

Control of P2X₂ Channel Permeability by the Cytosolic Domain

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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.

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Cloning, Expression, and Recording

The mP2X₂ cDNA was cloned from a mouse testis library using primers designed to the rat P2X₂ cDNA (antisense primer GAA TTC TCA AAG TTG GGC CAA ACC T, sense primer GGA TCC ATG GTC CGG CGC TTG G). Mouse P2X₂ cDNA were amplified using PCR Master (Roche) according to manufacturer's instructions. The PCR product was gel purified and cloned into TOPO TA pCR2.1 (Invitrogen) according to manufacturer's instructions. The five individual clones were sequenced (Roche Bioscience sequencing laboratory) on both strands. mP2X₂ and rP2X₂ cDNA were subcloned into pcDNA3.

The *C tail swap chimeras* were made by digesting rP2X₂ and mP2X₂ cDNA with HpaI (native site in both cDNAs) and XhoI (in the 3' polylinker) to produce two fragments: (a) the vector and coding region for the entire P2X₂ 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 mP2X₂ C tail domain was ligated into rP2X₂, and vice versa, and restriction analysis was used to confirm the construction.

The *C tail domain chimeras* were constructed in two PCR steps (High Fidelity PCR Master; Roche) using synthetic oligonucleotides (Caltech Polymer Synthesis Facility). In the first step, two PCR reactions produced overlapping pieces, one using rP2X₂ as the template and the other using mP2X₂ as the template. In the second step, the PCR products from the first steps were used as the template and the final product was a connected chimeric fragment spanning the DNA coding for TM2 through the cytoplasmic tale and into the polylinker. The chimeric fragment and wild-type DNA were digested with HpaI and XhoI. The pieces of the digestion were separated on an agarose gel and extracted. The chimeric fragment and the wild type vector were ligated. Sequencing confirmed the correct chimeras.

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 mMACHINE mMACHINE 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 MgCl₂ at pH 7.35–7.4, which was superfused over the oocytes by gravity flow at a rate of ~3 ml min⁻¹ (chamber volume was ~300 μl). In some experiments, equimolar substitutions of organic cations were made for Na⁺. The organic cations tested were dimethylammonium, 2-(methyl-amino)-ethanol, Tris⁺, 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 ramped 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 (>4). We employed the principles developed by Hille (Dwyer et al., 1980; Hille, 1992). A transform of the GHK voltage equation under bionic conditions from

$$E_{rev} = \frac{RT}{zF} \ln \frac{P_{X^+} [X^+]_o}{P_{Y^+} [Y^+]_i}$$

to

$$\frac{P_{X^+}}{P_{Y^+}} = ([Y^+]_i/[X^+]_o) \exp(zE_{rev}F/RT)$$

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

$$\frac{P_{X^+}}{P_{Y^+}} = ([X^+]_o/[Y^+]_o) \exp(zF(E_{rev}X^+ - E_{rev}Y^+)/RT)$$

where X⁺ is the organic cation and Y⁺ is Na⁺, with a reversal potential close to 0 mV for P2X₂ channels (see results). Under fixed ion concentrations this equation simplifies to

$$\frac{P_{X^+}}{P_{Na^+}} = \exp\left(\frac{\Delta E_{rev}F}{RT}\right),$$

where Δ*E*_{rev} 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 *Rc*, and ion X⁺ with a radius *Rx* (*RNa⁺* is the hydrated radius of Na⁺ at 2.15 Å) the *P*_{X⁺}/*P*_{Na⁺} is given by

$$\frac{P_{X^+}}{P_{Na^+}} = \left(\frac{Rc - Rx}{Rc - RNa^+}\right)^2.$$

A square root transformation gives

$$\sqrt{\frac{P_{X^+}}{P_{Na^+}}} = a - bRx,$$

where

$$a = \frac{Rc}{Rc - RNa^+} \quad \text{and} \quad b = \frac{1}{Rc - RNa^+}$$

and the diameter of the narrowest region of the pore is 2*a*/*b*. In the above equations *a* and *b* are obtained from linear regressions of plots of (*P*_{X⁺}/*P*_{Na⁺})^{1/2} versus *Rx* (as in Fig. 4). In such formalisms a convenient check is to determine the diameter of Na⁺, which is given by *RNa⁺* = (*a* - 1)/*b*, and using *a* = 1.57 Å⁻¹ and *b* = 0.28 (average from regressions of rP2X₂ I₁, mP2X₂ I₁, and I₂ shown in Fig. 4). This gives *RNa⁺* as 2.04 Å, which is close to the hydrated radius (Kielland, 1937) of Na⁺ 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⁺ (*Rx*) was determined as

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Data for Permeability Changes at rP2X₂ Channels for the Experiment Illustrated in Fig. 1

	I ₁	I ₂	I _{Na⁺}	n
Peak current at -60 mV (μA)	0.5 ± 0.1	-3.2 ± 1.2	-10.4 ± 0.5	9
E _{rev} (mV)	-66.7 ± 0.9	-35.1 ± 6.4	2.7 ± 0.9	9
Slope conductance (μS)	97 ± 3	136 ± 4	200 ± 20	9
P _{NMDG⁺} /P _{Na⁺}	0.07 ± 0.003	0.34 ± 0.1	-	9

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: I₁ 0.2, 0.7, 1.3, 0.5, 0.8, 0.1, 0.6, 0.6, 0.1 μA; I₂ -1.9, -1.1, -1.0, -0.5, -4.7, -0.9, -10, -1.3, -7.6 μA; and I_{Na⁺} -10.1, -10.2, -7.4, -9.2, -10.6, -10.5, -12.5, -11.4, -11.6 μA). The I_{Na⁺}/I₂ ratio was 7.3 ± 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).

$$Rx = \frac{\sqrt[3]{D1 \times D2 \times D3}}{2},$$

where D1, D2, and D3 are the three dimensions of the smallest box that will house the ion. The fold change (Δ) in P_{X⁺}/P_{Na⁺} is the ratio of P_{X⁺}/P_{Na⁺} for I₁ and I₂. Slope conductance values for I₁, I₂, and I_{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 I₂ there was a small difference between these values and those calculated from the average values of I and E_{rev(NMDG)} (as shown in Table I). These differences are expected with small numbers for the numerators, as in the NMDG⁺ data. For Na⁺ currents, inward rectification was quantified as G_{+60 mV}/G_{-60 mV}. Rate constants (1/τ; 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 rP2X₂ Channels

Fig. 1 illustrates permeability changes for rP2X₂ 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 mV and in extracellular solutions that contain NMDG⁺, application of ATP evokes an initial transient outward current (termed I₁) at a holding potential of -60 mV. Over a time course of seconds the currents become inward and reach steady-state values (I₂), but on switching to Na⁺ solutions the currents are larger (I_{Na⁺}; Fig. 1 A). The I_{Na⁺}/I₂ ratio calculated from the individual cells was 7.3 ± 1.9 ($n = 9$; Table I legend).

We also measured the reversal potential of the ATP-evoked currents between I₁, I₂, and I_{Na⁺} with voltage ramps (Fig. 1 B), and expressed these values as permeability ratios relative to Na⁺ (see MATERIALS AND METHODS). The inward I₂ current develops with a rate constant ($k_{+I} = 0.3 \pm 0.01$ s⁻¹) similar to that for the shift in reversal potential ($k_{+I} = 0.4 \pm 0.1$ s⁻¹) from -66.7 mV for I₁ to -35.5 mV for I₂, thus showing a

change in P_{NMDG⁺}/P_{Na⁺} from 0.07 for I₁ to 0.34 for I₂ (Table I). The rate constant for the increase in NMDG⁺ permeability is ~0.4 s⁻¹, similar to that reported previously in mammalian cells (Virginio et al., 1999b). Thus, with time NMDG⁺ becomes more permeable through rP2X₂ pores, but it does not become

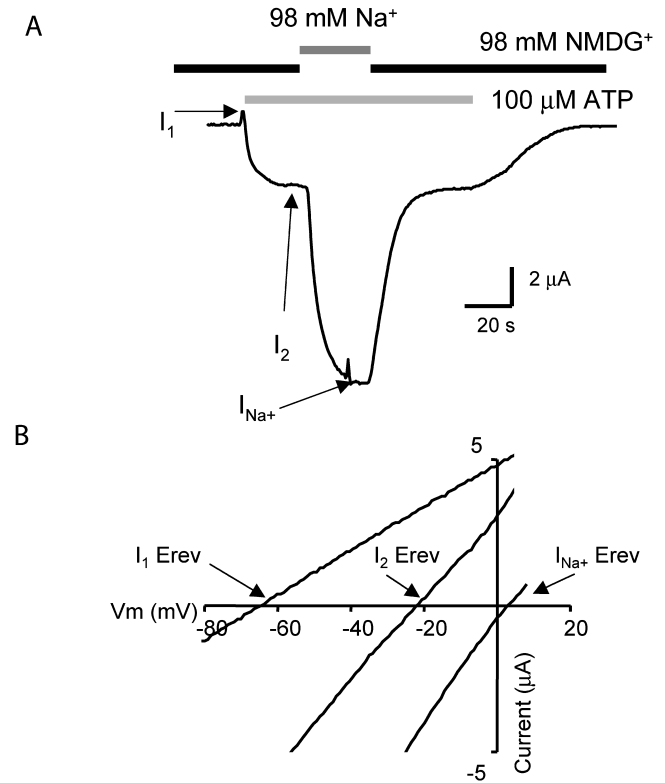


FIGURE 1. Properties of permeability changes at rP2X₂ channels. (A) Representative current waveform from a voltage-clamped oocyte (-60 mV). The cell was bathed in an NMDG⁺-containing extracellular solution and then at the indicated time ATP was applied, first with NMDG⁺ and then with Na⁺ in the extracellular solution. The record illustrates the three current phases, I₁, I₂, and I_{Na⁺}, the statistics for which are presented in Table I. (B) I-V relations determined at the peak of I₁, I₂, and at I_{Na⁺}: note that the reversal potentials differ. The ATP-evoked current reversal potential does not shift over time in extracellular solutions that contain only Na⁺ (Khakh et al., 1999).

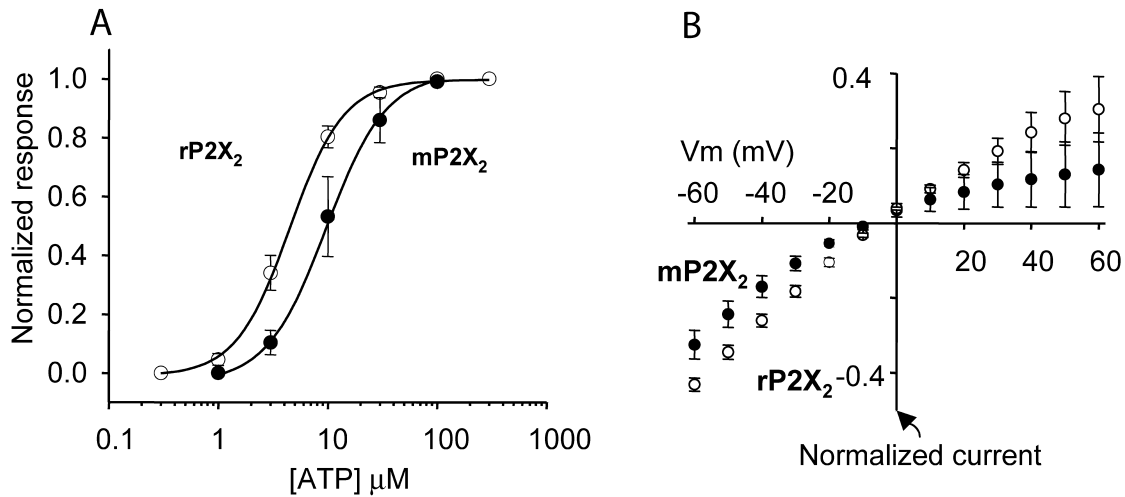


FIGURE 2. Basic properties of rP2X₂ and mP2X₂ channels. (A) Concentration-effect curves for ATP at rP2X₂ and mP2X₂. There is a significant difference in EC₅₀. (B) I-V relations from voltage steps (-60 to 60 mV in 10-mV steps) for mP2X₂ and rP2X₂. Note that rectification is approximately equal for both 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.

so permeable as Na⁺ (Erev 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_{NMDG+}/P_{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 I₁ to I₂, and the accompanying shifts in reversal potentials (Fig. 1, A and B).

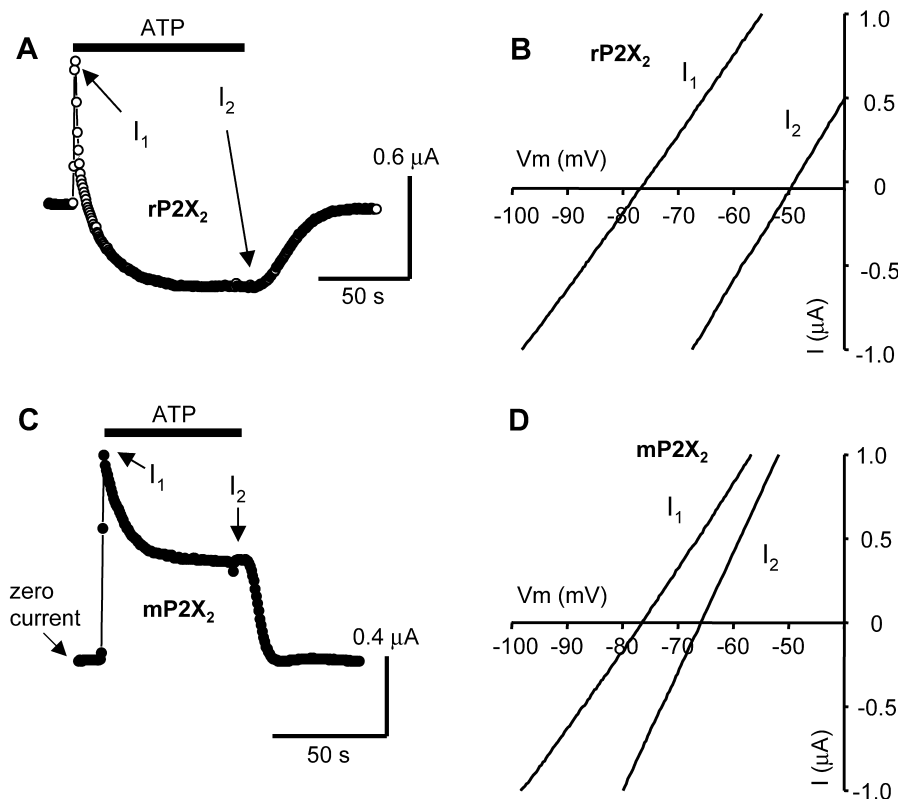


FIGURE 3. rP2X₂ and mP2X₂ channels differ with respect to permeability changes. (A) rP2X₂: steady-state current waveform (-60 mV) in NMDG⁺ extracellular solutions. ATP was applied when indicated and evoked an outward current that became inward over time. (B) Reversal potentials at the peak of I₁ and I₂ for rP2X₂. (C) mP2X₂: steady-state current waveform in NMDG⁺ 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 I₁ and I₂ 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 I_{Na+}, but the ATP EC₅₀s 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 I_{Na+} reversal potentials were close to 0 mV (−4.1 ± 0.1 and −6.5 ± 1.5 mV; *P* > 0.05) and the rectification indices (G_{+60 mV}/G_{−60 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 P_{NMDG⁺}/P_{Na⁺}} is 0.07 ± 0.002 for I₁ (500 ± 77 nA), and at the steady-state I₂ (−1241 ± 209 nA) the P_{NMDG⁺}/P_{Na⁺}} is 0.15 ± 0.007 (*P* < 0.01). In contrast for mP2X₂ channels at the initial I₁ peak (615 ± 92 nA) the P_{NMDG⁺}/P_{Na⁺}} is 0.07 ± 0.003, and at the steady-state I₂ current (−12 ± 140 nA) the P_{NMDG⁺}/P_{Na⁺}} 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

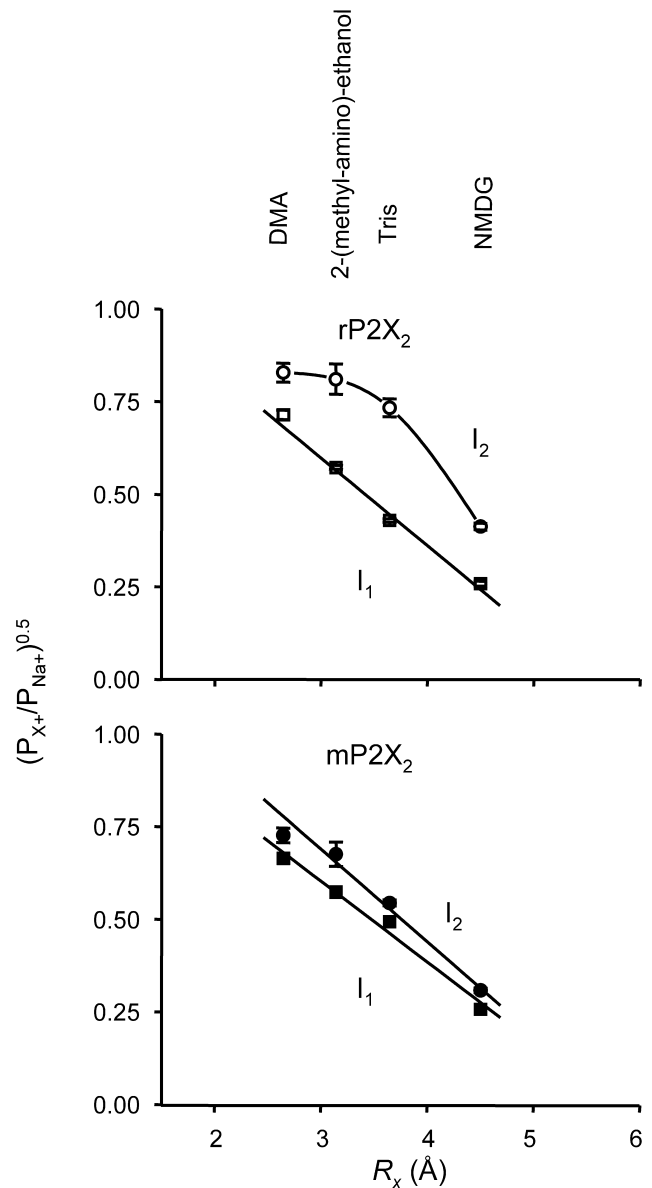


FIGURE 4. Pore sizes determined using EFT. Top and bottom panels show graphs used to determine the narrowest part of the channel pore for rP2X₂ and mP2X₂ channels, respectively, in I₁ (within 1s of applying ATP) and I₂ states (up to 30 s after applying ATP). The lines are linear regressions except for rP2X₂ I₂, which is a cubic spline. Please refer to MATERIALS AND METHODS for details of the pore size calculations. The ions (R_x = radius; see MATERIALS AND METHODS) in order of increasing size are: dimethylammonium (2.7 Å), 2-(methyl-amino)-ethanol (3.1 Å), Tris⁺ (3.7 Å), and NMDG⁺ (4.5 Å), and their mobilities in free solution are 51.8, 33.4, 29.4, 24.3, and 50.1 · 10^{−4} m² S mol^{−1}, for dimethylammonium, 2-(methyl-amino)-ethanol, Tris⁺, NMDG⁺, and Na⁺.

relative to Na⁺ (P_{X⁺}/P_{Na⁺}}). 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}

TABLE II

Experimentally Determined Parameters Used in EFT to Calculate Pore Sizes

	a (\AA^{-1})	b	$R_c = a/b$ (\AA)	R	n
rP2X ₂					
I ₁	1.3 ± 0.002	0.23 ± 0.005	5.7	0.99	>5
I ₂	-	-	-	-	-
mP2X ₂					
I ₁	1.6 ± 0.002	0.29 ± 0.01	5.5	0.99	>5
I ₂	1.8 ± 0.05	0.31 ± 0.01	5.8	0.99	>5

See MATERIALS AND METHODS for details, but briefly, R_c 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.

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 \AA (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 rP2X₂ pore in the I₁ state as 11.4 \AA , which is similar to the mP2X₂ pore I₁ states at 11.0 \AA , and 11.6 \AA diameter in the I₂ states (Table II).

Thus, both I₁ and I₂ states of the mP2X₂ channel, as well as the I₁ state of the rP2X₂ channel, have diameters equal to within 10%, but markedly smaller than the rP2X₂ I₂ 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 rP2X₂ I₂ state, whereas for the I₁ 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 I₁ 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 rP2X₂ I₂ state diameter is at least 3 \AA larger than the ~ 11 \AA that characterizes the rP2X₂ I₁ and mP2X₂ I₁ and I₂ states. This change increases the area across the narrowest region in the rP2X₂ pore by at least ~ 60 \AA^2 , for a circular filter, or ~ 76 \AA^2 for a square filter.

A Role for the C Tail in Permeability Changes

What is the structural basis for the differences between rP2X₂ and mP2X₂ with respect to I₂? 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 rP2X₂. An alignment for

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

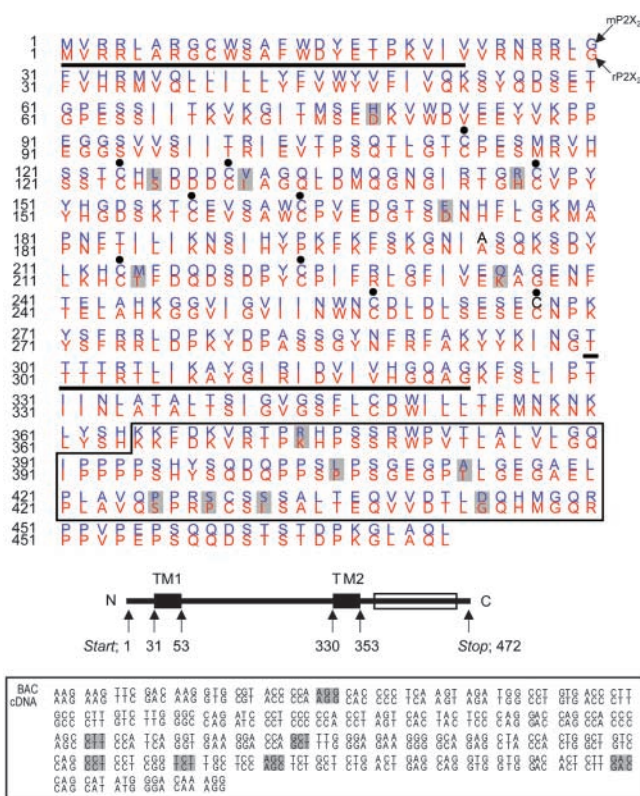
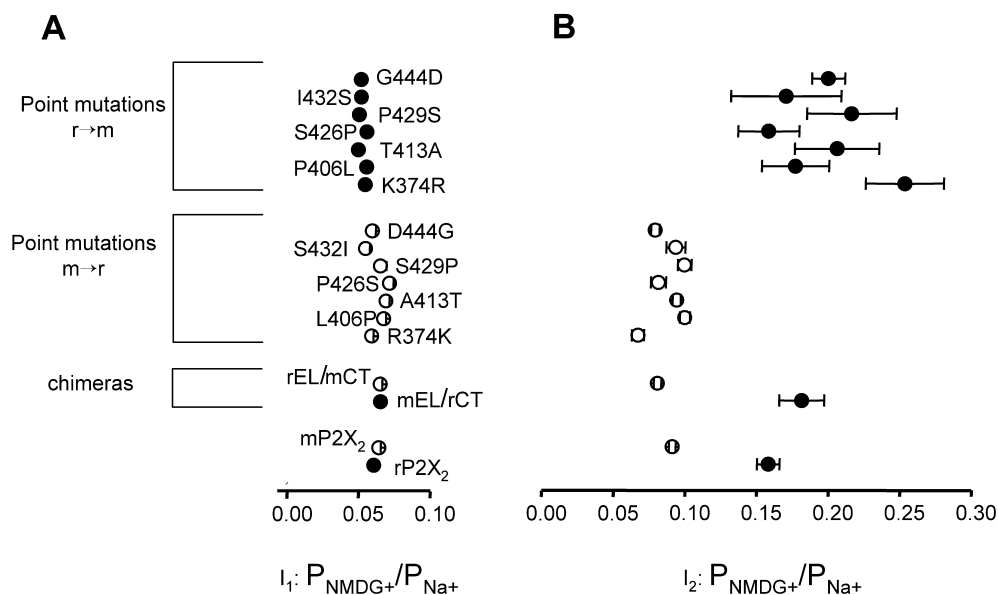


FIGURE 5. Comparison of rP2X₂ and mP2X₂ sequences. The top panel shows an alignment for the entire rP2X₂ and mP2X₂ protein sequences (black lines are above predicted transmembrane domains and the black dots indicate the conserved cysteine residues), whereas the middle panel shows a representation of P2X subunit topology. The bottom panel shows an alignment of the mP2X₂ cDNA used in this study, and the corresponding sequence of the mP2X₂ gene. The codons for the amino acids that differ between mP2X₂ and rP2X₂ are shown in gray. Note that the genomic sequence agrees with the cDNA sequence in each case, assuring that the mP2X₂ cDNA contains no PCR errors. The boxed regions correspond to identical sequence.

the rP2X₂ and mP2X₂ protein sequence is shown in Fig. 5. Of 472 residues, 14 differ between rP2X₂ and mP2X₂, but none are in the pore segment (Rassendren et al., 1997; Egan et al., 1998), near the presumed ATP binding sites (Ennion et al., 2000; Jiang et al., 2000) or in TM1 a region known to influence the link between ATP binding to gating of the pore (Haines et al., 2001; Jiang et al., 2001). Seven residue differences between rP2X₂ and mP2X₂ map to the extracellular loop and the other seven are in the 119-residue COOH-terminal cytosolic domain (Fig. 5). Although intact C tail domains are not critical for P2X function, rP2X₂ splice variants that have shorter C tail domains display faster desensitization (Simon et al., 1997; Koshimizu et al., 1998, 1999; Smith et al., 1999), and truncation of C tail domains impairs permeability in some P2X channels (Virginio et al., 1999b). We reasoned that a comparative study between rP2X₂ and mP2X₂ channels would

FIGURE 6. No single amino acid residue in the COOH-terminal domain accounts for the differences between mP2X₂ and rP2X₂ channels. The scatter graphs show data for wt mP2X₂, rP2X₂, mEL/rCT, rEL/mCT, and 14 single point mutant channels: (A) I₁ P_{NMDG+}/P_{Na+} and (B) I₂ P_{NMDG+}/P_{Na+}. ATP-evoked currents for all mutants, chimeric, and wild-type channels were similar in amplitude for recordings in Na⁺ solutions, indicating approximately equivalent membrane expression.



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_{NMDG+}/P_{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 functional responses that were indistinguishable from those of rP2X₂ with respect to I₁ P_{NMDG+}/P_{Na+} and I₂ P_{NMDG+}/P_{Na+} (Fig. 6). Conversely, transplanting the mP2X₂ C tail domain onto rP2X₂ (rEL/mCT) gave rise to responses that were identical to those of mP2X₂ channels. Seemingly, the differences between rP2X₂ and mP2X₂ reside entirely in the seven residue differences in the C tail domain, illustrated in Fig. 5. But which residues are involved?

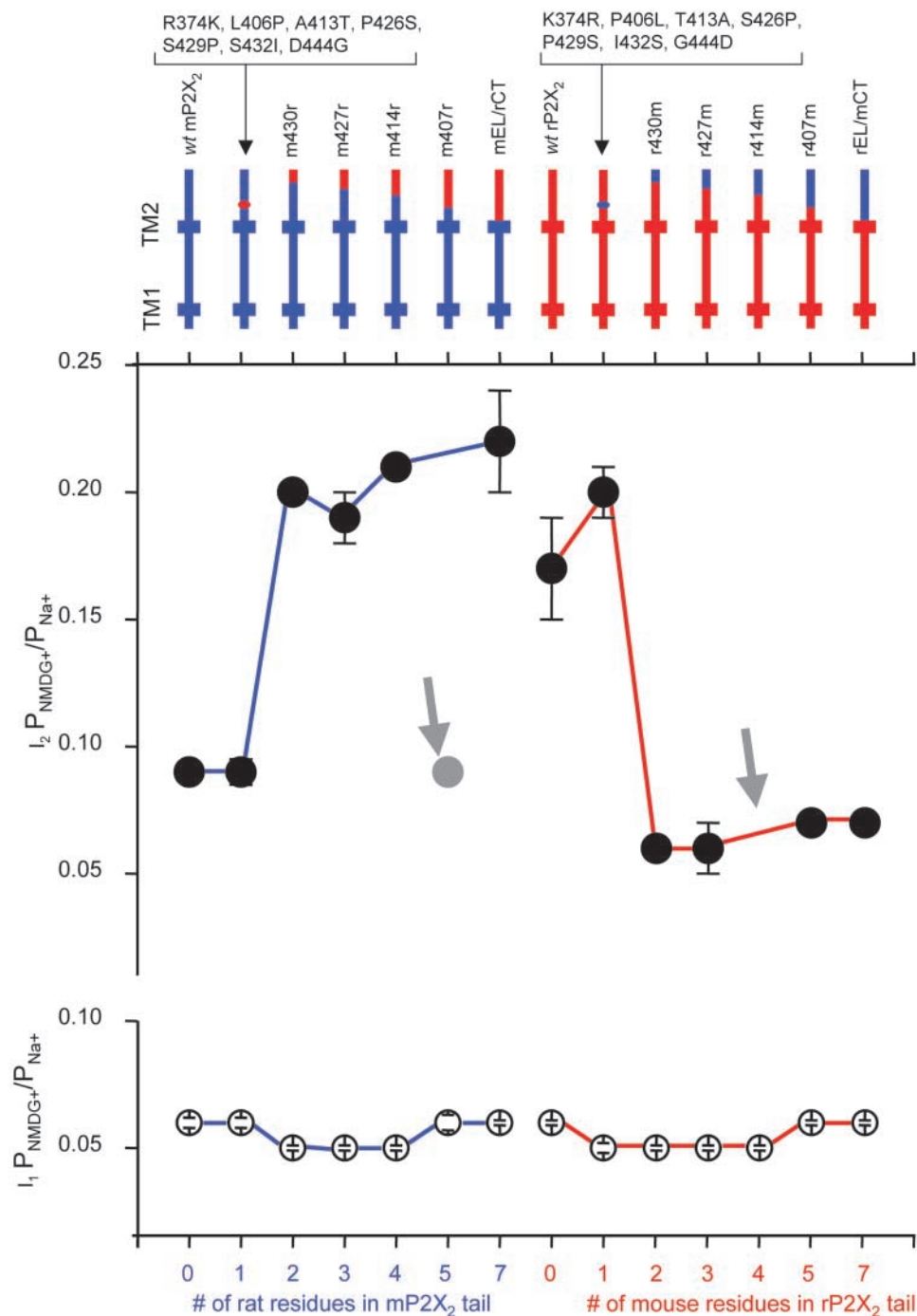
We next asked whether any single amino acid is sufficient to make mP2X₂ resemble rP2X₂, and vice versa. Analysis of the seven appropriate single-site mutants in mP2X₂ and the complementary seven mutants in rP2X₂ indicate that this is not the case (Fig. 6): for simplicity these mutants have been pooled 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.

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_{NMDG+}/P_{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_{NMDG+}/P_{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 chi-

FIGURE 7. Identification of C tail domain residues that govern permeability changes in the P2X₂ channel. The top panel illustrates the location of P2X₂ channel mutants, and chimeras with respect to the two TM domains. The bottom panel shows $P_{\text{NMDG}^+}/P_{\text{Na}^+}$ for the I₂ and I₁ states. Note that I₁ $P_{\text{NMDG}^+}/P_{\text{Na}^+}$ is constant for all channels, but I₂ $P_{\text{NMDG}^+}/P_{\text{Na}^+}$ varies depending on the composition of the C tail domain. Thus rP2X₂ channels undergo large changes between I₁ and I₂, whereas mP2X₂ channels do not. The differences can be reversed by transplanting the C tail domains (mEL/rCT and rEL/mCT) and by making chimeras that include the two most COOH-terminal rP2X₂ residues of the seven that differ in this domain (see Fig. 5). The gray arrows point to uninformative or outlier constructs.



mera, reversal potentials could not be measured at a time point when I₂ 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 I₂ 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 I₁, but Correlate with the Growth of I₂

The dataset presented in this study allows us to address a number of questions about the relationships between I₁ and I₂ states. For instance, is the extent of pore dilation in the I₂ states determined by the permeability of the preceding I₁ state? In other words, do channels with a lower $P_{\text{NMDG}^+}/P_{\text{Na}^+}$ in the I₁ state result in a lower $P_{\text{NMDG}^+}/P_{\text{Na}^+}$ for the I₂ state, and vice versa?

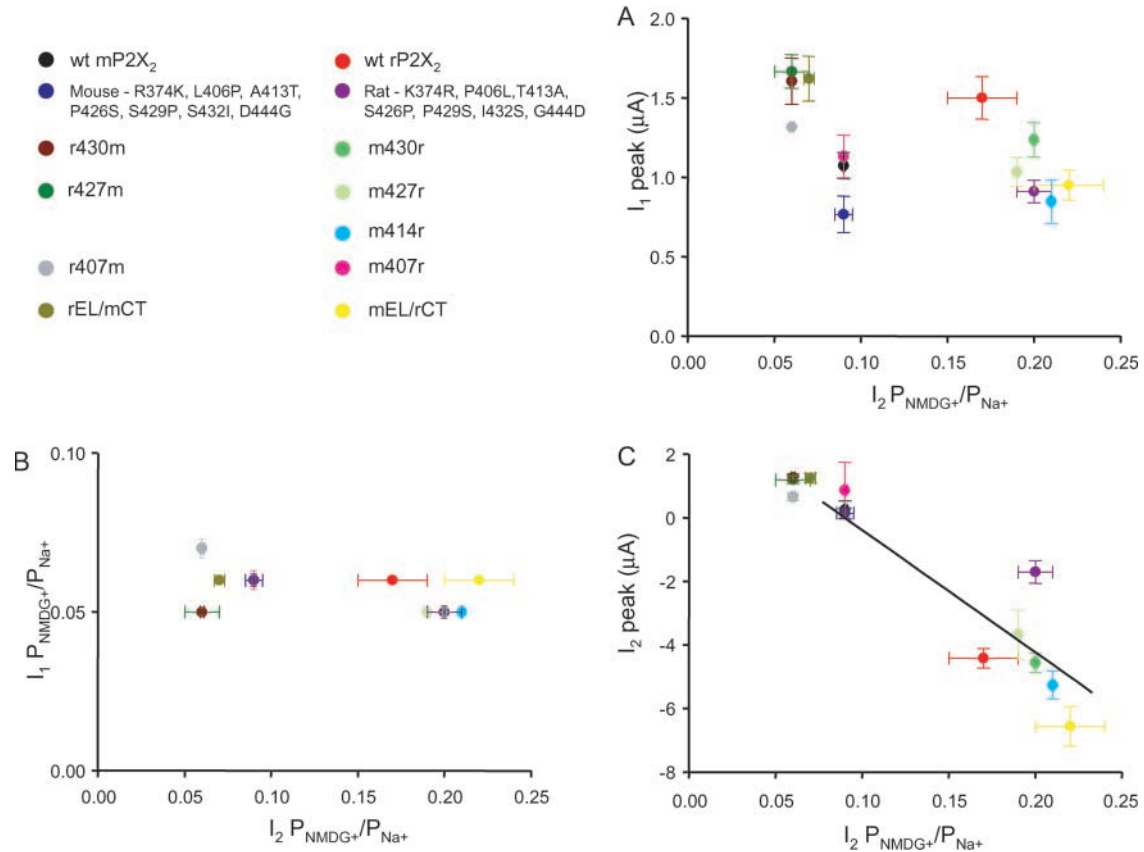


FIGURE 8. Relationship of permeability changes to steady-state currents, I_1 and I_2 . (A) The relationship between I_1 amplitude and $I_2 P_{\text{NMDG}^+}/P_{\text{Na}^+}$. (B) The relationship between $I_1 P_{\text{NMDG}^+}/P_{\text{Na}^+}$ and $I_2 P_{\text{NMDG}^+}/P_{\text{Na}^+}$. (C) The relationship between I_2 amplitude and $I_2 P_{\text{NMDG}^+}/P_{\text{Na}^+}$; the two parameters are well correlated ($r = 0.9$). We verified that slope conductance values followed a similar trend to current peak amplitudes for the majority of mutants where such data was available. For this figure, the single point mutants shown in Fig. 6 have been pooled, and there is no point for the r1414m chimera because there was no measurable I_2 .

Moreover, are $P_{\text{NMDG}^+}/P_{\text{Na}^+}$ 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 $I_2 P_{\text{NMDG}^+}/P_{\text{Na}^+}$ changes, but not between I_1 amplitude and $I_2 P_{\text{NMDG}^+}/P_{\text{Na}^+}$ (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 $I_1 P_{\text{NMDG}^+}/P_{\text{Na}^+}$ and $I_2 P_{\text{NMDG}^+}/P_{\text{Na}^+}$. 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 $P_{\text{NMDG}^+}/P_{\text{Na}^+}$, as well as for the growth from I_1 to I_2 for rP2X₂, mEL/rCT, and m430r channels. The rate constants for rP2X₂, mEL/rCT, and m430r channels are the same for $P_{\text{NMDG}^+}/P_{\text{Na}^+}$ changes and growth of I_2 from I_1 at -60 mV. Overall, these data show that (a) I_2 amplitude and $P_{\text{NMDG}^+}/P_{\text{Na}^+}$ 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) $P_{\text{NMDG}^+}/P_{\text{Na}^+}$ 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 $I_1 P_{\text{NMDG}^+}/P_{\text{Na}^+}$ 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

TABLE III
Rate Constants for Development of I_2 and Increase in $P_{\text{NMDG}^+}/P_{\text{Na}^+}$ for *wt* and Engineered P2X₂ Channels

	Rate		<i>n</i>
	Growth I_1 to I_2	Increase in $P_{\text{NMDG}^+}/P_{\text{Na}^+}$	
	s^{-1}	s^{-1}	
<i>wt</i> rP2X ₂	0.5 ± 0.09	0.5 ± 0.07	6
mEL/rCT	0.4 ± 0.03	0.3 ± 0.02	6
m430r	0.4 ± 0.06	0.4 ± 0.09	6

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 $P_{\text{NMDG}^+}/P_{\text{Na}^+}$ 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: I_2 .

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 rP2X₂/mP2X₂ chimeras, which show identical physiology in the I_1 state but completely different physiology in the I_2 state, allow us to conclude even more confidently that the I_2 state is not an artifact of voltage-clamp fidelity, Ca²⁺-activated conductances, or any other vaguely imaginable event. Instead, the I_2 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 I_2 state because two of its dimensions are larger than the I_1 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 I_1 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 I_1 to I_2 , or by increases in $P_{\text{NMDG}^+}/P_{\text{Na}^+}$. These data imply that YOPRO1 does not permeate the 11 Å I_1 states because of steric hindrance, but can permeate the dilated I_2 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 I_2 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 I_2 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 an 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 E469A polymorphism (Gu et al., 2001). In the simplest case, polymorphic E469A channels display impaired dye uptake through the I_2 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 E469A 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 I_1 state to a less selective I_2 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 I_2 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 $P_{\text{NMDG}^+}/P_{\text{Na}^+}$ range was 0.17–0.34 across all our experiments. The slope conductance for NMDG⁺ in the I_2 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 I₂ state. The pioneering single-channel studies of P2X₂ channels (Ding and Sachs, 1999a,b) have not systematically explored the optimal conditions for production of the I₂ state (Khakh et al., 1999; Virginio et al., 1999b): very low extracellular Ca²⁺ and concentrations of ATP >10 μM (Virginio et al., 1999b). Moreover, rP2X₂ 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 I₂ state recordings, we review several previously reported single-channel characteristics of the I₁ 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 a Na⁺ ion binding site ~20% of the distance through the electric field (from the outside), with an affinity of ~90 mM at -60 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 I₂ 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 I₂ amplitude and I₂ P_{NMDG⁺}/P_{Na⁺}} changes, but no effect on I₁ amplitude or I₁ P_{NMDG⁺}/P_{Na⁺}}. A lack of effect on I₁ 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 I₂ state. With this control dataset in hand we can conclude that residues in the C tail affect the I₂ 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

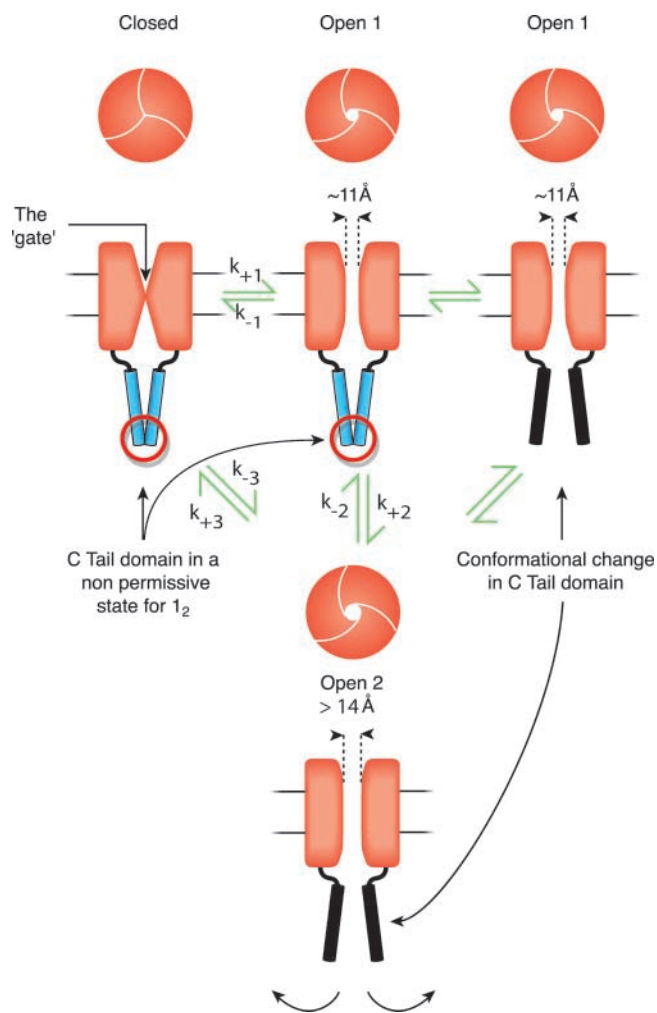


FIGURE 9. A summary of rP2X₂ permeability changes. The cartoon shows rP2X₂ channels in closed and open states of differing diameter across the narrowest region. The narrowest region is shown as the gate. In the Closed and Open 1 state the C tail domain is diagrammatically shown as in a “nonpermissive state” with respect to permeability changes. The present paper shows that a conformational change occurs in the tail as, or before, the pore dilates, and this is hypothetically shown as a motion of the C tail domain away from the inner aspect of the channel, as in MscL channel gating (Sukharev et al., 2001). The model implies the existence of a state where the tail has undergone a conformational change but the pore has not yet dilated. A direct transition from Closed to Open 2 was demonstrated previously for mutant channels (Khakh et al., 1999; Virginio et al., 1999b).

the transition from I₁ to I₂ increases the area across the narrowest region in the rP2X₂ pore by at least ~60 Å², for a circular filter, or ~76 Å² for a square filter. This implies an increase in the pore diameter by at least 3 Å, but an accurate estimate of the I₂ 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 (I_1 ; pore diameter 11 Å) and then to open 2 (I_2 ; 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 NH_2 and COOH termini are close to one another in functional P2X_2 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 P2X_2 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 P2X_7 channel (Kim et al., 2001), and it is possible that a similar situation exists for P2X_2 channels. Thus, permeability changes might involve interactions with other cytoplasmic partners, which may differ between mP2X_2 and rP2X_2 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 I_2 state. Indeed, P2X_2 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 I_2 would effectively 'lock' the C tail in a non-permissive state (Fig. 9). In such a model the transition from the I_1 to the I_2 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 contribu-

tion of the C tail domain to permeability changes at P2X_2 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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REFERENCES

- Barry, P.H., and J.W. Lynch. 1991. Liquid junction potentials and small cell effects in patch-clamp analysis. *J. Membrane Biol.* 121: 101–117.
- Boue-Grabot, E., V. Archambault, and P. Seguela. 2000. A protein kinase C site highly conserved in P2X subunits controls the desensitization kinetics of P2X_2 ATP-gated channels. *J. Biol. Chem.* 275:10190–10195.
- Cohen, B.N., C. Labarca, N. Davidson, and H.A. Lester. 1992. Mutations in M2 alter the selectivity of the mouse nicotinic acetylcholine receptor for organic and alkali metal cations. *J. Gen. Physiol.* 100:373–400.
- Ding, S., and F. Sachs. 1999a. Single channel properties of P2X_2 purinoceptors. *J. Gen. Physiol.* 113:695–720.
- Ding, S., and F. Sachs. 1999b. Ion permeation and block of P2X_2 purinoceptors: single channel recordings. *J. Membr. Biol.* 172: 215–223.
- Ding, S., and F. Sachs. 2000. Inactivation of P2X_2 purinoceptors by divalent cations. *J. Physiol.* 522:199–214.
- Dwyer, T.M., D.J. Adams, and B. Hille. 1980. The permeability of the endplate channel to organic cations in frog muscle. *J. Gen. Physiol.* 75:469–492.
- Egan, T.M., W.R. Haines, and M.M. Voigt. 1998. A domain contributing to the ion channel of ATP-gated P2X_2 receptors identified by the substituted cysteine accessibility method. *J. Neurosci.* 18: 2350–2359.
- Ennion, S., S. Hagan, and R.J. Evans. 2000. The role of positively charged amino acids in ATP recognition by human P2X_1 receptors. *J. Biol. Chem.* 275:29361–29367.
- Evans, R.J., C. Lewis, C. Virginio, K. Lundstrom, G. Buell, A. Surprenant, and R.A. North. 1996. Ionic permeability of, and divalent cation effects on, two ATP-gated cation channels (P2X receptors) expressed in mammalian cells. *J. Physiol.* 497:413–422.
- Gu, B.J., W. Zhang, R.A. Worthington, R. Sluyter, P. Dao-Ung, S. Petrou, J.A. Barden, and J. Wiley. 2001. A Glu-496 to Ala polymorphism leads to loss of function of the human P2X_7 receptor. *J. Biol. Chem.* 276:11135–11142.
- Haines, W.R., K. Migita, J.A. Cox, T.M. Egan, and M.M. Voigt. 2001. The first transmembrane domain of the P2X receptor subunit participates in the agonist-induced gating of the channel. *J. Biol. Chem.* 276:32793–32798.
- Hille, B. 1992. Ionic channels of excitable membranes. 2nd Edition.
- Jiang, L.H., F. Rassendren, V. Spelta, A. Surprenant, and R.A. North. 2001. Amino acid residues involved in gating identified in the first membrane-spanning domain of the rat P2X_2 receptor.

- J. Biol. Chem.* 276:14902–14908.
- Jiang, L.-H., F. Rassendren, A. Surprenant, and R.A. North. 2000. Identification of amino acid residues contributing to the ATP binding site of a P2X receptor. *J. Biol. Chem.* 275:34190–34196.
- Khakh, B.S., P.P. Humphrey, and A. Surprenant. 1995. Electrophysiological properties of P2X-purinoreceptors in rat superior cervical, nodose and guinea-pig coeliac neurones. *J. Physiol.* 484:385–395.
- Khakh, B.S., X. Bao, C. Labarca, and H.A. Lester. 1999. Neuronal P2X receptor-transmitter-gated cation channels change their ion selectivity in seconds. *Nat. Neurosci.* 2:322–330.
- Khakh, B.S., and H.A. Lester. 1999. Dynamic selectivity filters in ion channels. *Neuron.* 23:653–658.
- Khakh, B.S. 2001. Molecular physiology of P2X receptors and ATP signalling at synapses. *Nat. Rev. Neurosci.* 2:165–174.
- Kielland, J. 1937. Individual activity coefficients of ions in aqueous solutions. *J. Am. Chem. Soc.* 59:1675–1678.
- Kim, M., L.H. Jiang, H.L. Wilson, R.A. North, and A. Surprenant. 2001. Proteomic and functional evidence for a P2X7 receptor signalling complex. *EMBO J.* 20:6347–6358.
- Koshimizu, T., M. Koshimizu, and S.S. Stojilkovic. 1999. Contributions of the C-terminal domain to the control of P2X receptor desensitization. *J. Biol. Chem.* 274:37651–37657.
- Koshimizu, T.-A., M. Tomi, M. Koshimizu, and S.S. Stojilkovic. 1998. Identification of amino acid residues contributing to desensitization of the P2X₂ receptor channel. *J. Biol. Chem.* 273:12853–12857.
- Migita, K., W.R. Haines, M.M. Voigt, and T.M. Egan. 2001. Polar residues of the second transmembrane domain influence cation permeability of the ATP-gated P2X₂ receptor. *J. Biol. Chem.* 276:30934–30941.
- Miyazawa, A., Y. Fujiyoshi, M. Stowell, and N. Unwin. 1999. Nicotinic acetylcholine receptor at 4.6 Å resolution: transverse tunnels in the channel wall. *J. Mol. Biol.* 288:765–786.
- Ng, B., and P.H. Barry. 1995. The measurement of ionic conductivities and mobilities of certain less common organic ions needed for junction potential corrections in electrophysiology. *J. Neurosci. Methods.* 56:37–41.
- North, R.A. 1996. Families of ion channels with two hydrophobic segments. *Curr. Opin. Cell Biol.* 8:474–483.
- Rassendren, F., G. Buell, A. Newbolt, R.A. North, and A. Surprenant. 1997. Identification of amino acid residues contributing to the pore of a P2X receptor. *EMBO J.* 16:3446–3454.
- Sato, C., Y. Ueno, K. Asai, K. Takahashi, M. Sato, A. Engel, and Y. Fujiyoshi. 2001. The voltage-sensitive sodium channel is a bell-shaped molecule with several cavities. *Nature.* 409:1047–1051.
- Simon, J., E.J. Kidd, F.M. Smit, I.P. Chessell, R. Murrell-Lagnado, P.P. Humphrey, and E.A. Barnard. 1997. Localization and functional expression of splice variants of the P2X₂ receptor. *Mol. Pharmacol.* 52:237–248.
- Smith, F.M., P.P.A. Humphrey, and R.D. Murrell-Lagnado. 1999. Identification of amino acids within the P2X₂ receptor C-terminus that regulate desensitization. *J. Physiol.* 520:91–99.
- Sokolova, O., L. Kolmakova-Partensky, and N. Grigorieff. 2001. Three-dimensional structure of a voltage-gated potassium channel at 2.5 nm resolution. *Structure.* 9:215–220.
- Stoop, R., S. Thomas, F. Rassendren, E. Kawashima, G. Buell, A. Surprenant, and R. North. 1999. Contribution of individual subunits to the multimeric P2X₂ receptor: estimates based on methanethiosulfonate block at T336C. *Mol. Pharmacol.* 56:973–981.
- Sukharev, S., M. Betanzos, C.S. Chiang, and H.R. Guy. 2001. The gating mechanism of the large mechanosensitive channel MscL. *Nature.* 409:720–724.
- Surprenant, A., F. Rassendren, E. Kawashima, R.A. North, and G. Buell. 1996. The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor (P2X₇). *Science.* 272:735–738.
- Torres, G.E., T.M. Egan, and M.M. Voigt. 1998. Topological analysis of the ATP-gated ionotropic P2X₂ receptor subunit. *FEBS Lett.* 425:19–23.
- Unwin, N. 1995. Acetylcholine receptor channel imaged in the open state. *Nature.* 373:37–43.
- Virginio, C., A. MacKenzie, R.A. North, and A. Surprenant. 1999a. Kinetics of cell lysis, dye uptake and permeability changes in cells expressing the rat P2X₇ receptor. *J. Physiol.* 519:335–346.
- Virginio, C., A. MacKenzie, F.A. Rassendren, R.A. North, and A. Surprenant. 1999b. Pore dilation of neuronal P2X receptor channels. *Nat. Neurosci.* 2:315–321.