Control of P2X₂ Channel Permeability by the Cytosolic Domain

ANGELA N. EICKHORST, AMY BERSON, DEBRA COCKAYNE, HENRY A. LESTER, and BALJIT S. KHAKH

ABSTRACT ATP-gated P2X channels are the simplest of the three families of transmitter-gated ion channels. Some P2X channels display a time- and activation-dependent change in permeability as they undergo the transition from the relatively Na⁺-selective I₁ state to the I₂ state, which is also permeable to organic cations. We report that the previously reported permeability change of rat P2X₂ (rP2X₂) channels does not occur at mouse P2X₂ (mP2X₂) channels expressed in oocytes. Domain swaps, species chimeras, and point mutations were employed to determine that two specific amino acid residues in the cytosolic tail domain govern this difference in behavior between the two orthologous channels. The change in pore diameter was characterized using reversal potential measurements and excluded field theory for several organic ions; both rP2X₂ and mP2X₂ channels have a pore diameter of ~11 Å in the I₁ state, but the transition to the I₂ state increases the rP2X₂ diameter by at least 3 Å. The I₁ to I₂ transition occurs with a rate constant of ~0.5 s⁻¹. The data focus attention on specific residues of P2X₂ channel cytoplasmic domains as determinants of permeation in a state-specific manner.

KEY WORDS: ATP • ion channel • modulation • P2X • purinoceptor

INTRODUCTION

ATP-gated cationic P2X channels define one of the three major families of transmitter-gated ion channels (Khakh, 2001). Although there are seven distinct P2X subunits, much of our understanding is based on studies of the P2X₂ subunit which forms homomeric channels. Available evidence indicates that each P2X₂ subunit possesses two transmembrane domains, cytosolic NH₂ and COOH termini and a large cysteine-rich extracellular loop. There is strong evidence that transmembrane domain 2 (TM2) lines the pore, because mutations here affect organic cation permeability (Khakh et al., 1999; Virginio et al., 1999b) and Ca²⁺ permeability (Migita et al., 2001), and because substituted cysteines in TM2 are modified by extracellular hydrophilic methanethiosulphonate reagents when the channel opens (Rassendren et al., 1997; Egan et al., 1998). The gate appears to be close to a conserved glycine (G342), whereas a conserved aspartate (D349) is internal to it. P2X channel pores are cation selective with almost equal permeability to Na⁺/K⁺ and significant permeability to Ca²⁺.

Some P2X channels also display a time and activation-dependent increase in permeability to organic cations and fluorescent dye molecules before they desensitize. This phenomenon was first reported for, and considered unique to, the P2X₇ channel (Surprenant et al., 1996). But subsequent studies demonstrated permeability changes for various recombinant and natively expressed P2X channels in neurons (Khakh et al., 1999; Virginio et al., 1999b). The permeability change of the P2X₂ channel is just one example of the recent observations that some channels change their selectivity on the time scale of milliseconds in response to diverse stimuli, including membrane voltage, neurotransmitter binding, and second messengers (Khakh and Lester, 1999). The structural bases and biophysical properties of these changes are poorly understood.

The goal of this study was to provide a more complete understanding of the history-dependent changes in cation permeability at P2X₂ channels. We found that the previously reported permeability change of rat P2X₂ (rP2X₂) channels does not occur at mouse P2X₂ (mP2X₂) channels expressed in oocytes. We used domain swaps, chimeras, point mutations, reversal potential measurements, kinetics, and excluded field theory to track and quantify permeability changes. Surprisingly, we found that permeation of one P2X₂ state (I₂) is affected by two specific residues in the cytosolic tail domain, whereas the permeation of a preceding state (I₁) is not. We conclude that opening to the I₂ state requires conformational changes in the C tail domain, whereas opening to I₁ does not.
MATERIALS AND METHODS

Cloning, Expression, and Recording

The mP2X<sub>2</sub> cDNA was cloned from a mouse testis library using primers designed to the rat P2X<sub>2</sub> cDNA (antisense primer GAA TTC TCA AAG TTG GGC CAA ACC T, sense primer GGA TTC ATG GTC CGG CGC TTG G). Mouse P2X<sub>7</sub> was cloned from a mouse testis library using primers designed to the rat P2X<sub>7</sub> cDNA. The five individual clones were sequenced (Roche Biochemicals, Inc.). Electrodes were pulled from borosilicate glass and rinsed in physiological solutions described using described methods. The five individual clones were sequenced (Roche Biochemicals, Inc.). Thus, the mP2X<sub>2</sub> cDNA was cloned from a mouse testis library using primers designed to the rat P2X<sub>2</sub> cDNA and sequenced (Roche Biochemicals, Inc.). Electrodes were pulled from borosilicate glass and rinsed in physiological solutions described using described methods.

The C tail swap chimeras were made by digesting rP2X<sub>2</sub> and mP2X<sub>2</sub> cDNA with HpaI (native site in both cDNAs) and XhoI (in the 3' polynucleotide) to produce two fragments: (a) the vector and region coding for the entire P2X<sub>2</sub> cDNA, minus part of TM2 and the C tail, and (b) DNA coding for the remainder of TM2 and the COOH-terminal domain. The DNA fragments were separated by agarose gel electrophoresis and purified (QIAGEN gel extraction kit). Thus, the mP2X<sub>2</sub> C tail domain was ligated into rP2X<sub>2</sub>, and vice versa, and restriction analysis was used to confirm the construction.

Single site mutants were made using Quick Change Mutagenesis (Stratagene). DNA sequencing was used to confirm the mutation. All cDNAs were transcribed in vitro using the mMESSAGE mACHINE kit (Ambion).

Xenopus laevis oocytes were prepared and used for electrophysiological recordings described using described methods. Two-electrode voltage-clamp recording of oocytes was performed using the Geneclamp 500 amplifier (Axon Instruments, Inc.). Electrodes were pulled from borosilicate glass (Sutter Instrument Co.) and back filled with 3 M KCl to yield resistances of 1–2 MΩ. Recordings were made in solution consisting of 98 mM NaCl, 5 mM HEPES, and 1 mM MgCl<sub>2</sub> at pH 7.35–7.4, which was superfused over the oocytes by gravity flow. In some experiments, equimolar substitutions of organic cations were made for Na<sup>+</sup>. The organic cations tested were dimethylammonium, 2-(methyl-amino)-ethanol, Tris<sup>+</sup>, and N-methyl-d-glucamine. Solutions containing ATP were applied to the oocyte using a solenoid-operated solution switcher (General Valve Company); complete solution exchange around the oocyte occurred within 0.5–1.0 s. Voltage-clamp experiments were controlled by a Digidata 1200 interface and a personal computer running pCLAMP 7 or pCLAMP 8 software (Axon Instruments, Inc.). In some experiments, the voltage was clamped at a rate of 0.36–0.6 mV/ms. Data were filtered at 200–500 Hz and digitized at 3–5 times this rate. Current-voltage relation data were filtered at 1 kHz and digitized at 3 kHz. All experiments were performed at 18–20°C.

Data Analysis

Data were analyzed using Clampfit (Axon Instruments, Inc.) or Origin 5.0 (Microcal Software, Inc.), and appear in the text and graphs as mean ± SEM from n determinations as indicated (><). We employed the principles developed by Hille (Dwyer et al., 1980; Hille, 1992). A transform of the GHK voltage equation under bionic conditions from

\[ E_{rev} = \frac{RT}{2F} \ln \frac{P_X[X]}{P_Y[Y]}, \]

provides a way to measure the permeability, P, of ion X<sup>+</sup> with reference to ion Y<sup>+</sup>. However, under the assumption that the intracellular ion concentration is constant, the above equation can be used to measure the permeability of ion X<sup>+</sup> relative to Y<sup>+</sup>, as

\[ \frac{P_X}{P_Y} = \left(\frac{[X]}{[Y]}\right) \exp\left(\frac{zE_{rev}^{X} - E_{rev}^{Y}}{RT}\right) \]

where P<sub>X</sub>/P<sub>Y</sub> is the shift in reversal potential and F, R, and T have their usual meaning (Khakh et al., 1999). The size of the narrowest region of the pore was determined using excluded field theory (Dwyer et al., 1980; Cohen et al., 1992), which relates ion permeability ratios, and sizes of ions (treated as spheres) to the narrowest region of the pore, which is approximated as a cylinder with a narrow constriction. The theory does not provide information about the shape of the constriction, or the pore, but can be used to measure the dimensions of the narrowest region, as explained below. For a channel pore of radius R<sub>c</sub> and ion X<sup>+</sup> with a radius R<sub>x</sub> (R<sub>Na</sub><sup>+</sup> is the hydrated radius of Na<sup>+</sup> at 2.15 Å) the P<sub>X</sub>/P<sub>Na</sub><sup>+</sup> is given by

\[ \frac{P_X}{P_{Na^+}} = \left(\frac{R_{c} - R_{x}}{R_{c} - R_{Na^+}}\right)^2. \]

A square root transformation gives

\[ \frac{P_X}{P_{Na^+}} = a - bR_{x}, \]

where

\[ a = \frac{R_{c}}{R_{c} - R_{Na^+}} \quad \text{and} \quad b = \frac{1}{R_{c} - R_{Na^+}}, \]

and the diameter of the narrowest region of the pore is 2b/a. In the above equations a and b are obtained from linear regressions of plots of (P<sub>X</sub>/P<sub>Na</sub><sup>+</sup>)<sup>1/2</sup> versus R<sub>x</sub> (as in Fig. 4). In such formalisms a convenient check is to determine the diameter of Na<sup>+</sup>, which is given by R<sub>Na</sub><sup>+</sup> = (a – 1)/b, and using a = 1.57 Å<sup>-1</sup> and b = 0.28 (average from regressions of rP2X<sub>2</sub> I<sub>1</sub>, mP2X<sub>2</sub> I<sub>1</sub>, and I<sub>1</sub> shown in Fig. 4). This gives R<sub>Na</sub><sup>+</sup> as 2.04 Å, which is close to the hydrated radius (Kieland, 1937) of Na<sup>+</sup> in water at 2.15 Å. These numbers indicate that there is ~5% error in the calculations reported in this study. The radius of ion X<sup>+</sup> (R<sub>x</sub>) was determined as

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The change in $P_{\text{NMDG}}$ (as indicated, to the Hill equation. Statistical tests were performed on data. Concentration-effect curves were fitted where appropriate, and $P_{\text{NMDG}}/P_{\text{Na}^+}$ was calculated from the average values of I and $E_{\text{rev}}(\text{NMDG})$ (as shown in Table I). These differences are expected with small numbers for the numerators, as in the NMDG$^+$ data. For $\text{Na}^+$ currents, inward rectification was quantified as $G_{60 \text{ mV}}/G_{10 \text{ mV}}$. Rate constants ($1/\tau$, s$^{-1}$) were determined from single exponential fits to the data. Concentration-effect curves were fitted where appropriate, as indicated, to the Hill equation. Statistical tests were performed using the paired or unpaired Student’s $t$ test, as appropriate, and a $P < 0.05$ was taken to indicate significance.

**RESULTS**

**Initial Observations with rP2X$_2$ Channels**

Fig. 1 illustrates permeability changes for rP2X$_2$ channels expressed in oocytes, and introduces the measurements that are used throughout this paper. Table I contains data for various measurements from nine cells, from experiments of the type illustrated in the representative waveform shown Fig. 1 A. At a holding potential of $-60 \text{ mV}$ and in extracellular solutions that contain NMDG$^+$, application of ATP evokes an initial transient outward current (termed $I_1$) at a holding potential of $-60 \text{ mV}$. Over a time course of seconds the currents become inward and reach steady-state values ($I_2$), but on switching to $\text{Na}^+$ solutions the currents are larger ($I_{\text{Na}^+}$; Fig. 1 A). The $I_{\text{Na}^+}/I_2$ ratio calculated from the individual cells was $7.3 \pm 1.9$ ($n = 9$; Table I legend).

We also measured the reversal potential of the ATP-evoked currents between $I_1$, $I_2$, and $I_{\text{Na}^+}$ with voltage ramps (Fig. 1 B), and expressed these values as permeability ratios relative to $\text{Na}^+$ (see MATERIALS AND METHODS). The inward $I_1$ current develops with a rate constant ($k_{+} = 0.3 \pm 0.01$ s$^{-1}$) similar to that for the shift in reversal potential ($k_{\tau} = 0.4 \pm 0.1$ s$^{-1}$) from $-66.7 \text{ mV}$ for $I_1$ to $-35.5 \text{ mV}$ for $I_2$, thus showing a change in $P_{\text{NMDG}}/P_{\text{Na}^+}$ from 0.07 for $I_1$ to 0.34 for $I_2$ (Table I). The rate constant for the increase in NMDG$^+$ permeability is $\sim 0.4$ s$^{-1}$, similar to that reported previously in mammalian cells (Virginio et al., 1999b). Thus, with time NMDG$^+$ becomes more permeable through rP2X$_2$ pores, but it does not become

\[
R_x = \frac{3/D_1 \times D_2 \times D_3}{2},
\]

where $D_1$, $D_2$, and $D_3$ are the three dimensions of the smallest box that will house the ion. The fold change ($\Delta$) in $P_{\text{X}^+}/P_{\text{X}^+}$ is the ratio of $P_{\text{X}^+}/P_{\text{X}^+}$ for $I_1$ and $I_2$. Slope conductance values for $I_1$, $I_2$, and $I_{\text{Na}^+}$ were measured from current-voltage relationships over a range of 40 mV around the reversal potential. The values were calculated separately for each cell, before averaging. For $I_2$ there was a small difference between these values and those calculated from the average values of I and $E_{\text{rev}(\text{NMDG})}$ (as shown in Table I). These differences are expected with small numbers for the numerators, as in the NMDG$^+$ data. For $\text{Na}^+$ currents, inward rectification was quantified as $G_{60 \text{ mV}}/G_{10 \text{ mV}}$. Rate constants ($1/\tau$, s$^{-1}$) were determined from single exponential fits to the data. Concentration-effect curves were fitted where appropriate, as indicated, to the Hill equation. Statistical tests were performed using the paired or unpaired Student’s $t$ test, as appropriate, and a $P < 0.05$ was taken to indicate significance.

**TABLE I**

<table>
<thead>
<tr>
<th>Peak current at $-60 \text{ mV}$ ($\mu$A)</th>
<th>$I_1$</th>
<th>$I_2$</th>
<th>$I_{\text{Na}^+}$</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$0.5 \pm 0.1$</td>
<td>$-3.2 \pm 1.2$</td>
<td>$-10.4 \pm 0.5$</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>$E_{\text{rev}}$ (mV)</td>
<td>$-66.7 \pm 0.9$</td>
<td>$-35.1 \pm 6.4$</td>
<td>$2.7 \pm 0.9$</td>
<td>9</td>
</tr>
<tr>
<td>Slope conductance ($\mu$S)</td>
<td>$97 \pm 3$</td>
<td>$156 \pm 4$</td>
<td>$200 \pm 20$</td>
<td>9</td>
</tr>
<tr>
<td>$P_{\text{NMDG}}/P_{\text{Na}^+}$</td>
<td>$0.07 \pm 0.003$</td>
<td>$0.34 \pm 0.1$</td>
<td>$-9$</td>
<td></td>
</tr>
</tbody>
</table>

The slope conductances, calculated from current-voltage plots over a range of 40 mV around the reversal potential, were significantly different from each other when compared using analysis of variance at $P < 0.05$. For completeness, we also present the individual values for peak amplitudes: $I_1 = 0.2, 0.7, 1.3$, $0.5, 0.8, 0.1, 0.6, 0.6, 0.1 \mu$A; $I_2 = 1.9, -1.1, -1.0, -0.5, -4.7, -0.9, -10, -1.3, -7.6 \mu$A; and $I_{\text{Na}^+} = 10.1, -10.2, -7.4, -9.2, -10.6, -10.5, -12.5, -11.4, -11.6 \mu$A. The $I_{\text{Na}^+}/I_2$ ratio was $7.3 \pm 1.9$ (the ratio for each cell was $5.3, 9.3, 7.4, 18.4, 2.3, 11.7, 1.3, 8.8, 1.5$).
so permeable as Na\(^+\) (E<sub>rev</sub> 2.7 mV; \(n = 9\), mean values are shown in Table I).

In the present study rP2X2 channels underwent permeability changes in all batches of oocytes, but the extent of the change varied from batch to batch (for example our results show values for \(P_{\text{NMDG}^+}/P_{\text{Na}^+}\) between 0.17 and 0.34). These observations suggest the presence of uncontrolled variable(s) that influence the permeability change. To control for this variability, all comparisons between mutant and wt channels were made from the same batches. The remainder of this paper analyzes the nature of the changes that occur during the growth of the current from \(I_1\) to \(I_2\), and the accompanying shifts in reversal potentials (Fig. 1, A and B).

**Figure 2.** Basic properties of rP2X2 and mP2X2 channels. (A) Concentration-effect curves for ATP at rP2X2 and mP2X2. There is a significant difference in EC<sub>50</sub>. (B) I-V relations from voltage steps (-60 to 60 mV in 10-mV steps) for mP2X2 and rP2X2. Note that rectification is approximately equal for both channels, but that there is greater variability in the data for outward currents as compared with inward current. A similar trend for natively and heterologously expressed channels has been reported previously (Khakh et al., 1995; Evans et al., 1996). In this and all other figures the error bars are omitted when they are smaller than the symbols used.

**Figure 3.** rP2X2 and mP2X2 channels differ with respect to permeability changes. (A) rP2X2: steady-state current waveform (-60 mV) in NMDG<sup>+</sup> extracellular solutions. ATP was applied when indicated and evoked an outward current that became inward over time. (B) Reversal potentials at the peak of \(I_1\) and \(I_2\) for rP2X2. (C) mP2X2: steady-state current waveform in NMDG<sup>+</sup> extracellular solutions. ATP was applied for the period indicated and evoked an outward current that waned to a steady-state level. (D) Reversal potentials at the peak of \(I_1\) and \(I_2\) for mP2X2.
Species Differences Reveal a Rationale to Study the Basis of Permeability Changes

We cloned and expressed mP2X₂ channels in Xenopus oocytes. Rat and mouse P2X₂ channels were similar with respect to peak I₇Na⁺, but the ATP EC₅₀ differed, being 2 ± 1 and 12 ± 4 µM with Hill slopes of 1.9 ± 0.2 and 2.0 ± 0.2, respectively, under conditions where we expect the pore to have dilated to the I₂ state (n = 5; Fig. 2 A). Because of the difference in EC₅₀, and to minimize any errors on the linear part of the curve, all subsequent experiments were performed at saturating concentrations of ATP (≥100 µM). rP2X₂ and mP2X₂ channels displayed similar current-voltage relations in Na⁺ solutions: the I₇Na⁺ reversal potentials were close to 0 mV (−4.1 ± 0.1 and −6.5 ± 1.5 mV; P > 0.05) and the rectification indices (G₆₀ mV/G₉₀ mV) were 0.6 ± 0.2 and 0.5 ± 0.2 (P > 0.05), for rP2X₂ and mP2X₂ (Fig. 2 B). In short, there were no major differences between rP2X₂ and mP2X₂ channels in Na⁺ solutions.

When ATP was applied in extracellular solutions that contained NMDG⁺ in place of Na⁺ at −60 mV, a clear difference between rP2X₂ and mP2X₂ channels was apparent. Fig. 3 illustrates this distinction. In NMDG⁺ solutions application of ATP to mP2X₂ evoked an initial outward current (I₁), that decreases over 50 s to reach a steady-state value (I₂). In contrast, at rP2X₂ channels the current actually switches direction: I₁ is outward and I₂ is inward. Current-voltage relations based on voltage ramps (Fig. 3, B and D) show that the development of I₂ for both rP2X₂ and mP2X₂ channels is associated with shifts in reversal potential over time, but the shift is much greater for rP2X₂ than for mP2X₂. Thus, for rP2X₂ channels P₇NMDG⁺/PNa⁺ is 0.07 ± 0.002 for I₁ (500 ± 77 nA), and at the steady-state I₂ (−1241 ± 209 nA) the P₇NMDG⁺/PNa⁺ is 0.15 ± 0.007 (P < 0.01). In contrast for mP2X₂ channels at the initial I₁ peak (615 ± 92 nA) the P₇NMDG⁺/PNa⁺ is 0.07 ± 0.003, and at the steady-state I₂ current (−12 ± 140 nA) the P₇NMDG⁺/PNa⁺ is 0.09 ± 0.006 (P < 0.05). In summary, with time NMDG⁺ becomes much more permeable through rP2X₂ pores, but only slightly more permeable through mP2X₂ pores.

**Sizing the P2X₂ Pore States Using Excluded Field Theory**

For the above experiments we studied the permeability to NMDG⁺ (radius ~4.5 Å) because this was the standard protocol in previous work on rP2X₂ channels. We next tested the hypothesis that smaller cations also change their permeability during ATP activation of the rP2X₂ channel. To address this we used four organic cations (dimethylammonium, 2-(methyl-amino)-ethanol, Tris⁺ and N-methyl-D-glucamine) with radii between 2.7 and 4.5 Å and determined their permeability relative to Na⁺ (P₇X⁺/PNa⁺). While both mP2X₂ and rP2X₂ channels showed increased permeability in the I₂ state to all four test ions, the increases for rP2X₂ were much more dramatic. Fig. 4 shows the data plotted on graphs to determine the size of the narrowest part of
the channel pore, using excluded field theory (EFT).*

EFT was used previously to size the muscle nicotinic channel pore at a diameter of 6.5–8.4 Å (Dwyer et al., 1980; Cohen et al., 1992), which agrees well with the pore diameter revealed by imaging the open channel (Unwin, 1995). Using EFT we determined the diameter of the narrowest part of the rP2X$_2$ pore in the I$_1$ state as 11.4 Å, which is similar to the mP2X$_2$ pore I$_1$ states at 11.0 Å, and 11.6 Å diameter in the I$_2$ states (Table II).

Thus, both I$_1$ and I$_2$ states of the mP2X$_2$ channel, as well as the I$_1$ state of the rP2X$_2$ channel, have diameters equal to within 10%, but markedly smaller than the rP2X$_2$ I$_2$ state (Fig. 4); what is the size of the latter state? The data at hand do not give an accurate estimate because all tested organic cations acquired significant permeability. For example, the permeabilities (relative to Na$^+$) of Tris$^+$ and 2-(methyl-amino)-ethanol$^+$ were 0.5 and 0.7, are roughly proportional to their mobilities in free solution (0.6 and 0.7 for Tris$^+$ and 2-(methyl-amino)-ethanol$^+$, relative to Na$^+$) for the rP2X$_2$ I$_2$ state, whereas for the I$_1$ state the permeability values decline more steeply than mobility with $R_x$, as expected for ions that are slowed by interactions with the channel wall as they traverse the I$_1$ state pore (the free solution mobility values are presented in the legend for Fig. 4). On the basis of the plots shown in Fig. 4 we can state that the rP2X$_2$ I$_2$ state diameter is at least 3 Å larger than the I$_1$ state and mP2X$_2$ I$_1$ and I$_2$ states. This change increases the area across the narrowest region in the rP2X$_2$ pore by at least $\sim$60 Å$^2$, for a circular filter, or $\sim$76 Å$^2$ for a square filter.

**A Role for the C Tail in Permeability Changes**

What is the structural basis for the differences between rP2X$_2$ and mP2X$_2$ with respect to I$_2$? As expected, the mouse cDNA has all the hallmarks of a bona fide P2X channel (North, 1996; Khakh, 2001), including sequence identity in TM2 with rP2X$_2$. An alignment for

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*Abbreviation used in this paper: EFT, excluded field theory.

**TABLE II**

<table>
<thead>
<tr>
<th>Experimentally Determined Parameters Used in EFT to Calculate Pore Sizes</th>
<th>$a$ (Å$^{-1}$)</th>
<th>$b$</th>
<th>$Re = a/b$ (Å)</th>
<th>$R$</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>rP2X$_2$</td>
<td>I$_1$</td>
<td>1.3 ± 0.002</td>
<td>0.23 ± 0.005</td>
<td>5.7</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>I$_2$</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>mP2X$_2$</td>
<td>I$_1$</td>
<td>1.6 ± 0.002</td>
<td>0.29 ± 0.01</td>
<td>5.5</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>I$_2$</td>
<td>1.8 ± 0.05</td>
<td>0.31 ± 0.01</td>
<td>5.8</td>
<td>0.99</td>
</tr>
</tbody>
</table>

See MATERIALS AND METHODS for details, but briefly, $Re$ is the radius of the narrowest part of the channel pore, $r$ is the correlation coefficient for the linear regression, and – indicates that a regression was not attempted because the data were clearly nonlinear.

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**Figure 5.** Comparison of rP2X$_2$ and mP2X$_2$ sequences. The top panel shows an alignment for the entire rP2X$_2$ and mP2X$_2$ protein sequences (black lines are above predicted transmembrane domains and the black dots indicate the conserved cysteine residues), whereas the middle panel shows a representation of P2X subunit topology. The bottom panel shows an alignment of the mP2X$_2$ cDNA used in this study, and the corresponding sequence of the mP2X$_2$ gene. The codons for the amino acids that differ between mP2X$_2$ and rP2X$_2$ are shown in gray. Note that the genomic sequence agrees with the cDNA sequence in each case, assuring that the mP2X$_2$ cDNA contains no PCR errors. The boxed regions correspond to identical sequence.
represent a direct way to identify residues in the C tail domain that affect permeation.

Focusing on Residues that Are Permissive for Permeability Changes in the C Tail Domain

Fig. 5 highlights the differences in the C tail domains between rP2X2 and mP2X2. In our initial experiments, we asked which of the 14 amino acid differences between rP2X2 and mP2X2 determine the presence of I2 and the associated increases in $P_{\text{NMDG}^+/\text{Na}^+}$ in rP2X2, but not in mP2X2? Moreover, can rP2X2 I2-like phenotypes be imparted on to mP2X2 channels by swapping an appropriate domain? To this end we made chimeric channels. For example, transplanting the entire rP2X2 C tail domain onto mP2X2 (mEL/rCT) produced responses that were indistinguishable from those of mP2X2, and after residue 430 the sequence is rP2X2. Increasing the number of rP2X2 residues from two to four produces progressively smaller increases in the I2 phenotype, but clearly the largest jump occurs for transplantation of the last two residues. In rP2X2 they are I432 and G444 and for mP2X2 these residues are S432 and D444 (Fig. 5).

We next made a series of chimeras in the C tail domain with progressively increasing numbers of rP2X2 C tail domain residues in the mP2X2 C tail domain. This approach tests for effects of substituting sequential residues, beginning at the end of the tail. Results are shown in Fig. 7. Remarkably, from the data it is clear that the last (most COOH-terminal) two amino acids, of the seven differences between mP2X2 and rP2X2, are sufficient to confer rP2X2-like behavior on the mP2X2 channel with respect to the I2 and $P_{\text{NMDG}^+/\text{Na}^+}$ changes. We call this chimera m430r, because until residue 430 the sequence is mP2X2, and after residue 430 the sequence is rP2X2. Increasing the number of rP2X2 residues from two to four produces progressively smaller increases in the I2 phenotype, but clearly the largest jump occurs for transplantation of the last two residues. In rP2X2 they are I432 and G444 and for mP2X2 these residues are S432 and D444 (Fig. 5).

We also made the reverse series of chimeras, with progressively increasing numbers of mP2X2 C tail domain residues in the rP2X2 C tail domain. Here again a clear picture is apparent: the last two mP2X2 residues are sufficient to eliminate I2 and $P_{\text{NMDG}^+/\text{Na}^+}$ changes. Increasing the number of mP2X2 residues in the rP2X2 tail from two to four produces no greater effect than simply substituting two (Fig. 7).

There are two outliers to the trend (shown by gray arrows). Transplantation of five rP2X2 residues into mP2X2 (m407r) produces a channel that desensitizes rapidly within 1–2 s, whereas the other mutants resemble wt rP2X2 and desensitize negligibly over a time period of up to 60s. Similarly, transplanting four mP2X2 residues into rP2X2 produces a channel that expresses poorly and desensitizes completely. In this chimeristic approach, we asked whether any single amino acid is sufficient to make mP2X2 resemble rP2X2, and vice versa. Analysis of the seven appropriate single-site mutants in mP2X2 and the complementary seven mutants in rP2X2 indicated that this is not the case (Fig. 6): for simplicity these mutants have been pooled in Figs. 7 and 8. Thus, any one rP2X2 residue is not enough to endow the mP2X2 with I2, and any one mP2X2 residue is not enough to impair I2 in rP2X2 (Fig. 6). Between two and seven residues are needed to switch the I2 phenotype between mP2X2 and rP2X2.
mera, reversal potentials could not be measured at a time point when \( I_2 \) is expected to occur; therefore, these constructs are not informative with respect to changes in permeability. For these chimeras we expect that the channels close before spending appreciable time in the \( I_2 \) state. This behavior is similar to our previous data with rP2X1 and rP2X3, which also desensitize rapidly, and previous work with P2X2 channels that indicate mutants in the C tail domain affect desensitization (Smith et al., 1999).

Permeability Changes Are Unrelated to \( I_1 \), but Correlate with the Growth of \( I_2 \)

The dataset presented in this study allows us to address a number of questions about the relationships between \( I_1 \) and \( I_2 \) states. For instance, is the extent of pore dilation in the \( I_2 \) states determined by the permeability of the preceding \( I_1 \) state? In other words, do channels with a lower \( P_{\text{NMDG}^+/\text{Na}^+} \) in the \( I_1 \) state result in a lower \( P_{\text{NMDG}^+/\text{Na}^+} \) for the \( I_2 \) state, and vice versa?
Moreover, are P_{\text{PNMDG}}/P_{\text{Na}} changes related to I_1 amplitude, and therefore ion flow? There was a clear correlation for all point mutants, chimeras, tail swaps, and wt channels between I_2 amplitude and I_2 P_{\text{PNMDG}}/P_{\text{Na}} changes, but not between I_1 amplitude and I_2 P_{\text{PNMDG}}/P_{\text{Na}} (Fig. 8), arguing that the extent of pore dilation in the I_2 state is not related to ion flow in the I_1 state. There was also no correlation between I_1 P_{\text{PNMDG}}/P_{\text{Na}} and I_2 P_{\text{PNMDG}}/P_{\text{Na}}. We next asked, do permeability changes merely happen secondarily to the growth of I_2 or do they mirror it? Table III shows the rates for increases in P_{\text{PNMDG}}/P_{\text{Na}}, as well as for the growth from I_1 to I_2 for rP2X_2, mEL/rCT, and m430r channels. The rate constants for rP2X_2, mEL/rCT, and m430r channels are the same for P_{\text{PNMDG}}/P_{\text{Na}} changes and growth of I_2 from I_1 at -60 mV. Overall, these data show that (a) I_2 amplitude and P_{\text{PNMDG}}/P_{\text{Na}} provide measures of the same underlying phenomenon because they are well correlated and occur with the same rates across a range of mutants, and (b) P_{\text{PNMDG}}/P_{\text{Na}} changes are not a consequence of ion flow during the outward I_1. Moreover, the data clearly show that whereas the tail swaps and chimeras produce profound effects on I_2, they have no effect on I_1 amplitude or I_1 P_{\text{PNMDG}}/P_{\text{Na}} across experiments from 26 distinct channels (2 wild type channels, 14 point mutations, 10 chimeras; Figs. 6–8). A lack of effect on I_1 serves as an internal control for the experiments.

Table III: Rate Constants for Development of I_2 and Increase in P_{\text{PNMDG}}/P_{\text{Na}} for wt and Engineered P2X_2 Channels

<table>
<thead>
<tr>
<th></th>
<th>Growth I_1 to I_2</th>
<th>Increase in P_{\text{PNMDG}}/P_{\text{Na}}</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>s^{-1}</td>
<td>s^{-1}</td>
<td></td>
</tr>
<tr>
<td>wt rP2X_2</td>
<td>0.5 ± 0.09</td>
<td>0.5 ± 0.07</td>
<td>6</td>
</tr>
<tr>
<td>mEL/rCT</td>
<td>0.4 ± 0.03</td>
<td>0.3 ± 0.02</td>
<td>6</td>
</tr>
<tr>
<td>m430r</td>
<td>0.4 ± 0.06</td>
<td>0.4 ± 0.09</td>
<td>6</td>
</tr>
</tbody>
</table>

Rate constants (1/\tau) were determined from single exponential fits to the growth of steady state current from I_1 to I_2 as illustrated in the waveform of Fig. 1 A, and for the increase in P_{\text{PNMDG}}/P_{\text{Na}} over time they were determined from current-voltage relations like those illustrated in Figs. 1 B and 3.
against any effects on overall protein conformation. But more so the data sharply focus attention on amino acids that specifically affect permeation of a channel state: I₂.

DISCUSSION

There is convincing evidence that the P2X₂ channel COOH-terminal domain is cytosolic, including studies of functional concatenated subunits and access of antibodies raised to the cytosolic domain in permeabilized, but not non-permeabilized cells (Torres et al., 1998; Stoop et al., 1999). The main finding of the present study is a significant extension and sharpening of the view that the COOH-terminal cytosolic domain affects permeation of the P2X channels in a dilated state. We exploited the observation that rat P2X₂ (rP2X₂) channels display a much more robust history-dependent permeability change than do mouse P2X₂ (mP2X₂) channels. The present study shows that replacement of just two amino acid residues abolishes the change in permeability in rP2X₂, and just two residues allows mP2X₂ channels to undergo permeability changes. The “negative controls” of the mP2X₂ channel and of the various rP2Xₙ/mP2X₂ chimeras, which show identical physiology in the I₁ state but completely different physiology in the I₂ state, allow us to conclude even more confidently that the I₁ state is not an artifact of voltage-clamp fidelity, Ca²⁺-activated conductances, or any other vaguely imaginable event. Instead, the I₂ state results from a change in the pore, in a transition that is discussed below.

Our finding that the pore dilates by at least 3 Å is consistent with measurements using dye uptake studies (Khakh et al., 1999; Virginio et al., 1999a,b). Because cationic dye flux studies are interpreted in a binary fashion (the dye either permeates or not), they provide a cutoff for the pore size. For instance, YOPRO1 (16.8 × 12.8 × 8.2 Å) does not permeate P2X channels that lack the I₁ state because two of its dimensions are larger than the I₁ pore, which we estimated to be 11 Å in the present study for P2X₂ channels. However, YOPRO1 permeability through P2X₂ channels increases in a time-dependent manner during ATP application, reaching steady-state in 30–50 s. This time course is slower than opening to the I₁ state, which occurs in <300 ms (Fig. 1 A), but resembles the time for opening to the dilated state (Fig. 1 A), as measured either by the growth of I₁ to I₂, or by increases in Pₐ/NMDG⁺/Pₐ/Na⁺. These data imply that YOPRO1 does not permeate the 11 Å I₁ states because of steric hindrance, but can permeate the dilated I₂ state. These considerations imply that the pore of P2X channels must dilate by at least 2 Å, consistent with the electrophysiological data presented in this study indicating that the pore dilates by at least 3 Å. A more accurate estimate of I₂ pore size is required, but the present paucity of approximately spherical monovalent cations larger than NMDG⁺ vitiates further EFT experiments. Moreover, an attempt to use polymers of variable length has proved unfruitful (Virginio et al., 1999a). Perhaps novel approaches are required to accurately size the I₂ state.

We compare our data to observations on P2X₇ channels. Early studies of P2X₇ channels carrying a truncated C tail suggested a role for the C tail domain in permeability changes, but did not pinpoint individual or stretches of important residues (Surprenant et al., 1996). A single nucleotide polymorphism of the P2X₇ channel gene in a population of humans with deficits in lymphocyte and monocyte function results in functionally impaired, but appropriately membrane localized, P2X₇ channels carrying a E496A mutation in the COOH-terminal tail domain (Gu et al., 2001). These observations support our present findings that distinct COOH-terminal domain residues affect P2X channel function, but our data are enlightening because they suggest a molecular explanation for the functional deficits in humans with the P2X₇ channel E496A polymorphism (Gu et al., 2001). In the simplest case, polymorphic E496A channels display impaired dye uptake through the I₂ states (Gu et al., 2001) because the C tail domains are locked in a nonpermissive state, as revealed by the appropriate mutants and chimeras reported here. The two nonpermissive residues identified in this study (S432 and D444 in mP2X₂) and the E496A mutation in human polymorphic P2X₇ channels all locate to a region of the C tail domain that is ~100 residues from the pore-lining segment.

Permeability Measurements and Conductances

The present study adds quantitative details on the extent of the change from a relatively selective I₁ state to a less selective I₂ state, which permeates organic cations with ease. Our most complete dataset, for NMDG⁺, allows us to compare three related parameters for NMDG⁺ permeation in the rP2X₂ I₂ state: relative free solution mobility, relative conductance, and relative permeability. For the experiment specifically designed to compare permeability and conductance, these parameters for NMDG⁺ are, respectively, 0.49 (Barry and Lynch, 1991; Ng and Barry, 1995), 0.68 (Table I), and 0.34 (Table I), relative to Na⁺. As noted in results, the absolute magnitude of the permeability change varied somewhat between batches of oocytes, and the measured Pₐ/NMDG⁺/Pₐ/Na⁺ range was 0.17–0.34 across all our experiments. The slope conductance for NMDG⁺ in the I₁ state is therefore surprisingly high relative to that for Na⁺.

Conductance is governed by several mechanisms not directly related to permeability, which primarily reflects a single rate-limiting filter. The interestingly high ratio
of macroscopic NMDG\(^+\) to Na\(^+\) conductance calls for single-channel measurements of the I\(_2\) state. The pioneering single-channel studies of P2X\(_2\) channels (Ding and Sachs, 1999a,b) have not systematically explored the optimal conditions for production of the I\(_2\) state (Khakh et al., 1999; Virginio et al., 1999b): very low extracellular Ca\(^{2+}\) and concentrations of ATP \(\sim 10\ \mu\text{M}\) (Virginio et al., 1999b). Moreover, rP2X\(_2\) channels in excised patches display inactivation kinetics that are quite different to those in whole-cell mode, implying the loss of necessary cytosolic components (Ding and Sachs, 2000).

In the absence of single-channel I\(_2\) state recordings, we review several previously reported single-channel characteristics of the I\(_1\) state in monovalent metal cation solutions (NMDG\(^+\) single-channel currents are below the resolution of this state). At least two mechanisms decrease the macroscopic conductance (Ding and Sachs, 1999b). There is an Na\(^+\) ion binding site \(\sim 20\%\) of the distance through the electric field (from the outside), with an affinity of \(\sim 90\ \text{mM}\) at \(-60\ \text{mV}\) (Ding and Sachs, 1999a). Under our conditions, this site would be 50\% saturated. Single-channel recordings also show pronounced flickering; the maximal open probability is 0.6. One of these mechanisms might operate more strongly for Na\(^+\) than for NMDG\(^+\) in the I\(_2\) state, accounting for the interestingly high ratio of NMDG\(^+\) to Na\(^+\) slope conductance.

**Toward a View of P2X Channel Gating**

We observed profound effects for point mutants, chimeras, and tail swaps on I\(_2\) amplitude and I\(_2\) \(\text{P}_{\text{NMDG}^+}/\text{P}_{\text{Na}^+}\) changes, but no effect on I\(_1\) amplitude or I\(_1\) \(\text{P}_{\text{NMDG}^+}/\text{P}_{\text{Na}^+}\). A lack of effect on I\(_1\) across experiments from 26 distinct channels serves as a good internal control against effects on overall protein conformation, and strongly argues that the effect of the mutants and chimeras is specific to the permeation of the I\(_2\) state. With this control dataset in hand we can conclude that residues in the C tail affect the I\(_2\) state. But what are the structural details of this transition? P2X channels are the newest members of the transmitter-gated ion channels to be identified, they have little sequence homology with any other ion channels and relatively little is known about their structure-function relationships. Nonetheless, we present some plausible views.

Present evidence indicates that ATP binds to regions just extracellular to the transmembrane domains in P2X channels (Ennion et al., 2000; Jiang et al., 2000). In the present view, ATP binding causes motions in TM1 (Haines et al., 2001; Jiang et al., 2001), perhaps as the outer part of TM1 moves with respect to the outer part of TM2, that allow the pore to open at or near G342 in TM2 (Rassendren et al., 1997; Egan et al., 1998; Migita et al., 2001). Our data suggest (Fig. 9) that the transition from I\(_1\) to I\(_2\) increases the area across the narrowest region in the rP2X\(_2\) pore by at least \(\sim 60\ \text{Å}^2\), for a circular filter, or \(\sim 76\ \text{Å}^2\) for a square filter. This implies an increase in the pore diameter by at least 3 Å, but an accurate estimate of the I\(_2\) state pore size requires further work. The change is regulated by the disposition of side chains in a particular region of the cytoplasmic tail, but as previously demonstrated the change likely occurs at the pore (Khakh et al., 1999; Virginio et al., 1999b). Our data favor the model in
which channels go from closed to open 1 (I₁; pore diameter 11 Å) and then to open 2 (I₂; pore diameter >14 Å) during a conformational change(s) in the C tail domain. This conformational change(s) proceeds at a rate of 0.5 s⁻¹.

In the conventional view, the shape change in the permeation pathway during permeability changes is occurring on the axis of the multimeric channel, in a region where the cytoplasmic tails of several subunits approach the axis. Recent biochemical and electrophysiological evidence indicates that the NH₂ and COOH termini are close to one another in functional P2X₂ channels (Boue-Grabot et al., 2000), suggesting at the very least that the cytoplasmic regions are ordered and structured, and lending credence to the idea that these regions could contribute to the inner aspect of a conduction pathway. Consistent with this hypothesis are recent data on nicotinic channels (Miyazawa et al., 1999), Shaker (Sokolova et al., 2001), and Na⁺ channels (Sato et al., 2001), revealing the existence of cytoplasmic hanging baskets that split the conduction pathway. If such fenestrations also occur for P2X channels, then there might be multiple parallel and radially symmetric dilations of the conduction path.

The COOH-terminal tails of P2X₂ channels contain several Pro-rich regions, which often bind other proteins (Fig. 5). Work published since submission of this paper has identified protein partners for the P2X₇ channel (Kim et al., 2001), and it is possible that a similar situation exists for P2X₂ channels. Thus, permeability changes might involve interactions with other cytoplasmic partners, which may differ between mP2X₂ and rP2X₂ at the crucial two residues we have identified in this study. In particular, it has not escaped our attention that phosphorylation events on a time scale of milliseconds to seconds might participate in the transition to the I₂ state. Indeed, P2X₂ channel kinetics appear to be regulated by phosphorylation (Boue-Grabot et al., 2000). A further possibility is that the C tail domain itself may not line the pore directly, but may be involved in the conformational change that allows the pore to dilate. In this scenario, the residues that lead to absence of I₂ would effectively ‘lock’ the C tail in a non-permissive state (Fig. 9). In such a model the transition from the I₁ to the I₂ state might involve extensive changes in the C tail domain. A large shape change was recently suggested for the gating of the MscL channel (Sukharev et al., 2001): the location of the C tail domain would change during opening as it moves radially from the inner aspect of the pore. Because MscL and P2X channels have similar membrane topologies and both permeate large ions (Khakh, 2001), it is possible to consider that a similar gating mechanism may operate. The above hypotheses reveal several avenues of future experiments to better understand the contribution of the C tail domain to permeability changes at P2X₂ channels, but solving the channel structure at atomic resolution in the closed and open states, and deciphering the nature of the transitions, may provide the only completely satisfying view.

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