Acidic Amino Acids Impart Enhanced Ca\textsuperscript{2+} Permeability and Flux in Two Members of the ATP-gated P2X Receptor Family

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P2X receptors are ATP-gated cation channels expressed in nerve, muscle, bone, glands, and the immune system. The seven family members display variable Ca\textsuperscript{2+} permeabilities that are amongst the highest of all ligand-gated channels (Egan and Khakh, 2004). We previously reported that polar residues regulate the Ca\textsuperscript{2+} permeability of the P2X\textsubscript{2} receptor (Migita et al., 2001). Here, we test the hypothesis that the formal charge of acidic amino acids underlies the higher fractional Ca\textsuperscript{2+} currents (P\%\textsubscript{f}) of the rat and human P2X\textsubscript{2} and P2X\textsubscript{4} subtypes. We used patch-clamp photometry to measure the P\%\textsubscript{f} of HEK-293 cells transiently expressing a range of wild-type and genetically altered receptors. Lowering the pH of the extracellular solution reduced the higher P\%\textsubscript{f} of the P2X\textsubscript{1} receptor but had no effect on the lower P\%\textsubscript{f} of the P2X\textsubscript{2} receptor, suggesting that ionized side chains regulate the Ca\textsuperscript{2+} flux of some family members. Removing the fixed negative charges found at the extracellular ends of the transmembrane domains also reduced the higher P\%\textsubscript{f} of P2X\textsubscript{1} and P2X\textsubscript{2} receptors, and introducing these charges at homologous positions increased the lower P\%\textsubscript{f} of the P2X\textsubscript{2} receptor. Taken together, the data suggest that COO\textsuperscript{−} side chains provide an electrostatic force that interacts with Ca\textsuperscript{2+} in the mouth of the pore. Surprisingly, the glutamate residue that is partly responsible for the higher P\%\textsubscript{f} of the P2X\textsubscript{1} and P2X\textsubscript{4} receptors is conserved in the P2X\textsubscript{3} receptor that has the lowest P\%\textsubscript{f} of all family members. We found that neutralizing an upstream His\textsuperscript{165} increased P\%\textsubscript{f} of the P2X\textsubscript{3} channel, suggesting that this positive charge masks the facilitation of Ca\textsuperscript{2+} flux by the neighboring Glu\textsuperscript{46}. The data support the hypothesis that formal charges near the extracellular ends of transmembrane domains contribute to the high Ca\textsuperscript{2+} permeability and flux of some P2X receptors.

INTRODUCTION

A change in calcium concentration is an essential intracellular signal that initiates fundamental physiological processes such as secretion and contraction. To succeed in this role, the intracellular free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) is tightly controlled, and nature has devised an impressive array of proteins that regulate Ca\textsuperscript{2+} transport across cell membranes (Berridge et al., 2003). Neurotransmitter receptors play an essential role in this process. Metabotropic receptors increase [Ca\textsuperscript{2+}]\textsubscript{i}, by modulating voltage-gated Ca\textsuperscript{2+} channels or emptying internal Ca\textsuperscript{2+} stores (Ross et al., 2005). Ionotropic receptors, like the ATP-gated P2X receptor, take a more direct approach. P2X receptors are ligand-gated cation channels that increase [Ca\textsuperscript{2+}]\textsubscript{i} by forming a cation-permeable pore (Benham and Tsien, 1987). They are expressed in a diverse range of tissues and are particularly abundant in the nervous system (Khakh, 2001; Illes and Ribeiro, 2004; Khakh and North, 2006). Seven full-length subtypes (P2X\textsubscript{1–7}) are expressed in mammalian tissue (Pankratov et al., 2006), and mutagenesis studies indicate a role for the pore-lining second transmembrane segment (TM2) in the cation permeability (Migita et al., 2001) and Ca\textsuperscript{2+} current (Egan and Khakh, 2004) of at least one family member, the P2X\textsubscript{2} receptor. The first transmembrane segment (TM1) also lines the pore, and mutagenesis of this domain is known to alter permeability and gating (Samways et al., 2006).

We previously reported that ∼6% of the total current through the P2X\textsubscript{2} receptor is carried by Ca\textsuperscript{2+} (Egan and Khakh, 2004), and that the ability of this receptor to select amongst cations involves a Ca\textsuperscript{2+}-sensing
domain made of three polar amino acids of TM2 (Migita et al., 2001). This domain is absent in the two P2X receptors (P2X1 and P2X4) that display higher P% of 11–15%, suggesting that distinct loci underlie the divergent Ca2+ currents of different family members. The aim of the present study was to identify the site(s) responsible for the elevated Ca2+ current of the P2X1 and P2X4 receptors.

MATERIALS AND METHODS

Molecular Biology and Cell Culture

We used wild-type rat P2X6, human P2X1 (hP2X1), human P2X2 (hP2X2), and zebrafish P2X1, P2X2, and P2X4 receptors. C-terminal epitope-tagged rat P2X1 and P2X2 receptors (P2X1-EYPMPE, P2X2-DYKD) that were made and expressed using conventional techniques. Addition of epitope did not change the EC50 for ATP, the kinetics of the ATP-gated response (Torres et al., 1999), or, most importantly, the contribution of Ca2+ flux to total current through the channel pore of either parent receptor (Table 1). Point mutations were introduced with the Quikchange II site-directed mutagenesis kit (Stratagene) and verified by automated DNA sequencing (Retrogen, Inc.). Chimerae were made as previously described (Haines et al., 2001). All constructs were coexpressed with eGFP in human embryonic kidney-293 cells (HEK-293 or HEK-293T cells; American Type Culture Collection) using Effectene (Qiagen) or Lipofectamine (Invitrogen) according to the manufacturers protocols. Transfected cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% FBS (Invitrogen or HyClone), 2 mM glutamine, 50 U/ml penicillin G, and 50 μg/ml streptomycin (Invitrogen), and incubated at 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 coverslips (Gold Seal; Becton Dickinson) the night before the experiment.

Patch-Clamp Photometry

Our use of the dye-overload method (Schneggenburger et al., 1993) is described in detail in Egan and Khakh (2004). In brief, we measured fractional calcium current (P%) by simultaneously measuring total membrane current and fluorescence in cells loaded with a high concentration (2 mM) of the calcium-sensitive dye, Fura-2 (for examples, see Fig. 2A). Cells on coverslips were placed in a recording chamber mounted on the stage of a TE3000 epifluorescence microscope (Nikon Instruments). ATP-gated current was recorded from single cells held at −60 mV using borosilicate glass recording electrodes (1B150F, World Precision Instruments), an Axopatch 200B amplifier (Molecular Devices), ITC-16 data acquisition hardware (Instrutech), ICGOR Pro software (Waveformics), and a G4 computer (Apple Computers). Electrodes had open-tip resistances of 1–3 MΩ and contained the Quirk chamber by reflection from the surface of a 400-nm dichroic long pass mirror. Light emitted by fura-2 was gathered by the objective, passed through the dichroic mirror and a 510-nm bandpass filter, and measured by a Model 714 Photomultiplier Detection System (Photo Technology International). We controlled the day-to-day variation in the sensitivity of the microscope/PMT by normalizing the fura-2 signal to a “bead unit” (BU). One BU equaled the average fluorescence of seven Carboxy Bright Blue 4.6-μm microspheres (Polysciences) measured one at a time on the morning of that day’s experiment (Schneggenburger et al., 1993; Frings et al., 2000).

Subsequent measurements of ATP-evoked changes in the fura-2 fluorescence of living cells were recorded as multiples of the daily BU. Cells were loaded with fura-2 by passive diffusion through the tip of the recording electrode. The time course of diffusion was measured in a subset of cells by monitoring the increase in intracellular fluorescence that results from fura-2 entry (Pusch and Neher, 1988). The time constant of loading was 141 ± 16 s (n = 30), and a near steady-state concentration of intracellular fura-2 was reached within ~9–10 min of patch disruption. These measurements suggest that 10 min is the minimum time needed to equilibrate the intracellular compartment with the contents of the pipette, and we waited at least this long before acquiring data. The extracellular bath solution was (in mM) 140 NaCl, 2 CaCl2, 1 MgCl2, 10 glucose, and 10 HEPES, titrated to pH 7.4 with NaOH. HEPES was replaced by MEPS in experiments performed at pH 5. ATP was applied for 0.2–2.0 s once every 2–3 min using triple-barreled theta glass and a Perfusion Fast-Step System SF-77 (Warner Instruments). For each construct and experimental algorithm, we used a concentration of ATP that evoked an easily measured decrease in fluorescence of fura-2 excited at 380 nm (∆F380) but avoided the problem of dye saturation (Table I). As previously reported, we saw no effect of short applications of submaximal concentrations of ATP on the fura-2 fluorescence of mock-transfected cells using the methods described above (Egan and Khakh, 2004) and equaled 0.0185 ± 0.002 BU/μC (n = 12).

Ca2+ and Cl− Permeability Measurements

We measured the relative permeabilities of Ca2+ and Cl− to Cs+ (PCa/PCl) and PCl/PCa, respectively using a reversal potential-based method and the Goldman equations (Hille, 2004). Membrane current was recorded using an AxoPatch 200B amplifier, indifferent 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) 150 CsCl, 10 EGTA, and 10 HEPES, brought to pH 7.4 with CaOH. Open tip electrode resistances measured 0.7–2.0 MΩ. Giga-ohm seals were established in a standard bath solution of (in mM) 150 NaCl, 1 CaCl2, 1 MgCl2, 10 glucose, and 10 HEPES, pH 7.4 with NaOH, and subsequently switched to test solutions of different ionic compositions.

To measure PCa/PCl, 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), followed by one that contained predominantly Ca2+ (in mM: 112 CaCl2, 1 MgCl2, 10 glucose, 10 HEPES). We included 0.1 mM Ca2+ in the CsCl solution to retard pore dilation because this process causes a time-dependent change in cation permeability that could alter PCa/PCl (Khakh et al., 1999; Chaumont and Khakh, 2006); in theory, addition of 0.1 mM Ca2+ to a solution of 150 mM CsCl solution is expected to have a negligible effect on the reversal potential of ATP-gated current (Lewis, 1979). 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 measured the membrane voltage corresponding to the zero-current level (i.e., the $E_{\text{rev}}$) from the leak-subtracted currents. We calculated $P_{\text{Ca}}/P_{\text{Cl}}$ as

$$P_{\text{Ca}} = \frac{(\alpha_{\text{Ca}} \times [Ca^{2+}]) \times \exp(\Delta E_{\text{rev}} \times F/RT) \times (1 + \exp(\Delta E_{\text{rev}} \times F/RT))}{4 \times \alpha_{\text{Cl}} \times [Cl^{-}]}.$$ 

where $\alpha_{\text{Ca}}$ and $\alpha_{\text{Cl}}$ are the activity coefficients of $Ca^{2+}$ (0.75) and $Cl^{-}$ (0.25), respectively, $\Delta E_{\text{rev}} = E_{\text{m,ca}} - E_{\text{m,cl}}$, $T$ is temperature (295.15 K, equal to 22°C), and $F$ and $R$ are universal constants.

We used bath solutions containing different concentrations of $CaCl_2$ to measure $P_{\text{Cl}}/P_{\text{Ca}}$. The first solution had the same concentrations (154 mM) of $Ca^{2+}$ and $Cl^{-}$ as the pipette solution. We applied a voltage ramp to obtain $E_{\text{m}}$ of the ATP-gated current and calculated the junctional offset as the deviation of the measured $E_{\text{m}}$ from the expected zero current level of 0 mV. This was then
subtracted from the $E_o$, obtained in a “low” CsCl bath solution that contained (in mM) 30 CsCl, 0.1 CaCl$_2$, 1 MgCl$_2$, 210 sucrose, 10 glucose, and 10 HEPES. $P_f/P_o$ was calculated as

$$P_f = \frac{1}{P_o} \frac{[X]/[X_o]}{\exp(\Delta E_{rev} * F/RT) - [X]/[X_o]}$$

where $[X]/[X_o]$ is the ratio of the intracellular and extracellular ion activities (Mitchell et al., 1997). Activity coefficients were determined by interpolation of the tabulated data of Robinson and Stokes (1970) and equaled 0.82 and 0.72 for 30 and 150 mM CsCl, respectively.

Data Analysis
All data are presented as the mean ± SEM for the number of experiments stated in the text. Significant differences amongst groups were determined using InStat (GraphPad Software) by one-way ANOVA with Tukey’s post hoc, or Student’s t test where appropriate. The P values of individual datasets are quoted in the text; values ≤ 0.05 were considered significant. We used the pooled data of wild-type P2X$_1$ and P2X$_2$-EE receptors as controls for comparisons to mutated receptors because we measured no difference in the $P_f/%s$ of these two groups (Table I).

RESULTS
The percentage of ATP current carried by Ca$^{2+}$ varies significantly amongst members of the P2X receptor family. In the present study, we first focused on the P2X$_1$ and P2X$_2$ receptors because they transduce appreciably different Ca$^{2+}$ fluxes. We measured $P_f/%s$ of 11.8 ± 0.5 and 6.7 ± 0.2% for the rat P2X$_2$ and P2X$_4$ receptors, respectively (Table I). These values are significantly different (P < 0.001) from each other and in good agreement with previously published results (Rogers and Dani, 1995; Egan and Khakh, 2004). With this baseline established, we examined the effect of genetic manipulation of channel structure on $P_f$ to gain insight into the molecular basis of the variability in Ca$^{2+}$ flux throughout the P2X family.

To What Extent Do Individual Transmembrane Segments Influence Ca$^{2+}$ Flux?
Empirical data suggest that both TM1 and TM2 line the ion-conducting pore of the P2X$_2$ receptor (Samways et al., 2006). Although no such data exist for the P2X$_1$ receptor, ATP activates cationic currents through chimeric P2X$_1$/P2X$_2$ proteins with swapped transmembrane domains (Werner et al., 1996; Haines et al., 2001a), and therefore it is reasonable to assume that the pores of both channels share a roughly similar design. If so, then the disparity in Ca$^{2+}$ flux amongst receptors might reflect subtype-specific differences in the primary structures of their pore-lining domains. To determine the influence of individual segments, we investigated three chimeric channels in which one (P2X$_{2-x}$-TM1$_{x}$ and P2X$_{2-x}$-TM2$_{x}$) or both (P2X$_{2-x}$-TM1$_{x}$/TM2$_{x}$) of the transmembrane segments of the P2X$_x$ channel were replaced by equivalent stretches of the P2X$_1$ channel (Fig. 1, A and B). All three chimerae formed functional channels when expressed in HEK-293 cells (Fig. 1 C).

We found that swapping transmembrane segments had quantifiable effects on $P_f/%$ (Fig. 2). The chimeric channel containing the first transmembrane segment of the P2X$_1$ channel in the P2X$_2$ background (P2X$_{2-x}$-TM1$_{x}$) had a $P_f$ equal to 9.3 ± 0.6%, which was significantly greater (P < 0.001) than that of the wild-type P2X$_2$ channel. Substitution of both transmembrane segments (P2X$_{2-x}$-TM1/2$_{x}$) had no greater effect ($P_f$ of 8.9 ± 0.5%) than replacing TM1 alone. In stark contrast, the chimeric P2X$_2$ channel containing the TM2 of P2X$_1$, (P2X$_{2-x}$-TM2$_{x}$) exhibited a $P_f$ of 2.6 ± 0.3% that was significantly lower (P < 0.001) than both wild-type channels (Table I). These results suggest either that: (1) both transmembrane domains contribute to the regulation of Ca$^{2+}$ current, each in their own way; or (2) construction of the chimerae produced nonspecific changes in the pore-forming domains that affect $P_f$. To explore these possibilities, we used the less invasive methods described below.

The Ca$^{2+}$ Flux of the P2X$_1$ Receptor Is pH Sensitive
The considerable Ca$^{2+}$ fluxes of many ligand-gated channels result from an interaction with negatively charged acidic amino acids in the mouth of the pore (Keramidas et al., 2004; Jensen et al., 2005). We looked for an effect of ionized side chains by measuring the $P_f/%s$ of P2X$_1$ and P2X$_4$ receptors at a lower pH where a greater proportion of the COO$^-$ side chains would be protonated and hence neutral (Hille et al., 1975; Green and Andersen, 1991) (Fig. 3 A). The $P_f$ of the wild-type P2X$_1$ receptor at pH 5.0 was 5.8 ± 0.8%, a significant (P < 0.001) reduction when compared with that measured at pH 7.4. In contrast, the $P_f$ of the wild-type P2X$_4$ receptor was unaffected by changing pH (Fig. 3 D). These data suggest that the electrostatic attraction of Ca$^{2+}$ by one or more acidic amino acids is responsible for the high $P_f/%s$ of the P2X$_1$ receptor, and that titratable carboxylates are unlikely to play a role in Ca$^{2+}$ flux through the P2X$_2$ receptor.

Acidic Residues Regulate the Ca$^{2+}$ Flux of the P2X$_1$ Receptor
Next, we used site-directed mutagenesis to locate the acidic amino acid(s) responsible for the high Ca$^{2+}$ flux of the P2X$_1$ channel. The data obtained using chimeric receptors suggested that the search should first center on the transmembrane domains. We discounted the conserved aspartate of TM2 because it is present in all family members (bold black residues of the primary structure). Acidic residues at similar homologous positions in TM1 (Glu$^{52}$, P2X$_1$ and Glu$^{51}$, hP2X$_4$) and TM2 (Asp$^{327}$, P2X$_1$ and Asp$^{331}$, hP2X$_4$) that are missing in the P2X$_2$ channel (bold red residues of Fig. 1 B).
We engineered P2X₁ mutants in which one or both of these were replaced by the amino acids that occupy the equivalent positions in the P2X₂ receptor (Gln⁵² and Ser⁶²). Neutralizing fixed charge in TM1 (P2X₁-E₅₂Q) significantly reduced Pf% to 8.4 ± 0.4% (Fig. 3B and Table I). Removing the fixed charge in TM2 (P2X₁-D₃₂₇S) had no significant effect (Pf% equals 10.5 ± 0.5%). However, neutralizing both charges (P2X₁-DM) caused a greater reduction (6.1 ± 0.6%) than neutralizing the charge of either TM1 (P < 0.01) or TM2 (P < 0.001) alone, and the sum of the effects of the single mutations (42% reduction in Pf%) was roughly equal to the effect of the double mutation alone (48% reduction in Pf%). Further, lowering the pH to 5.0 significantly reduced the Pf% of P2X₁-E₅₂Q to 6.0 ± 0.7% but had no effect on P2X₁-DM (5.8 ± 1.6%), suggesting that the resident charge at
position 327 that is present in P2X₁-E52Q but missing in P2X₁-DM does affect Pf%. Taken together, these data argue that both Glu 52 and Asp 327 interact with Ca²⁺ in the mouth of the pore, although the relative contribution of each amino acid is different.

We also performed the converse experiment of placing carboxylates at appropriate sites in the P2X₂ receptor. We generated three mutant P2X₂ receptors in which one or both of Gln52 and Ser326 were replaced by glutamate or aspartate, respectively (Fig. 3, C and D). The Pf% for the single mutants P2X₂-Q52E and P2X₂-S326D were 11.2 ± 0.8 and 11.0 ± 0.9%, respectively. Both values are significantly greater (P < 0.001) than the wild-type P2X₂ receptor and approximately equal to the wild-type P2X₁ channel. Placing both mutations in a single construct (P2X₂-DM) did not increase Pf% more than either mutation alone. Lowering the pH to 5.0 significantly (P < 0.001) reduced the Pf% of P2X₂-DM to 6.6 ± 1.3%, (Fig. 3 A), suggesting that the increase in Pf% results from an electrostatic effect of the added charge and not an unintended change in protein structure.

The Pf% of the P2X₂ Receptor Decreases when Fixed Charge Is Removed
Next, we looked at the effects of mutagenesis on the hP2X₄ receptor because it shows the highest Pf% of all P2X receptors (Egan and Khakh, 2004) and, like the rat P2X₁ receptor, has fixed negative charge at the outer edges of its transmembrane segments. The Pf% of the wild-type hP2X₄ receptor was 16.0 ± 1.0% (Fig. 4). We found that neutralizing both of the fixed charges of the transmembrane segments by mutagenesis gave a receptor (hP2X₄-DM) with a significantly (P < 0.001) reduced Pf% of 8.6 ± 0.6%. In contrast, removing a single charge had no effect. Specifically, the hP2X₄-E51Q and hP2X₄-D332S mutants had Pf% of 14.6 ± 1.1 and 13.0 ± 0.6%, respectively, that were no different than the wild-type receptor. In this respect, the single charge deletion mutants of the hP2X₄ receptor resemble the single charge addition mutants of the P2X₂ receptor; the presence of one charge per subunit is enough to maintain a high Ca²⁺ flux through the pore.

As a final test, we measured the Pf% of the wild-type zebrafish P2X₁, receptor (zP2X₁) (Kucenas et al., 2003). It lacks the two relevant formal charges found in hP2X₄ receptors (Fig. 1 B) and therefore naturally replicates the charge reduction of P2X₁-DM. In keeping with our hypothesis, the Pf% of the zP2X₁ receptor at 7.3 ± 1.3% was significantly smaller than the hP2X₄ receptor and not different from the rat P2X₁ and hP2X₄-DM receptors (Fig. 4 B).

Neutralizing Fixed Charge Alters Pᵣ/Pₛ but Not Pᵣ/Pₛ
A caveat to using patch-clamp photometry to draw conclusions about the Ca²⁺ flux of cation channels is that it
the hP2X5 receptor because it shows a significant Cl− permeability, and under the conditions of our experiments (equimolar [Cl−] on either side of the membrane and a holding potential of −60 mV), such an effect would lead to an apparent inward current caused by efflux of Cl−, a larger Qp, and a reduction in Pf% that does not reflect a change in Ca2+ flux per se. To differentiate a change in Ca2+ permeability from a change in Cl− permeability, we acquired reversal potential data from ATP-gated currents obtained from cells bathed in a range of extracellular solutions (see Materials and methods).

Switching the bath solution from one that contained 150 mM CsCl to one that contained 112 mM CaCl2 caused a rightward shift in the reversal potential of the ATP-gated current of the wild-type P2X1 and hP2X1 receptors (Fig. 5 A) that translated to Pf%/Pf0 values of 3.6 ± 0.2 and 4.6 ± 0.4, respectively (Table II). The shifts were smaller for P2X1-DM and hP2X1-DM receptors that lack the relevant formal charges, and the resulting Pf%/Pf0 were significantly (P < 0.001) lower than the wild-type templates at 2.6 ± 0.1 and 2.8 ± 0.1, respectively. They were not significantly different from that of the P2X2 receptor that measured 2.9 ± 0.1 (Table II). Again, these data support our contention that formal charge influences the Ca2+ dynamics of P2X1 and P2X4 receptors.

In contrast, Cl− permeability was not affected by the mutagenesis (Fig. 5 B). We first measured the Pf%/Pf0 of the hP2X4 receptor because it shows a significant Cl− permeability (Bo et al., 2003). As expected, we found that switching to the 30 mM CsCl bath solution caused a ~6-mV shift in Em; the calculated Pf%/Pf0 was 0.66 ± 0.12 (Fig. 5 B). We then measured the Cl− permeability of wild-type and double mutant P2X1 and P2X4 receptors. Shifting the extracellular solution from one that contained 150 mM CsCl to one that contained 30 mM CsCl caused an approximately −30 mV shift in the ATP-gated current in all four constructs, demonstrating that both the wild-type and mutant receptors are largely impermeable to anions (Table II). These data support the hypothesis that the changes in Pf% that we measure after mutagenesis reflect specific effects on Ca2+ flux.

P2X3 Has Fixed Charge but a Relatively Low Pf%
The glutamate of TM1 is conserved in only one other P2X subtype as Glu46 of the P2X3 receptor. However, the P2X3 receptor exhibits the lowest Ca2+ flux of all family members (Egan and Khakh, 2004). Unlike P2X1 and P2X4 receptors, the P2X3 receptor contains a positively charged amino acid, His45, immediately upstream to the conserved Glu46 residue (green residues of Fig. 1 B). His45 may provide a countercharge in the pore that serves to partially shield the neighboring Glu46 or simply repel Ca2+, thus attenuating the ability of the carboxylate to attract Ca2+. To test this hypothesis, we constructed a mutant P2X3 receptor in which His45 was replaced by the tyrosine found at the homologous position of the P2X1 receptor. This produced a small but significant (P < 0.05) increase in Pf% to 6.7 ± 0.5% compared with the wild-type P2X3 control of 4.8 ± 0.3% (Table I).

We also generated mutant P2X1 receptors in which Tyr51 was replaced either by a basic histidine (P2X1-Y51H) or a neutral valine (P2X1-Y51V). We found that the Pf% of P2X1-Y51H was significantly reduced (6.3 ± 0.8%) compared with the wild-type P2X1 receptor. This decrease was unlikely to be caused by a gross disruption of channel structure because substituting neutral valine (the corresponding residue of P2X4) for Tyr51 had no effect on Pf% (Table I). Taken together, the data suggest that the identity of the amino acid just upstream of the conserved charges of P2X1 and P2X4 receptors helps to determine the magnitude of the Ca2+ flux through the channel pore.

**DISCUSSION**

We identified acidic residues at the outer ends of the transmembrane domains that contribute to the high
fractional Ca\(^{2+}\) current of ATP-gated P2X\(_1\) and P2X\(_4\) receptors. We found that acidification decreased the relatively large Ca\(^{2+}\) current of the P2X\(_1\) receptor but had no effect on the smaller Ca\(^{2+}\) current of the P2X\(_2\) receptor, suggesting that only the former uses acidic amino acids to discriminate amongst permeant cations. (We also looked at the effect of a lower pH on the hP2X\(_4\) receptor. The concentration–response curve of the P2X\(_4\) receptor shifts to the right at lower pHs, necessitating the use of a very high (>1 mM ATP) concentration of ATP to evoke a measurable ligand-gated current. At these high concentrations, applications of ATP evoked biphasic changes in intracellular calcium that did not follow the time course of the integrated current, making measurements of \(P_{f}%\) untenable.) The magnitude of the reduction is surprising when one considers the predicted degree of protonation of the COO\(^-\) side chains. We expected to measure a modest reduction in \(P_{f}%\) upon lowering the pH to 5.0, a value close to the \(pK_a\) of glutamate and aspartate in aqueous solution, and where half of the carboxyl groups are protonated and thus unable to attract Ca\(^{2+}\) (Falke et al., 1994). Instead, we found that acidification produced a \(P_{f}%\) equal to that of mutant receptors lacking both formal charges (i.e., P2X\(_1\)-DM). These data suggest that the \(pK_a\)s of Glu\(_{52}\) and Asp\(_{327}\) of the P2X\(_1\) receptor may be significantly higher than predicted from measurements of pure aqueous solutions of amino acids, an idea in keeping with published reports that the local microenvironment of a protein greatly influences the degree of ionization of its constituent amino acids (Fersht, 1985; Klockner et al., 1996; Seifert et al., 1999; Petsko and Ringe, 2004). Of particular interest is the positive charge provided by a conserved lysine (e.g., Lys\(_{53}\) of the P2X\(_1\) receptor) that sits immediately downstream of Glu\(_{52}\). Placing positive charge close to an acidic amino acid is enough to shift the latter’s \(pK_a\) to 7.0 or higher (Falke et al., 1994). We have not pursued a study of this lysine because it is fully conserved and therefore unlikely to play a role in determining the dissimilar Ca\(^{2+}\) fluxes measured across the family. However, its role in the absolute magnitude of the Ca\(^{2+}\) flux should be considered in future experiments.

The idea that individual P2X receptors use distinct domains for cation selection is supported by the marked changes in \(P_{f}%\) observed in P2X\(_1\)/P2X\(_2\) chimeras. Replacing the TM1 of the less Ca\(^{2+}\)-permeable P2X\(_2\) receptor with that of the more permeable P2X\(_1\) receptor
significantly enhanced Ca\(^{2+}\) flux, as did addition of a single formal negative charge to the extracellular end of TM1 (P2X\(_{2}\)-Q52E). These data are important for three reasons: first, they add weight to the proposition that TM1 lines the outer segment of the ion channel (Haines et al., 2001b); second, they show for the first time that TM1 makes a measurable contribution to cation selectivity; and third, they suggest that the divergent Ca\(^{2+}\) currents of the P2X\(_{1}\) and P2X\(_{2}\) subtypes result in part from differences in the primary sequences of their TM1s.

The case of TM2 is more complex. We found that adding fixed negative charge to TM2 of the P2X\(_{2}\) receptor increased \(Pf\)% only when Thr\(^{336}\), Thr\(^{339}\), and Ser\(^{340}\) are unperturbed (compare P2X\(_{2}\)-S326D to P2X\(_{2}\)-TM2\(_{X1}\)), a finding that supports previous reports that the polar residues of TM2 play an important role in Ca\(^{2+}\) flux in this receptor subtype (Egan and Khakh, 2004). Two of these are replaced by neutral amino acids in the P2X\(_{2}\)-TM2\(_{X1}\) chimera, thus depriving the parent P2X\(_{2}\) receptor of side chains that may provide the countercharge needed to partially dehydrate Ca\(^{2+}\) at a constriction within the pore (Migita et al., 2001). If so, then P2X\(_{2}\) receptors either use other means to overcome the energy barrier provided by the constriction or lack it entirely. An alternative explanation is that the decrease in \(Pf\)% in the P2X\(_{2}\)-TM2\(_{X1}\) chimera reflects nonspecific changes in the topology of the pore brought about by swapping one TM2 with another. This latter possibility is difficult to ignore, and additional experiments are needed to firmly establish a role for polar amino acids in ion permeation in some P2X receptors.

Many classes of ligand-gated cation channels use the formal charge of acidic amino acids positioned near the pore to facilitate Ca\(^{2+}\) transport into cells (Keramidas et al., 2004; Jensen et al., 2005). P2X receptors have three sites that could serve a similar function. One of these is fully conserved in TM2 (see bold black letters in Fig. 1B) and therefore unlikely to explain the differences in Ca\(^{2+}\) flux measured here. Two nonconserved sites are present in P2X\(_{1}\) and P2X\(_{4}\) receptors but not in the P2X\(_{2}\) receptor, and we found that removing both charges significantly decreased the \(Pf\)% and \(P_{Ca}/P_{Cs}\) without affecting \(P_{Cl}/P_{Cs}\). Thus, our data are consistent with a model that uses the oxygen atoms of COO\(^{-}\) side chains to form an electrostatic ring that attracts cations. The attraction is stronger for Ca\(^{2+}\) than for Na\(^{+}\), and therefore could explain the high Ca\(^{2+}\) permeability and flux of P2X\(_{1}\) and P2X\(_{4}\) receptors. The location of the ring in the permeation pathway is unknown because the inner and outer limits of the pore are poorly defined (Egan et al., 2006). We favor the hypothesis that it lies in the extracellular vestibule for the following two reasons. First, such a model is consistent with those that explain the high Ca\(^{2+}\) fluxes of other ligand-gated cation channels (Barry and Lynch, 2005). Second, models that incorporate the formal charges of aspartate and glutamate side chains into Ca\(^{2+}\) binding sites within the pore often result in channels that show Ca\(^{2+}\) block of monovalent current (Voets et al., 2004), and no such effect is reported for either the P2X\(_{1}\) or P2X\(_{4}\) receptors.

We suggest the simple hypothesis that the formal charges of both TM1 and TM2 influence the flux of Ca\(^{2+}\) through P2X\(_{1}\) and hP2X\(_{4}\) channels in a nonadditive fashion. We found that adding charge to either transmembrane domain significantly enhanced Ca\(^{2+}\) flux of the P2X\(_{2}\) receptor, whereas removing the charge of either Glu\(^{52}\) or Asp\(^{352}\) had only a small effect on the \(Pf\)% of the hP2X\(_{4}\) receptor. The results are therefore consistent, for together they suggest that (1) a single charge is sufficient to cause the elevated Ca\(^{2+}\) permeability noted in the P2X\(_{2}\)-DM and wild-type hP2X\(_{4}\) receptors; and (2) there is very little summation in terms of increasing \(Pf\)% when both charges are present in the outer parts of TM1 and TM2.

For the P2X\(_{1}\) receptor, it appears that Glu\(^{52}\) of TM1 might play a larger role than Asp\(^{352}\) of TM2. We found that removing Glu\(^{52}\) significantly reduced the \(Pf\)% of the P2X\(_{1}\) receptor, whereas removing Asp\(^{352}\) had no effect, as might be expected if Asp\(^{352}\) is innocuous. Although these findings seem to invalidate the dual-charge hypothesis discussed above, two experiments suggest that Asp\(^{352}\) does influence Ca\(^{2+}\) flux through the channel. First, subtracting both charges (i.e., P2X\(_{1}\)-DM) has a significantly greater effect on the \(Pf\)% than removing

| Protein   | \(\Delta E_{rev, Ca}\) | \(P_{Cl}/P_{Cs}\) | \(P_{Ca}/P_{Cs}\) | \(\Delta E_{rev, Cl}\) | \(P_{Cl}/P_{Cs}\) | \(P_{Ca}/P_{Cs}\) |
|-----------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| hP2X\(_{4}\) | 0.3 ± 0.1       | 2.6 ± 0.1       | 7.5 ± 0.1       | 0.0 ± 0.1       | 2.6 ± 0.1       | 7.5 ± 0.1       |
| hP2X\(_{1}\) | 3.0 ± 0.1       | 2.2 ± 0.1       | 5.8 ± 0.1       | 0.0 ± 0.1       | 2.2 ± 0.1       | 5.8 ± 0.1       |
| hP2X\(_{2}\) | 1.0 ± 0.1       | 2.2 ± 0.1       | 5.8 ± 0.1       | 0.0 ± 0.1       | 2.2 ± 0.1       | 5.8 ± 0.1       |
| hP2X\(_{3}\) | 4.5 ± 0.1       | 2.2 ± 0.1       | 5.8 ± 0.1       | 0.0 ± 0.1       | 2.2 ± 0.1       | 5.8 ± 0.1       |
| hP2X\(_{4}\) | 5.0 ± 0.1       | 2.2 ± 0.1       | 5.8 ± 0.1       | 0.0 ± 0.1       | 2.2 ± 0.1       | 5.8 ± 0.1       |

The control solution contained predominantly 150 mM CsCl, and the test solutions contained either 112 CaCl\(_{2}\) or 30 CsCl (see text for details). \(\Delta E_{rev, Ca} = E_{rev, 112 Ca} - E_{rev, 30 CaCl} = E_{rev, 112 Ca} - E_{rev, 30 CaCl}\)
either Glu$^{52}$ or Asp$^{327}$ alone. Second, acidification decreases the $P_f$% of P2X$_1$-E52Q but has no effect on P2X$_1$-DM, and the only difference between these two mutants is the presence of Asp$^{327}$. It is difficult to explain why eliminating the glutamate of TM1 significantly decreases the $P_f$% of P2X$_1$ but not P2X$_4$ receptors, except to say that structural differences in TM2 may make the Asp$^{327}$ of the P2X$_1$ receptor either less accessible to Ca$^{2+}$ or less charged than either the Asp$^{326}$ of the hP2X$_4$ receptor or the Asp$^{326}$ of the P2X$_4$-S326D mutant.

P2X$_1$, P2X$_2$, and P2X$_4$ receptors have remarkably different phenotypes and display unique agonist and antagonist profiles, rates of desensitization, Ca$^{2+}$ permeabilities, and trafficking (North, 2002). If function follows structure, then structural differences between these subtypes are to be expected.

Fig. 6 shows cartoon representations of P2X$_1$, P2X$_2$, and P2X$_4$ receptors that incorporate two different models of Ca$^{2+}$ transport. We assume that each channel is comprised of three subunits (Nicke et al., 1998; Aschrafi et al., 2004; Barrera et al., 2005; Mio et al., 2005), and that each subunit contributes two transmembrane segments to the pore (North, 2002). In P2X$_1$ and P2X$_4$ receptors, Ca$^{2+}$ current is enhanced by a ring of charge made of glutamate and aspartate residues positioned in the outer mouth of the pore. These residues could function to accumulate extracellular Ca$^{2+}$ in the permeation pathway by a simple electrostatic attraction, or form a binding site that selects Ca$^{2+}$ over monovalent cations (Hille, 2004; Jensen et al., 2005). As mentioned above, the latter hypothesis seems doubtful because, unlike P2X$_2$ receptors, Ca$^{2+}$ does not block monovalent cation current through P2X$_1$ receptors as expected for a binding site model (Evans et al., 1996). The extracellular ring of charge is missing in the P2X$_2$ receptor (Fig. 6, C and D). Instead, polar residues in the middle of the pore provide the countercharge needed to dehydrate Ca$^{2+}$ in a narrow part of the pore (Migita et al., 2001). In this model, the narrow constriction provides an energy barrier that impedes current flow through the channel, and the relative conductance of cations is determined by their ability to momentarily “bind” to the polar side chains as they shed water. The presence of an intrapore binding site in P2X$_2$ receptors is supported by two findings. First, ATP-gated currents saturate with increasing concentrations of extracellular Na$^+$ (Ding and Sachs, 1999b). Second, monovalent current is blocked by low millimolar concentrations of extracellular Ca$^{2+}$ (Nakazawa and Hess, 1993; Ding and Sachs, 1999a).

One question then remains: how well do these two mechanisms describe the Ca$^{2+}$ current of other family members? Fixed charge is conserved in the highly Ca$^{2+}$-permeable P2X$_1$ and P2X$_4$ receptors, but is also present in the less permeable P2X$_3$ and P2X$_7$ receptors. We have not investigated P2X$_7$ receptors so far, but experiments with P2X$_3$ receptors suggest again that each subunit uses a slightly different method to select amongst cations. P2X$_3$ receptors have a unique His$^{45}$ just upstream of Glu$^{46}$. The positive charge it supplies might serve to partially neutralize the negative charge of Glu$^{46}$ and hinder the electrostatic interaction with Ca$^{2+}$. However, it is worth noting that neutralizing His$^{45}$ did not elevate the $P_f$% of the P2X$_3$ receptor to a value equal to that of P2X$_1$ receptor, suggesting that additional factors contribute to the lower $P_f$% measured for P2X$_3$ receptor.
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