology), or anti-GAPDH (1:2,000; Sungene Biotech) antibodies diluted with 5% milk as ratio shown above. The membrane was further incubated with HRP-conjugated secondary antibodies for EGF tag (25°C, 1 h, 1:1,000; goat anti-rabbit IgG(HL)-HRP; Sungene Biotech) or GAPDH (25°C, 1 h, 1:3,000; goat anti-mouse IgG(HL)-HRP; Sungene Biotech), and finally exposed by using the ImageQuant RT ECL system (GE Healthcare) for 1–3 min by means of ECL solution (Thermo Fisher Scientific). Analysis of protein expression was repeated by at least three independent experiments.

Homology modeling

As described previously (52), program Modeler 9.9 (53) was used to create homology models of rP2X5 and its mutants on the basis of the crystal structures of zfP2X4 receptors in closed (Protein Data Bank code 4DW0) and open (Protein Data Bank code 4DW1) states (27, 28). The alignment of sequence was ordered and adjusted according to the published alignment (27). The raw models were further optimized and minimized by using the OPLS_2005 force field. Finally, the program ProCheck (54) was used to verify those deeply processing models.

MD simulations

As described previously (30, 31, 55), the program DESMOND (56) was used to perform all MD simulations with a constant number of particles, pressure (1 bar), and temperature (300 K) and periodic boundary conditions, which use a particular “neutral territory” method called the midpoint method to efficiently exploit a high degree of computational parallelism. A default OPLS_2005 force field together with the functional form of the OPLS-AA family of force fields were used for the P2X5 and ATP molecules. The starting structures for MD simulations were energy-minimized by DESMOND. A suitable membrane system in which the transmembrane domain of the rP2X5 might be buried was generated with a large constructed 1-palmitoyl-2-oleoylphosphatidylcholine bilayer, this system was subsequently deposited in simple point charge water molecules. The subsequent addition of counter ions made up for the net negative charge of the system. NaCl (150 mM) was thrown into a simulation box that represents background salt at physiologic conditions. All of the MD simulations ran on the DAWNING TC2600 system. Preparation, analysis, and visualization were executed on a DELL T7500 graphic workstation. In light of protocols we used previously (31, 55), the distance and dihedral angle among the atoms were analyzed using the simulation event analysis (SEA) module of Desmond; the r.m.s. fluctuation analysis of protein and ligand and interaction analysis between ATP and rP2X5 (WT and mutated receptors) were carried out in the simulation interaction diagram (SID) module; the pair distributions of the COM among various domains during MD simulations were performed in the radial distribution function module of Desmond.

Data analysis

All results are expressed as mean ± S.D. Statistical comparisons were made using the value of t test, pair t test, or one-way ANOVA as a standard, 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 activated by different ATP concentrations, and data were collected from 3–8 cells. Current collected from various ATP concentrations was normalized by the maximal current; these data then were fitted to the Hill equation, \[ I/I_{\text{max}} = 1/(1/(EC_{50}/[\text{ATP}])^n) \], where I is the normalized current at a given concentration of ATP, \( I_{\text{max}} \) is the maximum normalized current, \( EC_{50} \) is the concentration of ATP yielding a current that is half of the maximum, and n is the Hill coefficient.

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