Polar Residues of the Second Transmembrane Domain Influence Cation Permeability of the ATP-gated P2X₂ Receptor*

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P2X receptors are simple polypeptide channels that mediate fast purinergic depolarizations in both nerve and muscle. Although the depolarization results mainly from the influx of Na⁺, these channels also conduct a significant Ca²⁺ current that is large enough to evoke transmitter release from presynaptic neurons. We sought to determine the molecular basis of this Ca²⁺ conductance by a mutational analysis of recombinant P2X₂ receptors. Wild type and 31 mutant P2X₂ receptors were expressed in HEK-293 cells and studied under voltage-clamp. We found that the relative Ca²⁺ permeability measured from the reversal potentials of ATP-gated currents was unaffected by neutralizing fixed charge (Asp¹³¹, Asp³⁴⁹) near the mouths of the channel pore. By contrast, mutations that changed the character or side chain volume of three polar residues (Thr³³⁹, Thr³⁴⁰, Ser³⁴⁰) within the pore led to significant changes in P₉₄/P₇₄. The largest changes occurred when Thr³³⁹ and Ser³⁴⁰ were replaced with tyrosine; these mutations almost completely abolished Ca²⁺ permeability, reduced P₉₄/P₇₄ by about one-half, and shifted the relative permeability sequence of Cs⁺, Rb⁺, K⁺, and Na⁺ to their relative mobility in water. Our results suggest that the permeability sequence of the P2X₂ receptor arises in part from interactions of permeating cations with the polar side chains of three amino acids located in a short stretch of the second transmembrane domain.

We know more about the biophysics of homomeric P2X₂ receptors than other family members primarily because this subunit shows a relatively slow rate of desensitization; the slow desensitization results in sustained responses to applications of ATP that are easy to study under voltage-clamp. Like all family members, P2X₂ receptors are freely permeable to small cations, impermeable to anions, and display a strong preference for Ca²⁺ over Na⁺ (11, 12). Furthermore, they show a conductance sequence to alkali metal cations that differs from the relative mobility of these ions in water, suggesting that the receptor distinguishes among similarly sized ions (13). The molecular basis of this selection is unknown. Each P2X₂ subunit has two transmembrane-spanning domains (14), and scanning cysteine mutagenesis studies demonstrate that the second of these, TMD₂, forms a hydrated surface of the channel pore (15, 16). In or near TMD₂ are two conserved aspartates (Fig. 1) that may facilitate cation transport through the pore by altering the local concentrations of cations and anions. In addition, TMD₂ contains a number of pore-lining, polar amino acids that may regulate cation current by solvating ions within the pore (16).

To identify the specific amino acids of TMD₂ that play a role in monovalent cation and Ca²⁺ permeability, we measured relative permeability sequences from the reversal potentials of ATP-gated currents. Acidic and polar amino acids within TMD₂ of the P2X₂ receptor were mutated one at a time to alter the charge, polarity, and/or volume of the side chain. P₉₄/P₇₄ was measured first because the high Ca²⁺ permeability suggests that sites within the pore confer a favorable electrostatic profile for Ca²⁺ transport. Then, relative monovalent cation permeabilities were measured for all mutants displaying altered Ca²⁺ permeabilities. Our results show that point mutations of polar residues within a short stretch of TMD₂ altered monovalent cation and Ca²⁺ permeability in a manner consistent with an effect on a narrow section of the channel pore. This short stretch of TMD₂ may form part of the ion selectivity filter that regulates Ca²⁺ permeability by providing favorable solvation of partially dehydrated ions as they pass through the channel pore.

EXPERIMENTAL PROCEDURES

Molecular Biology and Heterologous Expression of Recombinant Receptors—Site-directed mutagenesis of acidic and polar amino acids of the rat P2X₂ receptor was performed using the overlap-primer extension method (17). Polymerase chain reaction was carried out using Vent polymerase (New England Biolabs), and all mutations were verified by

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1 The abbreviations used are: TMD₂, second transmembrane domain; WT, wild-type; HEK-293, human embryonic kidney; TEA, tetraethylammonium; NMDG, N-methyl-D-glucamine; P₉₄/P₇₄, the permeability of Ca²⁺ in relation to that of Cs⁺; P₇₄/P₉₄, the permeability of X⁻ in relation to that of Cs⁺, where X⁻ is Ca²⁺, Na⁺, K⁺, Li⁻, Rb⁺, TEA⁺, or NMDG⁻.

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The amino acid sequence of the P2X<sub>2</sub> subunit from Asp<sup>315</sup> to Asp<sup>329</sup>. TMD2 is underlined. The charged aspartates, Asp<sup>315</sup> and Asp<sup>329</sup>, are positionally conserved in all members of the P2X receptor family. Starred letters denote charged or polar amino acids that were mutated in this study.

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Reversal potentials measured in different extracellular solutions were compared with that measured in 154 mM extracellular Ca<sup>2+</sup> in the same cell. P<sub>Ca</sub>/P<sub>Na</sub> was calculated as P<sub>Ca</sub>/P<sub>Na</sub> = exp((V<sub>rev</sub> <sup>Ca</sup> - V<sub>rev</sub> <sup>Na</sup>)/FRT), where V<sub>rev</sub> <sup>Ca</sup> is the relative permeability of X<sup>2+</sup> to Ca<sup>2+</sup>, and V<sub>rev</sub> <sup>Na</sup> is the difference in the reversal potentials measured in the two solutions. F is Faraday’s constant, R is the universal gas constant, and T is the absolute temperature. Relative Ca<sup>2+</sup> permeability was calculated as P<sub>Ca</sub>/P<sub>Na</sub> = (α<sub>Ca</sub>/α<sub>Na</sub>)*exp((V<sub>rev</sub> <sup>Ca</sup> - V<sub>rev</sub> <sup>Na</sup>)/FRT)/(1 + exp((V<sub>rev</sub> <sup>Ca</sup> - V<sub>rev</sub> <sup>Na</sup>)/FRT))/4α<sub>Na</sub>α<sub>Ca</sub>, where α<sub>Na</sub> and α<sub>Ca</sub> are the activity coefficients of Na<sup>+</sup> (0.75) and Ca<sup>2+</sup> (0.25), respectively. Each experiment was repeated at least five times. The data were pooled, analyzed by one-way analysis of variance, and evaluated using the Fisher’s protected least significance difference post hoc test of Statview 5.0 (SAS Institute, Cary, NC).

The Effect of Mutating Acidic Residues on Ca<sup>2+</sup> Permeability—All P2X receptors contain two conserved aspartates (Asp<sup>315</sup> and Asp<sup>349</sup>) of the P2X<sub>2</sub> subunit that are, in theory, appropriately positioned to influence ionic current. They may do so by attracting cations and repelling anions near the mouths of the pore or by forming anionic binding sites that have a high affinity for Ca<sup>2+</sup> (21). Asp<sup>315</sup> occupies a site about 20 amino acids upstream of the start of TMD2 (22), and Asp<sup>349</sup> is within TMD2 on the intracellular side of channel gate (16). These two residues were mutated to lysine, glutamate, and aspartagine to test the role of fixed charge on Ca<sup>2+</sup> permeability.

Lysine-substituted mutants (D315K, D349K) had dramatically altered responses to ATP. No ligand-gated current was recorded in cells transfected with D315K regardless of the concentration of ATP applied (0.03–1 mM), and peak currents evoked by 30 μM ATP through D349K receptors were significantly smaller (5.5 ± 1.5 pA/pF, n = 5) than those of D349N (60.6 ± 10.3 pA/pF, n = 12) or D349E (75.3 ± 14 pA/pF, n = 13). These differences did not result from either a rightward shift in the ATP concentration-response curve of the lysine-substituted receptors (23) or a change in the amount of P2X receptor protein targeted to the cell surface membrane. The small or negligible responses of the lysine-substituted mutants made accurate measurements of ATP-gated current reversal potentials problematic, and these two mutants were not investigated further.

In other studies, we measured a progressive change in reversal potential in about 10% of the cells exposed to an extracellular solution containing 154 mM NMDG and 0 mM divalent cations; no difference in P<sub>NMDG</sub>/P<sub>Na</sub> was noted in the remaining 90% of the cells (data not shown).

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Cells expressing glutamate- or asparagine-substituted receptors (D315E, D315N, D349E, D349N) gave ATP-gated currents that were no different than those of the WT receptor in either size or shape of the current. Additionally, PCa/PCs was unchanged even when the fixed negative charge of the carboxylate was removed by mutation to asparagine (Table I). This result argues against a role for either D315 or D349 in determining relative Ca\(^{2+}\) permeability.

The Effect of Mutating Asn\(^{333}\) on Ca\(^{2+}\) Permeability—Next, we investigated the contribution of the uncharged polar amino acid Asn\(^{333}\) to ion selectivity because the results of two studies suggest it may play a role in this process. First, P2X\(_2\) receptors are predicted to contain a saturable binding site for Na\(^{+}\) that is located about 20% of the electrical distance from the extracellular surface (13). Asn\(^{333}\) is appropriately positioned to form such a site if TMD2 passes through the membrane as a straight \(\alpha\) helix.\(^{5}\) Second, mutant N333I receptors have reduced single channel conductances, a result that suggests that the negative pole of Asn\(^{333}\) facilitates cationic current either by concentrating cations near the extracellular mouth of the channel or by influencing the molecular dimensions of a narrow part of the channel pore (23).

PCa/PCs was measured for five mutant receptors in which the polarity and/or volume of the side chain at position Asn\(^{333}\) were changed. In the first series of experiments, we introduced mutations (N333Q, N333Y) that predominately affected side chain volume. N333Q resulted in a modest increase in side chain volume but had no effect on PCa/PCs (Table II). N333Y further increased side chain volume and decreased PCa/PCs by 27%, although the decline was not large enough to reach statistical significance. These findings demonstrate that the WT receptor is wide enough at position Asn\(^{333}\) to tolerate moderate changes in side chain volume without significantly affecting Ca\(^{2+}\) permeability.

Next, we examined the effect of a change in hydrophobicity. Increasing hydrophobicity was without effect when side chain volume decreased (N333C) or remained constant (N333V). The lack of effect of the N333V mutation on relative Ca\(^{2+}\) permeability is particularly important because it is inconsistent with the hypothesis that the negative pole of asparagine facilitates Ca\(^{2+}\) current by producing a high local Ca\(^{2+}\) concentration near the extracellular end of the ion channel (24). By contrast, PCa/PCs was significantly reduced when isoleucine replaced Asn\(^{333}\) (see Table II), a result in keeping with the reduced single channel conductance of N333I previously reported by Nakazawa et al. (24). The side chain of isoleucine is both larger and more hydrophobic than asparagine, and our results suggest that both parameters must change before an effect on PCa/PCs is measured.

The Effect of Substituting Hydrophobic for Polar Residues on Ca\(^{2+}\) Permeability—The P2X\(_2\) receptor contains five additional uncharged polar amino acids (Thr\(^{330}\), Thr\(^{336}\), Thr\(^{339}\), Ser\(^{340}\), Ser\(^{345}\)) in TMD2. We investigated the role these amino acids play in Ca\(^{2+}\) current by measuring PCa/PCs in a series of mutants containing hydrophobic substitutions. Native residues were replaced by valine or alanine to reduce polarity without causing a dramatic change in side chain volume (Table III). A defined pattern of reactivity was apparent. The two polar amino acids (T330V, N333V) thought to be positioned closest to the extracellular mouth of the ion channel were unaffected by valine replacement. Next to these is a stretch of residues (Thr\(^{336}\), Thr\(^{339}\), Ser\(^{340}\)) that imparted a roughly equally (55%) reduction in PCa/PCs when side chains were rendered hydrophobic (see Table III). The last polar amino acid, Ser\(^{345}\), which may lie adjacent to the intracellular side of the channel gate (16), was not affected by mutation to alanine. A simple hypothesis that explains these results is that the pore is narrower between Thr\(^{336}\) and Ser\(^{340}\) than it is at Asn\(^{333}\) or Ser\(^{345}\). If so, then hydrophobic substitutions within this domain may disrupt Ca\(^{2+}\) permeability by removing the countercharge required by the partially dehydrated ion as it permeates the channel pore.

The Effect of Increasing Side Chain Volume on Ca\(^{2+}\) Permeability—We tested the hypothesis that the channel pore narrows between Thr\(^{336}\) and Ser\(^{340}\) by mutating all uncharged polar residues in TMD2 to tyrosine to increase side chain volume without changing hydrophobicity. All tyrosine-substituted mutants except T336Y and T339Y resembled WT receptor in the size and shape of ATP-gated currents (Fig. 3). HEK-293 cells transfected with cDNA encoding the T336Y mutant receptor failed to respond to 30–100 \(\mu\)M ATP regardless of the direction or magnitude of the electrical potential across the membrane.\(^{6}\) The reason for the loss of function is unknown. T339Y was functional but displayed a more profound desensitization/inactivation in response to ATP than did control (Fig. 3); however, the rate of current decline of T339Y was still compatible with the voltage-ramp method used to measure reversal potentials, and recovery from desensitization/inactivation was complete within 3 min.

The same residues that responded to hydrophobic substitutions with a decrease in PCa/PCs responded to tyrosine substitutions in a similar fashion. Uncharged polar residues outside of the stretch from Thr\(^{336}\) to Ser\(^{340}\) were not affected, whereas tyrosine-substituted mutants of the adjoining residues Thr\(^{339}\) and Ser\(^{340}\) became almost impermeable to Ca\(^{2+}\) (Table IV). Again, the results suggest that the pore diameter is relatively small at Thr\(^{339}\)–Ser\(^{340}\) and that permeating ions can sense the composition of the wall in this domain.

The Effect of Other Mutations at Thr\(^{339}\) and Ser\(^{340}\) on Ca\(^{2+}\) Permeability—We looked in more detail at the effects of changing the character of the side chains at positions Thr\(^{336}\) and Ser\(^{340}\). Table V presents all data of Thr\(^{339}\)- and Ser\(^{340}\)-substituted mutants. A few general trends are clear. First, an increase in hydrophobicity at either position leads to a decrease in PCa/PCs regardless of the effect of the mutation on side chain volume (compare T339C, T339Y, S340A, and S340F). Second, the largest decreases in PCa/PCs occur when side chain volume dramatically increases (T339Y, S340F, S340Y). For example, both the S340F and S340Y mutations result in an almost complete loss of Ca\(^{2+}\) permeability (PCa/PCs = 0.24 and 0.08, respectively) despite the fact that substituting tyrosine for serine does not change side chain hydrophobicity. The situation is more complicated at position Thr\(^{339}\), where bulky substitutions also altered gating kinetics. Specifically, the T339Y re-
Effect of site-directed mutagenesis of Asn<sup>333</sup> on relative calcium permeability

| Clone      | \(D_{V_{rev}}\) mV | \(P_{Na}/P_{Ca}\) | \(n\) | \(p\) value | \(\Delta Vol\) | \(\Delta Hydro\) |
|-----------|---------------------|-------------------|------|-------------|--------------|----------------|
| WT        | 4.6 ± 1.7           | 2.74 ± 0.25       | 12   |             |              | 0.16           |
| N333Q     | 3.84 ± 3.2          | 2.76 ± 0.44       | 5    | 0.97        | 25.9         | 0.01           |
| N332Y     | −2.8 ± 2.9          | 2.02 ± 0.18       | 7    | 0.07        | 68.4         | 2.2            |
| N333C     | 2.3 ± 3.6           | 2.80 ± 0.51       | 5    | 0.62        | −29.6        | 6.0            |
| N333V     | 2.3 ± 1.6           | 2.43 ± 0.39       | 5    | 0.55        | 6.5          | 7.7            |
| N333I     | −10.5 ± 2.4         | 1.21 ± 0.17<sup>a</sup> | 7 | 0.006  | 33.6 | 8.0 |

<sup>a</sup> Ratio is significantly different (<i>p</i> < 0.01) from WT.

Effect of increased hydrophobicity on relative calcium permeability

| Clone      | \(D_{V_{rev}}\) mV | \(P_{Na}/P_{Ca}\) | \(n\) | \(p\) value | \(\Delta Vol\) | \(\Delta Hydro\) |
|-----------|---------------------|-------------------|------|-------------|--------------|----------------|
| WT        | 4.6 ± 1.7           | 2.74 ± 0.25       | 12   |             |              | 0.16           |
| T330V     | 4.6 ± 3.5           | 2.74 ± 0.25       | 5    | 0.98        | 19.5         | 4.9            |
| N332V     | 3.3 ± 3.6           | 2.43 ± 0.39       | 5    | 0.55        | 6.5          | 7.7            |
| T336V     | −3.6 ± 2.8          | 1.57 ± 0.24<sup>ab</sup> | 9 | 0.005 | 19.5 | 4.9 |
| T339V     | −5.0 ± 2.8          | 1.61 ± 0.21<sup>a</sup> | 4 | 0.03  | 19.5 | 4.9 |
| S340A     | −7.8 ± 3.8          | 1.49 ± 0.08<sup>bc</sup> | 5 | 0.01  | −7.6 | 2.6 |
| S345A     | 5.9 ± 1.4           | 3.00 ± 0.27       | 5    | 0.59        | −7.6         | 2.6            |

<sup>a</sup> Ratio is significantly different from WT (<i>p</i> < 0.01).<br><sup>b</sup> Ratio is significantly different from WT (<i>p</i> < 0.05).

Fig. 3. ATP-gated currents recorded from tyrosine-substituted mutants of the P2X<sub>3</sub> receptor. ATP (30 μM) was used to evoke inward currents in HEK-293 cells expressing either WT receptor or one of a range of tyrosine-substituted mutants (T330Y, N333Y, T339Y, S340Y, and S345Y). Currents are normalized to give equal-sized peak current amplitudes. The holding voltage was −60 mV. Currents through T339Y mutant receptors declined faster than did those through WT and other mutant receptors.

The TMD2 and Cation Selectivity of P2X Receptors

Mean ± S.E.; <i>n</i>, number of experiments.

charge in a narrow part of the channel increases Ca<sup>2+</sup> permeability, which is not surprising, as a similar effect is reported for glutamate-substituted mutants of voltage-gated sodium channels (25).

The Effect of Mutating Polar Residues on Monovalent Cation Selectivity Sequences—In a final series of experiments, we measured relative monovalent cation permeability for group 1A cations (Na<sup>+</sup>, K<sup>+</sup>, Cs<sup>+</sup>, Rb<sup>+</sup>, Li<sup>+</sup>), TEA<sup>+</sup>, and NMDG<sup>−</sup> in WT and mutant receptors. Only those mutants that showed altered Ca<sup>2+</sup> were studied in detail.

The WT receptor discriminated poorly among most alkali metal ions, and only a slight difference in P<sub>Na</sub>/P<sub>Ca</sub> was measured for Rb<sup>+</sup>, Cs<sup>+</sup>, and Na<sup>+</sup> (Fig. 4c). Li<sup>+</sup> was the exception, in that it was about 40% more permeable than the other group 1A cations (Table VI). In keeping with previous published results, the WT receptor showed limited permeability to the large organic cations, TEA<sup>−</sup> (P<sub>TEA</sub>/P<sub>Ca</sub> = 0.13 ± 0.02) and NMDG<sup>−</sup> (P<sub>NMDG</sub>/P<sub>Ca</sub> = 0.08 ± 0.01) when the extracellular solution contained mM concentrations of divalent cations (26).

The effects of changing side chain volume on relative monovalent cation permeabilities are shown in Fig. 5. The largest effects occurred when side chain volume increased at positions Thr 339 and Ser 340. However, the introduction of a positive charge (Thr 339Glu) caused only a minor change (Li<sup>+</sup> > Rb<sup>+</sup> > K<sup>+</sup> > Cs<sup>+</sup> > Na<sup>+</sup>) in relative permeabilities of the alkali metal ions. By contrast, hydrophobic substitutions at positions Thr<sup>339</sup> and Ser<sup>340</sup> led to an increase in P<sub>Na</sub>/P<sub>Ca</sub> for all group 1A cations except Li<sup>+</sup>, which retained its already high permeability (see Table VI). P<sub>Na</sub>/P<sub>Ca</sub> changed the most, so that the relative permeability sequences became Li<sup>+</sup> > Na<sup>+</sup> > K<sup>+</sup> > Rb<sup>+</sup> > Cs<sup>+</sup>. This is equivalent to type XI Eisenman sequence indicative of a decrease in the size of an intrapore interaction site. Changing charge at position Thr<sup>339</sup> had a similar effect (T339E, T339K; see Fig. 4c), as did the bulky, hydrophobic mutation of Asn<sup>333</sup> to isoleucine. The smaller, hydrophobic substitution of valine at position Asn<sup>333</sup> was without effect (data not shown).

The effects of changing side chain volume on relative monovalent cation permeabilities are shown in Fig. 5. The largest effects occurred when side chain volume increased at positions
Thr\textsuperscript{339} and Ser\textsuperscript{340}, all three mutants (T339Y, S340F, and S340Y) showed a significant decrease in $P_{\text{NMDG}}/P_{\text{Ca}}$ (0.05 ± 0.01, 0.02 ± 0.01, 0.02 ± 0.01, respectively, see Fig. 5) and $P_{\text{Li}}/P_{\text{Ca}}$ (Table VI), indicating that these mutations narrowed the side chain of Asp315 and Asp349 to asparagine; we found no effect of S340A or S340G on the relative permeability sequences of the other test cations (see Table VI); these mutations also had minor effects on the relative permeability sequences of the other test cations (see Fig. 5). Only T339K and T339E showed a significant ($p < 0.01$) increase in $P_{\text{NMDG}}/P_{\text{Ca}}$, which may be due to an increase in pore diameter caused by mutual repulsion of closely spaced fixed charge contributed by adjoining subunits.

**DISCUSSION**

Although major advances in locating the pore-forming domains of the P2X\textsubscript{2} protein have been made in the last few years (15, 16, 27), very little is known about how these domains determine the permeation and gating properties of the receptor-channel complex. The results reported here are the first to define a part of the protein that influences the relative cation selectivity of a member of the P2X receptor family. We used site-directed mutagenesis to locate a short stretch of TMD2 that influences the ability of cations to permeate the channel. We found that mutations that changed the size or character of the side chains of three polar amino acids (Thr\textsuperscript{336}, Thr\textsuperscript{339} and Ser\textsuperscript{340}) within this stretch altered relative Ca\textsuperscript{2+} permeability in a predictable manner. The greatest changes occurred when Thr\textsuperscript{339} and Ser\textsuperscript{340} were replaced with tyrosine. These mutations reduced $P_{\text{Ca}}/P_{\text{Ca}}$ to less than 10% of that of control and shifted the relative permeability sequence of the alkali metal cations toward their relative mobility in water. Both results are consistent with an effect on the selectivity filter.

There are three well documented mechanisms of selection used by ionic channels. First, fixed charge may dictate the local ion concentrations available for passage through the pore (28–31). We tested this hypothesis by mutating the conserved aspartates Asp\textsuperscript{315} and Asp\textsuperscript{349} to asparagine; we found no effect of this mutation on $P_{\text{Ca}}/P_{\text{Ca}}$. Thus, although it is possible that the negative pole of asparagine can substitute to a degree for the fixed negative charge of Asp\textsuperscript{315} and Asp\textsuperscript{349}, our results clearly demonstrate that the relatively high Ca\textsuperscript{2+} permeability of the WT receptor does not result from an obligatory electrostatic interaction between Ca\textsuperscript{2+} and the -COOH side chain of Asp\textsuperscript{315} or Asp\textsuperscript{349}. Second, a narrow constriction within the pore may act as a sieve to restrict the flow of ions larger than some critical size (32). The P2X\textsubscript{2} receptor-channel prefers smaller cations (32, 33). We tested the hypothesis that cation selectivity among small cations of similar size suggests that other mechanisms of ion selection must also come into play. Third, selection among cations may reflect the ability of protein domains within the pore to preferentially solvate different species of permeating ions (28–31, 32). We tested the hypothesis that cation selectivity reflects the barrier to movement of partially hydrated ions in a narrow part of the channel pore. If so, then relative ionic permeability would arise from the ability of the pore wall to provide suitable solvation of different species of permeating ions. Our results support such a role for the polar residues of TMD2.

**Table IV**

| Clone   | $\Delta V_{rev}$ (mV) | $P_{\text{rev}}$ | $P_{\text{rev}}$ | $p$ value | $\Delta V$ (Å) | $\Delta$Hydro |
|---------|----------------------|------------------|------------------|-----------|----------------|----------------|
| WT      | 4.6 ± 1.7            | 2.74 ± 0.25      | 12               |           |                |                |
| T330Y   | 4.6 ± 1.1            | 2.76 ± 0.2       | 7                | 0.96      | -55.8          | 0.3            |
| T339C   | -12.5 ± 3.4          | 1.5 ± 0.12       | 9                | 0.0009    | -16.6          | 3.2            |
| T339V   | -5.0 ± 2.8           | 1.61 ± 0.21      | 5                | 0.03      | 19.5           | 4.9            |
| T339F   | ND                   | ND               | 9                |           | 81.3           | 3.5            |
| T339Y   | -36.0 ± 3.5          | 0.33 ± 0.05      | 5                | <0.0001   | 81.4           | -0.6           |
| T339E   | 16.2 ± 2.3           | 6.14 ± 0.75      | 8                | <0.0001   | 32.9           | -2.8           |
| T339K   | -10.9 ± 1.9          | 1.16 ± 0.12      | 8                | 0.0002    | 49.2           | -3.2           |
| S340A   | -7.8 ± 3.8           | 1.49 ± 0.08      | 5                | 0.01      | -7.6           | 2.6            |
| S340G   | -4.4 ± 1.0           | 1.62 ± 0.05      | 5                | 0.02      | -32.7          | 0.4            |
| S340F   | -40.2 ± 6.5          | 0.24 ± 0.09      | 8                | <0.0001   | 104.3          | 3.6            |
| S340Y   | -67.2 ± 1.3          | 0.08 ± 0.01      | 5                | <0.0001   | 104.5          | -0.5           |
| T339K   | -12.0 ± 2.3          | 7.8 ± 0.25       | 5                |           | 72.2           | -4.7           |

* Ratio is significantly different from WT ($p < 0.01$).

**Table V**

| Clone   | $\Delta V_{rev}$ (mV) | $P_{\text{rev}}$ | $P_{\text{rev}}$ | $p$ value | $\Delta V$ (Å) | $\Delta$Hydro |
|---------|----------------------|------------------|------------------|-----------|----------------|----------------|
| WT      | 4.6 ± 1.7            | 2.74 ± 0.25      | 12               |           |                |                |
| T339G   | 4.6 ± 1.1            | 2.76 ± 0.2       | 7                | 0.96      | -55.8          | 0.3            |
| T339C   | -12.5 ± 3.4          | 1.5 ± 0.12       | 9                | 0.0009    | -16.6          | 3.2            |
| T339V   | -5.0 ± 2.8           | 1.61 ± 0.21      | 5                | 0.03      | 19.5           | 4.9            |
| T339F   | ND                   | ND               | 9                |           | 81.3           | 3.5            |
| T339Y   | -36.0 ± 3.5          | 0.33 ± 0.05      | 5                | <0.0001   | 81.4           | -0.6           |
| T339E   | 16.2 ± 2.3           | 6.14 ± 0.75      | 8                | <0.0001   | 32.9           | -2.8           |
| T339K   | -10.9 ± 1.9          | 1.16 ± 0.12      | 8                | 0.0002    | 49.2           | -3.2           |
| S340A   | -7.8 ± 3.8           | 1.49 ± 0.08      | 5                | 0.01      | -7.6           | 2.6            |
| S340G   | -4.4 ± 1.0           | 1.62 ± 0.05      | 5                | 0.02      | -32.7          | 0.4            |
| S340F   | -40.2 ± 6.5          | 0.24 ± 0.09      | 8                | <0.0001   | 104.3          | 3.6            |
| S340Y   | -67.2 ± 1.3          | 0.08 ± 0.01      | 5                | <0.0001   | 104.5          | -0.5           |
| T339K   | -12.0 ± 2.3          | 7.8 ± 0.25       | 5                |           | 72.2           | -4.7           |

* Ratio is significantly different from WT ($p < 0.01$).

* Ratio is significantly different from WT ($p < 0.05$).
We suggest that ion selection in the P2X channel occurs in the following manner. Our data predict that the extracellular vestibule is relatively wide at Thr330 because substitutions that change the hydrophobicity or volume of the side chain have no effect on the ability of Ca\(^{2+}\) to permeate the channel (Fig. 6a).

The channel then begins to narrow near Asn333 where tyrosine substitutions begin to affect \(P_{\text{Ca}}/P_{\text{Cs}}\). The pore must be large enough at Asn333 to ignore the effects of changing hydrophobicity (N333V) or volume (N333Y) alone. At the same time, it must be small enough to allow permeating Ca\(^{2+}\) to sense the moderately larger hydrophobic side chain of N333I. Although others have shown that some mutations at Asn333 alter single

**FIG. 4.** The effect of changing hydrophobicity or charge on relative monovalent cation permeability. Figure shows relative monovalent cation permeabilities of mutant P2X\(_2\) receptors. a, raw (left side) and tabulated (right side) data for the WT receptor. b, tabulated data for mutants in which the amino acid substitution resulted in a change in hydrophobicity. c, data for a change in charge.

![Graphs showing relative monovalent cation permeability](image)

**TABLE VI**

| Clone | \(\Delta V_{\text{rev}}\) \(\text{mV}\) | \(P_{\text{Ca}}/P_{\text{Cs}}\) | \(\Delta\text{Vol}\) \(\text{Å}^3\) | \(\Delta\text{Hydro}\) |
|-------|----------------|-----------------|----------------|----------------|
| WT    | 8.1 ± 1.2      | 1.38 ± 0.06     | 81.4           | −0.6           |
| T330Y | 13.4 ± 0.8     | 1.72 ± 0.1      | 88.4           | 4.0            |
| N333I | 5.1 ± 3.1      | 1.25 ± 0.14     | 33.6           | 8.0            |
| N333Y | 9.2 ± 2.9      | 1.53 ± 0.17     | 68.4           | 2.2            |
| T336Y | 7.5 ± 3.2      | 1.36 ± 0.17     | 19.5           | 4.9            |
| T336Y | ND             | ND              | 81.4           | −0.6           |
| T339V | 6.4 ± 5.8      | 1.42 ± 0.13     | 19.5           | 4.9            |
| T339V | 8.3 ± 1.8      | 1.38 ± 0.03     | 32.9           | −2.2           |
| T339K | 9.7 ± 2.4      | 1.46 ± 0.05     | 49.2           | 3.2            |
| T339G | 12.6 ± 2.3     | 1.64 ± 0.06     | −55.8          | 0.3            |
| T339F | ND             | ND              | 81.3           | 3.5            |
| T340Y | −13.6 ± 1.0    | 0.59 ± 0.02\(^a\) | <0.0001        | 81.4           | −0.6 |
| S340A | 7.2 ± 4.9      | 1.35 ± 0.1      | −7.6           | 2.6            |
| S340G | 7.8 ± 2.6      | 1.36 ± 0.05     | −32.7          | 0.4            |
| S340F | −2.0 ± 4.2     | 0.9 ± 0.05\(^a\) | 104.3          | 3.6            |
| S340Y | −14.6 ± 2.9    | 0.69 ± 0.09\(^a\) | <0.0001        | 104.5          | −0.5 |
| S340E | ND             | ND              | 56             | −4.3           |
| S340K | ND             | ND              | 72.2           | −4.7           |

\(^a\) Ratio is significantly different from WT (\(p < 0.01\)).

We suggest that ion selection in the P2X channel occurs in the following manner. Our data predict that the extracellular vestibule is relatively wide at Thr330 because substitutions that change the hydrophobicity or volume of the side chain have no effect on the ability of Ca\(^{2+}\) to permeate the channel (Fig. 6a). The channel then begins to narrow near Asn333 where tyrosine substitutions begin to affect \(P_{\text{Ca}}/P_{\text{Cs}}\). The pore must be large enough at Asn333 to ignore the effects of changing hydrophobicity (N333V) or volume (N333Y) alone. At the same time, it must be small enough to allow permeating Ca\(^{2+}\) to sense the moderately larger hydrophobic side chain of N333I. Although others have shown that some mutations at Asn333 alter single
channel conductance (24), our data do not support a major role for this residue in control of selectivity. Specifically, relative permeability is unaffected by replacing the polar side chain of asparagine with the hydrophobic side chain of valine, an unexpected result if Asn\textsubscript{333} acts to solvate the permeating cations. Instead, we believe that a permeating ion makes its first meaningful interaction with the wall of the pore at Thr\textsubscript{336}. Here, the diameter of the channel is small enough that hydrophobic substitutions that have little effect on side chain volume significantly alter Ca\textsuperscript{2+} permeability. We assume that if the diameter of the pore is small enough to allow permeating ions to notice fairly minor changes in the hydrophobicity of the pore lining, then it is possible that the hydroxyl side chain of the native threonine is properly positioned to help cations shed water as they approach the narrowest stretch of the channel pore. In our model, this stretch occurs at Thr\textsubscript{339} and Ser\textsubscript{340}, where tyrosine replacements significantly reduce P\textsubscript{NMDG}/PCs and PCa/PCs. The crystal radius of Ca\textsuperscript{2+} is smaller than that of Cs\textsuperscript{+}, and it is therefore unlikely that the reduction in Ca\textsuperscript{2+} permeability results solely from a change in the size of the pore. Rather, Thr\textsuperscript{339} and Ser\textsuperscript{340} may form an intrapore ligand-binding site that prefers Ca\textsuperscript{2+} to most monovalent cations. This hypothesis is supported by the fact that tyrosine substitutions at these positions also disrupt Li\textsuperscript{+} permeability. Lithium’s high permeability (P\textsubscript{Li}/PCs = 1.38 ± 0.06) contrasts with its low single channel chord conductance (\(\gamma_{\text{Li}}/\gamma_{\text{Cs}} = 0.65\), from Ding and Sachs (Ref. 13)); such a finding is predicted to occur when permeation involves a binding step that limits current through the channel. Mutating Thr\textsuperscript{339} or Ser\textsuperscript{340} to tyrosine reduces

\section*{Δ side chain volume}

![Graphical representation of channel conductance](image)

**FIG. 5.** The effect of changing side chain volume on relative monovalent cation permeability. Figure shows relative monovalent cation permeabilities of mutant P2X\textsubscript{2} receptors, and tabulated data for mutants in which the amino acid substitution resulted in a change in side chain volume. WT receptor is shown for comparison.

**FIG. 6.** a, the schematic shows a model of the shape of the pore suggested by the present results. The pore may have relatively wide outer and inner vestibules separated by a narrow stretch of TMD2 formed by Asn\textsubscript{333}–Ser\textsubscript{340}. The channel is narrowest at Ser\textsubscript{340}. Although the location of the gate is unproven, it may lie just interior to Ser\textsubscript{340}. b, helical wheel model of TMD2 from Ser\textsubscript{326} to Asp\textsubscript{349}. Shaded positions are those at which at least one amino acid substitution led to a change in P\textsubscript{Cs}/P\textsubscript{Cs}.
P_{Ca}/P_{Na} to less than 1 (see Table VI), perhaps by decreasing the apparent affinity of Li$^+$ for the same site that binds Ca$^{2+}$. Although some data suggest that TMD2 may not be a stable α helix (16), it is interesting to note that Asn$^{333}$, Thr$^{336}$, Thr$^{339}$, and Ser$^{340}$ all lie on the same side of a helical wheel model of TMD2 (Fig. 6b); selective mutagenesis at each site can lead to a fall in relative Ca$^{2+}$ permeability (see Table V) and/or a reordering of the monovalent cation permeability sequence. One helical turn inward from Ser$^{340}$ lies Val$^{343}$, a residue that sits at the apex of a fairly well conserved sequence of three amino acids (GXXGA), where X equals the small, hydrophobic amino acids alanine, valine, and leucine) common to all functional homomeric P2X receptors. This domain may form a flexible part of the channel pore that opens and closes during gating. If so, then both the ionic selectivity filter and the channel gate may be cast from the same general area of TMD2. The proximity of the gate and the filter may help to explain the changes in gating kinetics and desensitization/inactivation that we noticed with some Thr$^{339}$-substituted receptors.

P2X receptors form a family of seven members that display high sequence homology but little identity in their two putative transmembrane domains. We have identified a domain in the P2X$_2$ subunit that influences Ca$^{2+}$ and monovalent cation permeability. It is tempting to speculate that the range of relative Ca$^{2+}$ to monovalent cation permeabilities (P$_{Ca}$/P$_{Na}$ = 1.2–5; Ref. 1) reported for different family members reflects the sequence variability in this same domain. Site-directed mutagenesis of other family members combined with permeability measurements like those reported here may help answer this question in the near future.

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