On the Role of the First Transmembrane Domain in Cation Permeability and Flux of the ATP-gated P2X2 Receptor*

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P2X receptors are a family of seven ligand-gated ion channels (P2X1–P2X7) that open in the presence of ATP. We used alanine-scanning mutagenesis and patch clamp photometry to study the role of the first transmembrane domain of the rat P2X2 receptor in cation permeability and flux. Three alanine-substituted mutants did not respond to ATP, and 19 of the 22 functional receptors resembled the wild-type receptor with regard to the fraction of the total ATP-gated current carried by calcium or the permeability of calcium relative to cesium. The remaining three mutants showed modest changes in calcium dynamics. Two of these occurred at sites (Gly40 and Phe43) that are unlikely to interact with permeating cations in a meaningful way. The third was a conserved tyrosine (Tyr43) that may form an inter-pore binding site for calcium. The data suggest that, with the possible exception of Tyr43, the first transmembrane domain contributes little to the permeation properties of the P2X2 receptor.

The notoriety of ATP-gated P2X receptors continues to grow. In large part, this reflects the discovery of an increasing number of physiological roles that can be ascribed to these ligand-gated ion channels, including such fundamental tasks as osmoregulation in amoeba (1) and sensation in mammals (2). By contrast, a reliable description of the biophysics of the channel is less forthcoming, due largely to the fact that the structure of the pore is unsolved. Each P2X receptor is a complex of three subunits arranged around an intrinsic ion channel permeable to Na+, K+, Ca2+, and, in some cases, Cl− (3, 4). Individual subunits have two transmembrane segments, called TM1 and TM2, both of which may line the permeation pathway (5).

In this study, we consider the role of TM1 in the cation permeability and flux of the P2X2 receptor. Our study is based on the assumption that both transmembrane segments form the wall of the pore. There is general agreement that TM2 contributes, because published data show that some of its side chains form a water-accessible surface (6, 7) that participates in control of cation conductance (8), permeability (9), and flux (10). The suggestion that TM1 lines the pore is controversial (11). Recently, we showed that removing the fixed negative charge of a glutamate at the extracellular end of TM1 decreases the Ca2+ flux of the highly Ca2+-permeable P2X1 and P2X4 receptors (12), thus providing a precedence for the involvement of TM1 in ion permeation. The TM1 of the P2X2 receptor lacks this glutamate but still has a Ca2+ flux that is higher than expected for the molar ratios of Ca2+ and Na+ in the extracellular solution (4). Here, we use a combination of whole-cell voltage clamp technology, site-directed mutagenesis, and fluorescence microscopy to study the role of TM1 in Ca2+ permeability and flux. We measured the fraction of the total current carried by Ca2+, called the Pf% (fractional Ca2+ current) (13), and the permeability of Ca2+ relative to Cs+, called the Pca/Cs, of wild-type P2X2 receptors and mutants with altered TM1s. With one possible exception, we found that systematic mutagenesis produced no easily explainable changes in either Pf% or Pca/Cs, suggesting that TM1 plays a limited role in controlling cation permeability and flux through the P2X2 pore.

EXPERIMENTAL PROCEDURES

Molecular Biology and Cell Culture—We used rat P2X2 receptors with the FLAG-epitope (DYKDDDDK) fused to their C terminus. Addition of epitope had no effect on the pharmacology of the receptor (14, 15) or the function of the channel (12). Point mutations were introduced with the QuickChange II site-directed mutagenesis kit (Stratagene) and verified by automated DNA sequencing (Retrogen). All constructs were co-expressed with a fluorescent reporter protein in human embryonic kidney 293 cells (HEK-293) using Effectene (Qiagen). Transfected cells were maintained in a standard culture medium and incubated for 24–48 h at 37 °C in a humidified, 5% CO2 atmosphere. They were subsequently replated at low density onto poly-L-lysine-coated glass gold seal® coverslips (BD Biosciences) the night before the experiment.

Fractional Calcium Current—The fractional calcium current (Pf%) was measured using patch clamp photometry as described previously (10, 12). Hardware included a Nikon TE3000 epifluorescence microscope, HMC 40X ELWD Plan Fluor objective (Modulation Optics), a model 714 photomultiplier detection system (Photo Technology International), and an Axoclamp 200B amplifier (Molecular Devices). Briefly, HEK-293 cells were loaded with the calcium-sensitive dye,

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3 The abbreviations used are: TM1, first transmembrane domain; TM2, second transmembrane domain; MTSET, (2-trimethylammonium)ethyl]methanethiosulfonate HBr; MTSM, methylmethanethiosulfonate; YFP, yellow fluorescent protein.
fura-2 (Invitrogen), by passive diffusion through the tip of low resistance (1–3 megohms) recording electrodes containing (in mM) the following: 140 CsCl, 10 tetraethylammonium Cl, 10 HEPES, 2 KCl, fura-2, adjusted to pH 7.4 by addition of 4 CsOH. ATP (3–10 μM) was then applied for 0.5 s once every 2–3 min using triple-barreled glass and a Perfusion Fast-Step System SF-77 (Warner Instruments), whereas membrane current and fura-2 fluorescence (excitation 380 nm; emission 510 nm) were simultaneously measured. P% was calculated as shown in Equation 1,

\[ P\% = \frac{Q_{ca}}{Q_T} \times 100 \]  

(Eq. 1)

Q_T is the total charge, and equal to the integral of the leak-subtracted ATP-gated transmembrane current. Q_{ca} is the part of Q_T carried by Ca^{2+} and is equal to \( \Delta F_{180} \) (\( \Delta F_{180} \), the change in fura-2 fluorescence) divided by an experimentally derived calibration factor, \( F_{max} \). Day-to-day variation in the sensitivity of the fluorescence recordings was controlled by normalizing the fura-2 signal to a “bead unit” equal to the average fluorescence of seven carboxyl bright blue 4.6-μm microspheres (Polysciences) measured one at a time on the morning of that day’s experiment (13, 16). The extracellular bath solution was (in mM) as follows: 140 NaCl, 2 CaCl₂, 1 MgCl₂, 10 glucose, and 10 HEPES, adjusted to pH 7.4 by addition of 4 NaOH. ATP had no effect on the fura-2 fluorescence of mock-transfected cells using the methods described above, as reported previously (10, 12).

Relative Permeability Measurements—We recorded membrane current using indirect electrodes suspended in 3 M KCl agar bridges in contact with the bath solution, and the broken patch configuration of the whole-cell voltage clamp technique. The solution in the recording pipette was (in mM) as follows: 150 CsCl, 10 EGTA, 10 HEPES, and 4 CsOH, pH 7.4. The bath solution was first changed to one that contained predominantly CsCl (in mM: 150 CsCl, 0.1 CaCl₂, 1 MgCl₂, 10 glucose, 10 HEPES, 4 mM CsOH, pH 7.4), followed by one that contained either an additional monovalent cation (in mM: 150 XCl, 0.1 CaCl₂, 1 MgCl₂, 10 glucose, 10 HEPES, brought to pH 7.4 using 4 mM XOH, where X is the monovalent cation) or Ca^{2+} (in mM: 110 CaCl₂, 1 MgCl₂, 10 glucose, 2 CaOH, and 10 HEPES, at pH 7.4). We included 0.1 mM Ca^{2+} in the monovalent solutions to retard progression to the larger I₂ state, because this process causes a time-dependent change in cation permeability that might alter \( P_{ca}/P_{cs} \) (17, 18). In the absence of pore dilation, addition of 0.1 mM Ca^{2+} has a negligible effect on the reversal potential of ATP-gated current when \([Cs^+]_o\) and \([Cs^+]_i\), both equal 154 mM (19). We changed the membrane voltage of cells bathed in each solution from −80 to 60 mV at a constant rate (1.4 V/s) before and during applications of ATP, and we measured the zero current level (E_{rev}) from the leak-subtracted currents. We calculated the relative permeability of the monovalent cation to Cs^{+} (\( P_X/P_{ca} \)) from the bi-ionic potential (20). We calculated the permeability of Ca^{2+} relative to Cs^{+} (\( P_{ca}/P_{cs} \)) as shown in Equation 2,

\[ \left( a_{ca} \times [Cs]_i \right) \exp\left( \frac{\Delta E_{rev} \times F/RT}{4 \times a_{ca} \times [Ca]_o} \right) \]

where \( a_{ca} \) and \( a_{cs} \) are the activity coefficients of Cs⁺ (0.75) and Ca^{2+} (0.25), respectively, and \( \Delta E_{rev} \) is \( E_{rev,ca} - E_{rev,cs} \) (21). F, R, and T are universal constants.

RESULTS

Alanine-scanning Mutagenesis—Activation of wild-type P2X₂ receptors with 3 μM ATP evoked a P% of 6.6 ± 0.2% (n = 22; Fig. 1A), a value in keeping with published results (10, 12). To identify individual residues within TM1 that regulate Ca^{2+} flux, we first investigated the effect of systematically substituting each residue from Gly³⁰ to Ser⁴⁴ with an alanine (Fig. 1B). Alanine is generally well tolerated at solvent-accessible surfaces (22), and we used it to eliminate the side chain beyond the β-carbon of residues in TM1 without altering the main chain conformation of the protein (23). Twenty of the 25 mutants formed functional receptors with measurable P% values when expressed in HEK-293 cells. Three mutants (P2X₂-V45A, P2X₂-Y47A, and P2X₂-V51A) did not respond to ATP (1 mM). These three residues are not positioned on a water-accessible surface (24, 25) and are buried in the protein (14, 26); thus, they are unlikely to contribute to the pore wall and were not investigated further. Two other mutants, P2X₂-Y43A and P2X₂-F44A, were constitutively active in the absence of ATP, as reported previously (14). Although they respond to ATP with measurable currents, we were unable to determine P% because the Ca^{2+} that entered the cell through the ATP-independent standing current saturated the intracellular fura-2 before our measurement commenced. Nonetheless, as described below, the standing current did not preclude measurement of \( P_{ca}/P_{cs} \) and we used this parameter to determine the effect of swapping alanine for Tyr⁴³ and Phe⁴⁴ on the Ca^{2+} dynamics of the P2X₂ receptor.

Of the 20 mutants tested using the P% method, only the P2X₂-G30A receptor exhibited a Ca^{2+} flux that was different from the wild-type receptor, having a modestly increased P% of 9.1 ± 0.6% (n = 8; Fig. 1, C and D). The effect on P% of swapping the hydrogen of Gly³⁰ with the methyl group of alanine is surprising. Gly³⁰ is fully conserved in all P2X receptors and therefore may play a role in receptor function. However, our data imply that this role is not the regulation of cation flux through the pore. Replacing Gly³⁰ with alanine produced a gain-of-function measured as an increase in P%, suggesting that the native Gly³⁰ is unlikely to underlie the already high Ca^{2+} flux of the wild-type P2X₂ receptor. Glycine adopts a wide range of torsion angles in peptides because it lacks a side chain (27). For this reason, we cannot rule out the possibility that the
TABLE 1
The effect of fixed charge in TM1
For the wild-type receptor (WT), the number of experiments is presented in parentheses. ND indicates not determined.

| n  | I_{ATP} (pA/pF) | I_{ATP} Pf% | Pf% | P_{Ca}/P_{CS} |
|----|----------------|------------|-----|---------------|
| WT | −2.6 ± 1.1     | 301 ± 39   | 6.6 ± 0.2 (22) | 3.2 ± 0.3 (10) |
| H33E 6 | −8.0 ± 1.9     | 277 ± 95   | 8.9 ± 0.6    | 4.5 ± 0.1    |
| R34E 4 | −4.92 ± 1.8    | −6.6 ± 2.6 | ND            | ND           |
| Q37E 4 | −12.9 ± 4.0    | 162 ± 8    | 7.4 ± 0.8    | ND           |

FIGURE 1. Pf% of wild-type and alanine-substituted P2X2 receptors. A, representative traces of current (black), integrated current (equal to O, red), and F_{380} (gray) resulting from a 0.5-s application of 3 mM ATP (solid blue bar). The time courses of O and F_{380} are identical, as would be expected if ΔF_{380} is due only to Ca\(^{2+}\) entering the cell through the P2X\(_2\) channel (13). B, sequence alignment of TM1s of all seven members of the rat P2X receptor family. Conserved residues are indicated by pink shading. Residues that influence the Pf% of P2X\(_2\), P2X\(_3\), and P2X\(_6\) receptors are indicated by blue shading. C, O_{LOF} derived from F_{380} as described in the text, was re-sampled at 100 Hz, smoothed using a box filter of size 40, divided by the maximum O, and multiplied by 100%. The plateau of each trace is then equal to the Pf% of the respective receptors. The graph shows that the Pf% of the P2X2-G30A receptor is larger than that of the wild-type (WT) receptor. D, bar graph of data from wild type (gray bar) and 25 mutants (black bars). LOF indicates loss-of-function; ND indicates not determined. The dotted red line indicates the Pf% of the wild-type receptor equal to 6.6%.

carbonyl oxygen of P2X\(_2\)-G30A faces the permeation pathway and facilitates Ca\(^{2+}\) transport by providing a favorable electrostatic environment. However, empirical data suggest that the this oxygen faces away from the pore (28), and it therefore seems more likely that the small increase in Pf% is secondary to a positional change in another part of the protein involved in regulating Ca\(^{2+}\) flux. Definitive crystallographic analyses of the structures of wild-type and P2X\(_2\)-G30A channels are needed to determine whether this hypothesis is correct.

Do the Side Chains of Hydrophilic Residues in TM1 Face the Pore?—The fact that alanine substitutions of the hydrophilic residues, His\(^{33}\) and Arg\(^{34}\), had no effect on Pf% also came as a surprise, because cysteine-scanning mutagenesis suggest they line the pore (24). This is in stark contrast to the large effects on Pf% of mutagenesis of the pore-lining hydrophilic residues of TM2 (Asn\(^{133}\), Thr\(^{136}\), Thr\(^{139}\), and Ser\(^{140}\)) (9, 10). The current results of alanine-scanning mutagenesis suggest the following:

(i) either the side chains of His\(^{33}\) and Arg\(^{34}\) in fact do not face the permeation pathway, or (ii) these residues face the pore but do not interact with Ca\(^{2+}\). To provide an additional test of the hypothesis that they line the pore, we mutated three hydrophilic residues of TM1 to glutamate, one at a time (P2X\(_2\)-H33E, P2X\(_2\)-R34E, and P2X\(_2\)-Q37E). We included Gln\(^{37}\) because its amide group is polar, and because P2X\(_2\)-Q37C reacts to a thiol-reactive compound with a change in the size of the ATP-gated current (28). Glutamate is generally found on the surface of proteins (22) where, in the case of an ion channel, it supports cation flux by providing a negative electrostatic milieu in the pore (20). For example, replacing the pore-lining Thr\(^{339}\) of TM2 with glutamate produces a mutant P2X\(_2\)-T339E receptor that displays a significant elevation in P_{Ca}/P_{CS} and Pf% (9, 10).

Our results with TM1 were mixed (Table 1). First, we found that P2X\(_2\)-R34E receptors were functional but responded to ATP with currents that were too small (6.6 ± 2.6 pA/pF, n = 4) to derive meaningful Pf% values. This mutant was not studied further. Second, substitution of Gln\(^{37}\) with glutamate produced a channel with significant ATP-gated current but had no effect on the Pf% (7.1 ± 0.8%, n = 4) relative to the wild-type P2X\(_2\) receptor. This experiment supports the conclusion that Gln\(^{37}\) does not line the permeation pathway, as originally suggested by Jiang et al. (28). Third, the ATP-gated current through the P2X\(_2\)-H33E receptor was large (Table 1), and the Pf% was significantly increased to 8.9 ± 0.6% (n = 5). The rise in Pf% was accompanied by a significant increase in P_{Ca}/P_{CS} from 3.2 ± 0.2 (n = 17) for the wild-type P2X\(_2\) receptor to 4.5 ± 0.1 (n = 5) for the P2X\(_2\)-H33E mutant, measured using a conventional bi-ionic approach (20). The simplest case that explains this result places the side chain in the pore where it is properly aligned to influence ionic currents, thus confirming the original hypothesis that His\(^{33}\) lines the channel (24). A similar effect is seen when glutamate is substituted for Gln\(^{37}\) of the P2X\(_2\) receptor, as reported previously (12). Although this may be so, the fact that the P2X\(_2\)-H33A and P2X\(_2\)-Q52A receptors have normal Pf% values suggests that the native side chain of His\(^{33}\) and Gln\(^{52}\) play little or no role in regulating the Ca\(^{2+}\) flux of the wild-type P2X\(_2\) receptor. Taken together, these data suggest that the putative pore-lining, hydrophilic residues of TM1 do not interact with Ca\(^{2+}\) in a meaningful way.

Alanine Substitutions at Tyr\(^{43}\) and Phe\(^{44}\) Attenuate Ca\(^{2+}\) Permeability—Two functional receptors, P2X\(_2\)-Y43A and P2X\(_2\)-F44A, could not be studied using the Pf% method.
Tyr43 is fully conserved in all P2X receptors. We chose to pur-}
from the much larger currents (28). We attached YFP to the C terminus of the P2X2-Y43C receptor to further examine its cellular distribution. We were surprised to find that cells expressing P2X2-Y43C-YFP displayed larger than normal standing currents in the absence of ATP, a characteristic they share with cells expressing other Tyr43 mutants, and had small but measurable transmembrane currents when challenged with ATP (Table 2). We do not know why addition of the YFP tag to the C terminus permits expres-
sion of a functional channel.5 We assume that it reflects an increase in the stability of the fusion protein in the membrane and not a change in the properties of the channel because addition of YFP or GFP to wild-type P2X receptors has no effect on the properties of ATP-gated current (29–32). With a functional cysteine-substituted receptor in hand, we were able to revisit the issue of whether this position is accessible to thiol-modifying methanethiosulfonate reagents (Fig. 3). We measured the size of the currents through the P2X2-Y43C-YFP receptor before and during application of 1 mM MTSET. The peak amplitude of the ATP-gated current measured after 9 min in MTSET was 89.6 ± 19.6% (n = 5) of that measured before MTSET. This small change was not significantly different from that measured at the same time point in control experiments (83.0 ± 12.6%, n = 5). The standing current of the P2X2-Y43C-YFP receptor was similarly unaffected (100 ± 16.2%). In parallel experiments (Fig. 3), we found that MTSET had no effect on the ATP-gated current through wild-type receptors (90.3 ± 15.6%, n = 3) but did inhibit the current of cells expressing mutant P2X2-I328C receptors (18.2 ± 1.7%, n = 3), consistent with previous published data and showing proof of concept (7). Furthermore, we used an identical protocol to measure the effect of the smaller methylmethanethiosulfonate (1 mM), and we saw no effect on the sizes of the standing (115.4 ± 6.4%, n = 6) and ATP-gated (87.9 ± 18.7%, n = 5) currents of the P2X2-Y43C-YFP receptor. Methylmethanethiosulfonate did cause a significant reduction of the ATP-gated current through the P2X2-F44C mutant receptor (28.6 ± 6.9%, n = 4), as expected from published results (28).

because the standing leak of Ca2+ into the cell made fura-2 fluorescence measurements untenable. To determine the effect of mutagenesis on these receptors, we instead measured relative Ca2+ permeability (i.e. \( P_{Ca}/P_{Cs} \)) by subtracting the smaller standing currents (~20 pA/pF) seen in the absence of ATP from the much larger currents (~200 pA/pF) measured in its presence (Table 2). Because both currents flow through the same receptor population, subtraction reduced the slope of the I-V curve but did not affect its reversal potential (Fig. 2A). The \( P_{Ca}/P_{Cs} \) values of P2X2-Y43A and P2X2-F44A measured in this way were 1.6 ± 0.1 and 1.0 ± 0.2, respectively. Both values are significantly smaller than that of the wild-type P2X2 receptor (Fig. 2B).

The decrease in permeability measured in the P2X2-Y43A and P2X2-F44A receptors suggests that the relevant sites may interact with Ca2+ in the pore. Although Phe44 is a large hydrophobic residue, it could conceivably influence \( P_{Ca}/P_{Cs} \) through an electrostatic attraction between the \( \pi \) electrons of its aromatic ring and Ca2+. Likewise, the side chain of Tyr43 contains a hydroxyl group that could facilitate Ca2+ flux by acting as a surrogate water molecule in a narrow part of the pore.

**Mutation of Tyr43 Attenuates Ca2+ Permeability and Flux**—
Tyr43 is fully conserved in all P2X receptors. We chose to pur-
sue the study of Tyr43 because of the following: (i) conservation implies function; and (ii) it may serve the same function as the hydroxyl-bearing amino acids in TM2 that regulate the Ca2+ permeability (9) and flux (10) of the P2X2 receptor. To broaden our characterization of Tyr43, we used additional site-directed mutagenesis to alter the size of the side chain alone (P2X2-Y43S) or both size and hydrophobicity (P2X2-Y43F, P2X2-V45A, P2X2-V51A).4 All three mutants displayed large inward currents in response to ATP and had reduced Ca2+ permeabilities ranging from 1.1 to 2.1 (Fig. 2B). Furthermore, we were able to measure \( Pf\% \) because the ATP-independent standing currents of these three mutants were smaller than those of the P2X2-Y43A receptor (Table 2). We found that P2X2-Y43F and P2X2-Y43W receptors had decreased \( Pf\% \) values of 3.7 ± 0.5 and 3.5 ± 0.6%, respectively (Fig. 2, C and D). In contrast, the \( Pf\% \) of the P2X2-Y43S receptor (6.3 ± 0.6%) was not significantly different from the wild-type receptor, despite the lower \( P_{Ca}/P_{Cs} \) value measured for this mutant. The side chain of serine is smaller than that of tyrosine, although both contain a hydroxyl group. Our results suggest that the presence of a hydroxyl is required to maintain Ca2+ flux through the channel and that the size of the side chain influences relative permeability. This could be explained if Tyr43 forms a weak binding site for Ca2+ in the channel pore.

Collectively, these data suggest the following: (i) either the Tyr43 of TM1 regulates Ca2+ current, perhaps by acting in con-
cert with the Thr386, Thr389, and Ser340 of TM2 to partially dehydrate cations in a narrow part of the pore, or (ii) mutating Tyr43 leads to a nonspecific change in channel structure that indirectly affects Ca2+ current.

**Does the Side Chain of Tyr43 Face into the Channel Pore?**—As a next step in our investigation of Tyr43, we took advantage of an unexpected consequence of tagging P2X2-Y43C with YFP. Previously, we and others used sulfhydryl-reactive reagents and cysteine-scanning mutagenesis in an attempt to identify pore-
lining residues in both TM1 and TM2 (5). Tyr43 presented a problem because the cysteine-substituted mutant (P2X2-Y43C) was nonfunctional, despite being targeted to the cell membrane (28). We attached YFP to the C terminus of the P2X2-Y43C receptor to further examine its cellular distribution. We were surprised to find that cells expressing P2X2-Y43C-YFP displayed larger than normal standing currents in the absence of ATP, a characteristic they share with cells expressing other Tyr43 mutants, and had small but measurable transmembrane currents when challenged with ATP (Table 2). We do not know why addition of the YFP tag to the C terminus permits expres-
sion of a functional channel.5 We assume that it reflects an increase in the stability of the fusion protein in the membrane and not a change in the properties of the channel because addition of YFP or GFP to wild-type P2X receptors has no effect on the properties of ATP-gated current (29–32). With a functional cysteine-substituted receptor in hand, we were able to revisit the issue of whether this position is accessible to thiol-modifying methanethiosulfonate reagents (Fig. 3). We measured the size of the currents through the P2X2-Y43C-YFP receptor before and during application of 1 mM MTSET. The peak amplitude of the ATP-gated current measured after 9 min in MTSET was 89.6 ± 19.6% (n = 5) of that measured before MTSET. This small change was not significantly different from that measured at the same time point in control experiments (83.0 ± 12.6%, n = 5). The standing current of the P2X2-Y43C-YFP receptor was similarly unaffected (100 ± 16.2%). In parallel experiments (Fig. 3), we found that MTSET had no effect on the ATP-gated current through wild-type receptors (90.3 ± 15.6%, n = 3) but did inhibit the current of cells expressing mutant P2X2-I328C receptors (18.2 ± 1.7%, n = 3), consistent with previous published data and showing proof of concept (7). Furthermore, we used an identical protocol to measure the effect of the smaller methylmethanethiosulfonate (1 mM), and we saw no effect on the sizes of the standing (115.4 ± 6.4%, n = 6) and ATP-gated (87.9 ± 18.7%, n = 5) currents of the P2X2-Y43C-YFP receptor. Methylmethanethiosulfonate did cause a significant reduction of the ATP-gated current through the P2X2-F44C mutant receptor (28.6 ± 6.9%, n = 4), as expected from published results (28).

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4 We also made the P2X2-Y43E mutant, but this showed no ATP-gated current.

5 The three nonfunctional mutants, P2X2-V45A, P2X2-V47A, and P2X2-V51A, also displayed ATP-gated currents when tagged with YFP.

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**TABLE 2**

Mutating Tyr43 affects Ca2+ current

|           | \( I_{ATP} \) (pA/pF) | \( I_{ATP} \) (pA/pF) | \( Pf\% \) | \( P_{Ca}/P_{Cs} \) |
|-----------|------------------------|------------------------|------------|----------------------|
| WT        | -26 ± 1.1              | -301 ± 39              | 6.6 ± 0.2  | 10                    |
| Y43A      | -208 ± 3.6             | -196 ± 85              | ND         | 1.6 ± 0.1             |
| Y43F      | -10.4 ± 3.4            | -453 ± 121             | 3.7 ± 0.5  | 1.2 ± 0.1             |
| Y43S      | -11.1 ± 1.7            | -190 ± 76              | 6.3 ± 0.6  | 2.1 ± 0.2             |
| Y43W      | -10.4 ± 2.7            | -283 ± 63              | 3.5 ± 0.6  | 1.1 ± 0.3             |
| Y43C-YFP  | -54 ± 4.3              | -65.6 ± 18             | ND         | ND                    |
| F44A      | 18.4 ± 4.5             | 153 ± 45               | 1.0 ± 0.2  | 5                     |

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**TM1 and Ca2+ Current through P2X2 Receptor**
TM1 and Ca\(^{2+}\) Current through P2X\(_2\) Receptor

**As a group, the experiments failed to confirm the hypothesis that the side chain of Tyr\(^{43}\) faces into the pore. However, these results must be considered with caution because the inability of the methanethiosulphonates to affect current can be explained by outcomes other than inaccessibility (33).**

**Site-directed Mutagenesis of Tyr\(^{43}\) Has No Effect on Monovalent Cation Permeability or the Size of the Pore**—We considered the possibility that mutation of Tyr\(^{43}\) causes a broad change in channel architecture that leads to a nonspecific effect on pore dynamics. To test this hypothesis, we focused our interest on P2X\(_2\)-Y43F, which exhibits a significantly reduced Ca\(^{2+}\) permeability and flux, and yet only differs from the wild-type receptor in that the phenol ring of Tyr\(^{43}\) is deprived of its hydroxyl group. First, we measured the relative permeabilities of the P2X\(_2\) receptor and the P2X\(_2\)-Y43F mutant to a range of monovalent organic cations of varying size. In both cases (\(P_{\text{X}}/P_{\text{Cs}}\))\(^{0.5}\) decreased in a linear fashion as the size of the permeant ion increased. When plotted on X-Y coordinates, the X intercept of the resulting graph provides an estimate of the diameter of the narrowest part of the pore (Fig. 4). We found that the minimum diameter of the mutant P2X\(_2\)-Y43F receptor was approximately the same size (8.0 Å) as others (34–36), and we measured for wild-type P2X receptors (7.7 Å; Fig. 4). From these results, we conclude that substituting phenylalanine for Tyr\(^{43}\) does not change the size of the narrowest constriction of the channel.

**DISCUSSION**

Our goal was to identify sites in TM1 that regulate ionic flux through P2X\(_2\) receptors. As a first step, we substituted each and every TM1 residue for alanine. Our data were largely negative in that only 3 of the 22 functional alanine mutants showed a
Substituting alanine at three sites altered $P_f^\text{Na}$ and/or $P_{\text{Ca}}/P_{\text{Na}}$. One of these is Gly$^{30}$, which lacks a side chain capable of attracting cations and therefore is unlikely to regulate $\text{Ca}^{2+}$. We are less sure of the roles of the other two sites, Tyr$^{43}$ and Phe$^{44}$. Most P2X receptors have nonaromatic amino acids at positions homologous to Phe$^{44}$; therefore, the putative cation-π interaction that we put forward as a possible regulator of $\text{Ca}^{2+}$ flux cannot be a general property of all P2X receptors. In contrast, Tyr$^{43}$ is fully conserved, and we found that altering the side chain of Tyr$^{43}$ significantly reduces $\text{Ca}^{2+}$ permeability and flux. The phenylalanine-substituted mutant that retains the phenyl ring of the side chain but lacks the hydroxyl illustrates this best. P2X$_2$-Y43F receptors showed a significant reduction in both $P_f^\text{Na}$ and $P_{\text{Ca}}/P_{\text{Na}}$. Such an effect could conceivably be due to the −OH group directly interacting with $\text{Ca}^{2+}$ in the manner proposed for certain hydroxyl group-bearing residues in TM2 (9, 10). Our finding that mutagenesis has no effect on the relative monovalent permeability or the minimum size of the pore would tend to support this hypothesis.

This said, a cautious approach is warranted for two reasons. First, the inability of the methanethiosulfonates to reduce either the ATP-independent standing current or the ATP-dependent inward current runs counter to the argument that Tyr$^{43}$ is positioned in the permeation pathway. Second, mutating Tyr$^{43}$ also affects gating (14, 40).

If we conclude for the sake of argument that the side chain of Tyr$^{43}$ does not face the pore, then how do we explain the profound effect of mutagenesis on conduction? One possible explanation is that Tyr$^{43}$ is crucial to the correct arrangement of subunits around the channel pore, which would certainly explain why this residue is so well conserved across the receptor family. Indeed, mutating this residue to alanine affects both gating and permeability, in that P2X$_2$-Y43A channels are constitutively active and have a reduced $P_{\text{Ca}}/P_{\text{Na}}$. Is it possible that mutating Tyr$^{43}$ causes an unintended change in the pore architecture that affects the ability of the channel to select among permeant ions? If so, then the change must be subtle, because the measured effect on cation flux is limited to an action on $\text{Ca}^{2+}$. Furthermore, the selective nature of the effect of mutagenesis on $\text{Ca}^{2+}$ handling seems to indicate that the outcome is not because of a secondary change in the orientation of TM2, because the mutations in TM2 that affect $P_f^\text{Na}$ and $P_{\text{Ca}}/P_{\text{Na}}$ also decrease $P_f^\text{Pf}/P_{\text{Na}}$ (9), whereas mutating Tyr$^{43}$ does not. Finally, the fact that $\text{Ca}^{2+}$ permeability and flux are attenuated but $P_f^\text{Pf}/P_{\text{Na}}$ is not might indicate that $\text{Ca}^{2+}$ selection involves residues separate from those involved in monovalent cation selection.

In Fig. 5, we present a model of the TM1 of the P2X$_2$ receptor that incorporates the results of a number of indirect measurements of secondary structure and primary function. The model shows how the available data support the hypothesis that TM1 forms a regular α-helix across the plasma membrane. In Fig. 5, blue denotes amino acids positions identified in cysteine scanning studies as being accessible to methanethiosulfonates and/or Ag$^+$, and green denotes amino acids identified in alanine scanning studies that are thought to be involved in protein-protein packing and repacking during gating (5). Homologous positions were identified in a scanning tryptophan analysis of
the P2X4 receptor (26). In Fig. 5, yellow denotes a residue identified in both cysteine and alanine scans. The model shows that positions that respond to thiol-reactive reagents with a change in ATP-gated current tend to line one side of the putative TM1 helix. Although Gln52, shown in red in Fig. 5, was not identified as pore lining in SCAM studies, the analogous acidic residue in P2X1 and P2X4 receptors is important in regulating Ca\(^{2+}\) permeability and flux; it is probably positioned at a wide part of the ion-permeating pathway where it functions to concentrate calcium in the mouth of the pore (12). Residues involved in protein packing generally line the same side of the TM1 helix as those that are accessible to water-soluble thiolating reagents, which could be explained if TM1 moves during gating such that side chains initially facing protein become reoriented toward the aqueous environment. This hypothesis is supported by the observation that the mutant P2X\(_2\)-V48C is only blocked by MTSET modification when the receptor is in the open state (28). Some positions (white residues) were not identified in either the alanine or cysteine scans. These may line the opposite side of the helix, where they may face the lipid environment of the plasma membrane. Tyr\(^{43}\) is positioned at the boundary of lipid and protein/water, which might explain why its side chain is not accessible to modification by water-soluble reagents. If so, then Tyr\(^{43}\) must move during gating to be readily accessible to Ca\(^{2+}\).

In conclusion, our data suggest that TM1 is not a primary determinant of permeability and flux in the P2X\(_2\) receptor. In this respect, it resembles the trimeric ASIC1 channel that also has a TM1 that contributes little to permeation despite lining the pore (39). The innocuous nature probably reflects the following: (i) the TM1 of the P2X\(_2\) receptor lacks the fixed negative charge that underlies the higher Pf\% values of the P2X1 and P2X4 receptors; and (ii) the hydrophilic residues in TM1 contribute little to an electrostatically favorable environment for cation flux. The one exception may be Tyr\(^{43}\), which appears to influence Ca\(^{2+}\) permeability and flux in an as yet undetermined manner. These data provide a framework for understanding the role of TM1 in permeation, and will be useful in assigning function to structure when a definitive picture of the pore architecture becomes available from crystal studies.

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