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INVITED REVIEW

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Biophysics of P2X receptors

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Abstract The P2X receptor is the baby brother of the ligand-gated ion channel super-family. An understanding of its role in human physiology is still developing, and no one truly knows how it works to transport ions across the membrane. In this study, we review some aspects of P2X channel biophysics, concentrating on ion permeation and gating. P2X channels transport both small and large cations and anions across cell membranes in a manner that depends on both the subunit composition of the receptor and the experimental conditions. We describe the pore properties of wild-type receptors and use the altered phenotypes of mutant receptors to point the way towards a structural model of the pore.

Keywords ATP · Ionotropic receptor · Purinergic receptor · P2X · Permeability · Conductance · Gating

Introduction

Simply put, the function of all ATP-gated P2X receptors is to transport water-soluble molecules across the lipid barrier of membranes. In most cases, the relevant membrane is the plasmalemma that separates the cytoplasm from the extracellular space, and the molecules are simple ions like sodium, potassium, chloride, and calcium (Fig. 1). However, there are exceptions. For example, $P2X_7$ receptors are found in the nuclear envelope of hippocampal neurons where they serve an unknown function [1] (but see [2]), and some P2X receptors transport larger and more complex ions like glutamate [3, 4], nucleotides [5], and perhaps even ATP itself [6]. In this review, we examine the biophysics of P2X receptors. By definition, the science of biophysics seeks to explain biological function in terms of molecular structure.[1] The atomic scale structure of a P2X receptor is still unknown and, therefore, strictly speaking, it is impossible to accurately describe the receptor biophysics. At present, the best we can do is use the data gathered from site-directed mutagenesis to compose a reasonable model of the structure of the channel and then extend this model to suggest a structural basis for function. In this paper, we do just that by considering the two basic properties that P2X receptors share with all ion channels, ion selectivity and gating.

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The receptor as a channel

P2X receptors are a family of seven [7] or more [8] isoforms designated $P2X_1$ to $P2X_7$. Six of the seven form functional homomeric receptors and heteromeric assemblies are also possible [9–12]. Each receptor is made of three [13–15] or more [16] subunits arranged in a "head-to-tail" order [17]. Individual subunits have intracellular N- and C-termini, a large extracellular loop containing the ATP binding site, and two putative transmembrane segments [18, 19]. Both the first (TM1) and second (TM2) transmembrane segments are thought to line an integral ion pore [20–23]. If so, then the channel is made of six transmembrane domains, two from each of the three subunits, an arrangement that is unique in the ion channel literature. Each transmembrane domain plays an active role in the transport of ions. For example, both TM1 [24–26] and TM2 [25–28] participate in the repacking that occurs during gating, and it appears that fairly dramatic changes occur in the structure of the pore as the channel opens. Furthermore, TM2 interacts with permeating ions and regulates specific properties of ion flow including conductance [29], permeability [30], and Ca^{2+} flux [31]. A similar role for TM1 in regulating conductance and/or permeability is not established although it is easy to imagine that such a role exists [32]. The diameter of the narrowest

[1] From, "What is Biophysics". Online educational resources of the Biophysical Society.

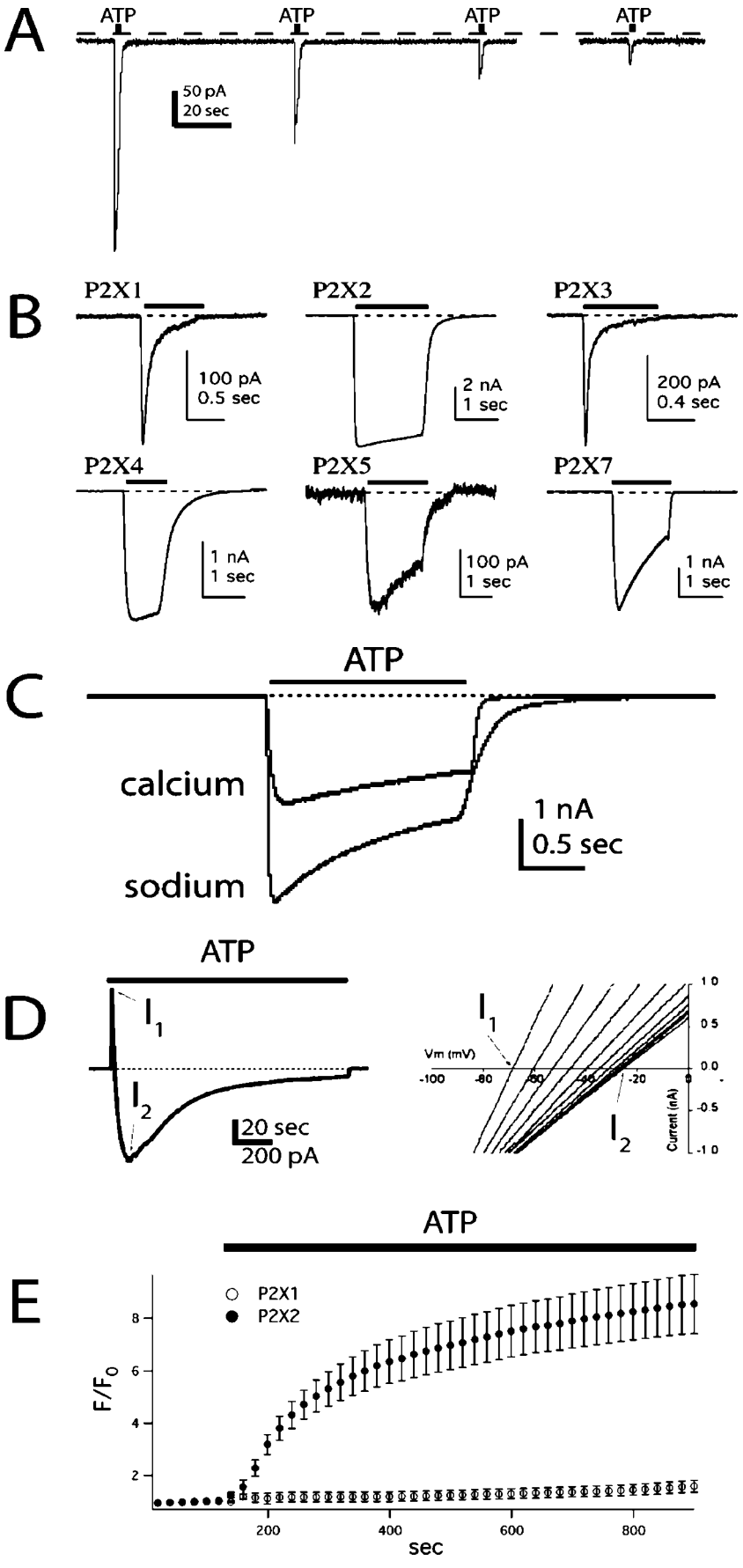


Fig. 1 P2X receptors are ion channels. **a** ATP currents recorded from a single guinea-pig atrial myocyte held at −60 mV. The figure shows three applications of ATP (50 μM) spaced 1 min apart and a fourth application applied 15 min later. The successive applications produce smaller currents. The subunit composition of the atrial receptor is unknown (unpublished data of Yum and Egan). **b** ATP-gated current in voltage-clamped HEK293 cells expressing recombinant P2X receptors (from [32]). **c** Applying ATP dissolved in an external solution that contains either 154 mM Na^+ or 110 mM Ca^{2+} evoked inward currents showing that the channel is permeable to both ions (unpublished data of Egan). **d** The permeability of the $P2X_2$ receptor changes during long applications of ATP. The *left panel* shows ATP-gated whole-cell current in a low-divalent extracellular solution containing 154 mM $NMDG^+$ and held at −60 mV. The intracellular solution contained 154 mM Cs^+. ATP causes a rapid outward Cs^+ current (called I_1) followed by a slower inward $NMDG^+$ current (called I_2). The *right panel* shows that E_{rev} shifts from −70 to −25 mV as P_{NMDG}/P_{Cs} increases (from [97]). **e** Some P2X receptors are permeable to large ions when extracellular Ca^{2+} is low. We applied 600 μM ATP to cells bathed in a low divalent extracellular solution that contained the nucleotide dye, YO-PRO-1. YO-PRO-1 shows minimal fluorescence outside the cell but begins to fluoresce as it enters the cell and binds nucleotides. The figure shows that ATP evokes YO-PRO-1 fluorescence HEK-293 cells expressing $P2X_2$ but not $P2X_1$ receptors (unpublished data of Lipka and Egan)

part of the open pore is thought to be about 8–20 Å [33–36], a size that is large enough to permit small monovalent cations to pass through the channel [35, 37]. It is too small, however, to permit appreciable conduction of larger organic cations like *N*-methyl-*d*-glucamine ($NMDG^+$) and quinolinium-4-[(3-methyl-2(3H)-benzoxazo-lylidene) methyl]-1-[3-(trimethyl-am-monio)-propyl] diiodide (YO-PRO-1) [33]. Some P2X subtypes show an increase in the diameter of the pore during sustained applications of ATP to the point where $NMDG^+$ and YO-PRO-1 are freely permeable [27, 28]. The mode of the channel that is permeable to small ions only is called the "O_1 state", and current through O_1 is called "I_1". The mode of the channel that is permeable to both small and large ions is called the "O_2 state", and current through O_2 is called "I_2" [27]. A very simple kinetic model that describes this behavior is:

$$C \underset{ATP(fast)}{\rightleftharpoons} O_1 \underset{ATP(slow)}{\rightleftharpoons} O_2$$

The channel is closed in the absence of ATP, opens immediately to O_1, and then moves slowly to O_2 in the continued presence of agonist. As we shall see, this model grossly underestimates the complexity of gating, and it is presented here only to give the reader a framework from which to understand the permeability changes described in the next few sections. The molecular mechanism(s) underlying the change in selectivity from O_1 to O_2 are unknown and remain a popular and exciting avenue of investigation [7, 38].

Ion selectivity

All P2X receptors are permeable to sodium, potassium, and calcium [39]. A few also show significant chloride permeability [40–42]. Furthermore, when the conditions are right, some develop a new open state (i.e., O_2) that permits relatively large cations and anions to traverse the pore [5, 43, 44]. Each case is considered separately below.

Selectivity is determined by measuring either the relative permeability or the relative conductance of the channel to a range of ions. *Relative permeability* is calculated from the reversal potentials of currents measured in solutions of known composition using the Goldman–Hodgkin–Katz equation. In channels that incorporate a binding site within the pore, the ion that binds the tightest would be the most permeable. *Relative conductance*, a measure of how well ions pass through the pore, is determined by measuring the amplitudes of single channel currents obtained in comparable concentrations of permeant ions. In theory, a channel may display different permeability and conductance sequences if the affinity of the ion for the binding site is high enough to slow its travel through the pore. Therefore, channel behavior is best described by obtaining both types of data for a single channel and then forming an appropriate model of the pore by comparing the permeability and conductance sequences [45]. The P2X literature lacks such a comparison. Although several groups describe the relative permeabilities of native and recombinant P2X receptors [30, 40, 46–53], only one details relative conductance [35, 37], and no group has performed both measurements using a single receptor subtype expressed in a single type of cell. The reason for this deficiency is simple: permeability is determined from undemanding measurements of reversal potentials whereas relative conductance is determined from the more problematic measurement of single channel current amplitude. The

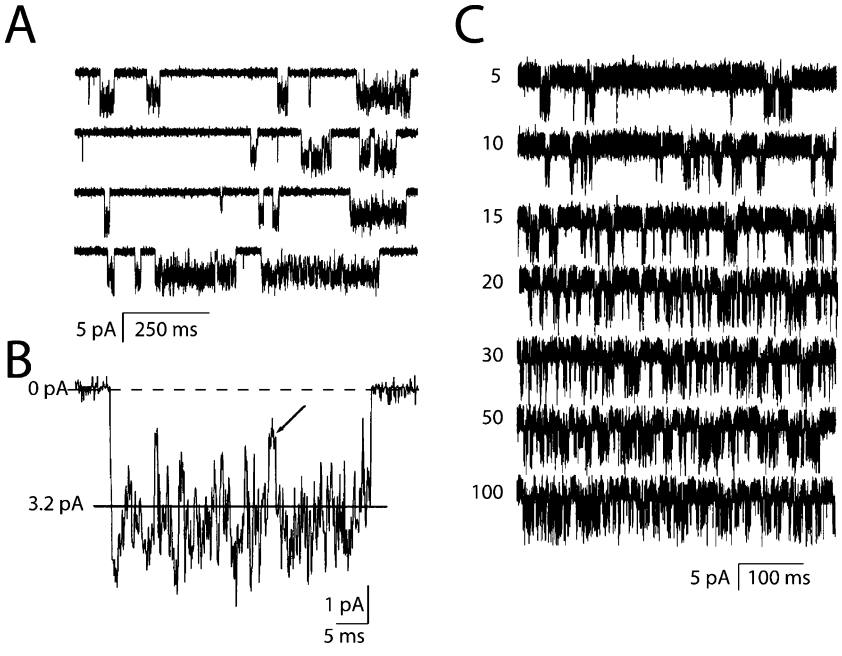


Fig. 2 Single channel currents recorded from outside-out patches of membrane containing recombinant $P2X_2$ receptors. **a** The channel opens in burst in the presence of 1.5 μM ATP. **b** Each burst contains many poorly defined openings and closings. Subconductance levels may be present but are difficult to resolve (*arrow*). **c** The open channel probability is a function of the concentration of ATP (modified from [37] with the permission of the authors)

channel currents of P2X receptors are particularly difficult to quantify because they flicker open and close at rates that exceed the bandwidth of the recording equipment (Fig. 2) [48, 54] and they rundown in excised membrane patches [37].

Despite these problems, Ding and Sachs [37] completed a series of single channel experiments that significantly advanced our understanding of the inner workings of the pore. Published in 1999, this paper remains the single best description of P2X channel biophysics available today, and we will refer to it extensively throughout this review.

Using outside-out membrane patches from *Xenopus laevis* oocytes and HEK293 cells expressing recombinant $P2X_2$ receptors, Ding and Sachs recorded channel openings that appeared as flickery bursts of poorly defined openings and closings separated by relatively longer periods of silence. The inability to accurately measure the peak amplitudes of single channel currents made a precise determination of conductance levels impossible. Instead, the average chord conductance measured at a holding potential of −100 mV was estimated from the Gaussian fit of the open component of the all-points histogram. Calculated in this way, the mean conductance was 32 pS, a value that probably underestimates the peak chord conductance of the fully open $P2X_2$ channel by as much as 40% [37]. Other groups report single channel conductances that range from about 10–60 pS depending on membrane holding potential, identity of the subunit under investigation, and the composition of the extracellular solution [54–60]. By comparison, recombinant $5\text{-}HT_{3A/B}$ [61], α4β2 nicotinic [62], and NR1/NR2A glutamate receptors [63] have slope conductances of 16, 31, and 51 pS, respectively. As reported previously for whole-cell current, the single $P2X_2$ channel current exhibited a strong inward rectification [37]. The molecular basis of this effect is unknown, although it does not involve block by Mg^{2+} or polyamines and does not result from an unequal distribution of permeant ions across the membrane [58]. Rather, it may be inherent property of the channel that results from a voltage-dependent change in gating and/or conductance [58, 64]. The degree of rectification varies from cell to cell [33, 65] and depends on receptor density [66]. The molecular basis of the variability is also unknown.

Permeability to group 1 monovalent cations (alkali metals) P2X channels are often called "cation non-selective" ion channels, but this is not strictly true. First, as described in more detail below, some subtypes show significant anion permeability. Second, the channels are not entirely cation non-selective. If so, then the conductance sequence to a range of ions should follow the relative mobility of these ions in water. To address the mechanism of ion selection, Ding and Sachs measured $P2X_2$ channel conductance in cells bathed in a range of alkali metals. The conduction sequence was $K^+ > Rb^+ \geq Cs^+ > Na^+ > Li^+$ which corresponds to a type IV Eisenman equilibrium ion exchange sequence ([35, 37]; see also Fig. 4 of [32]). This sequence is different from the relative mobility in water and suggests that cations interact with an anionic binding site of low to moderate field strength located somewhere in the permeation pathway [45, 67]. It should be noted that the disparity in mean conductances measured in equimolar solutions of K^+, Rb^+, and Cs^+ differed by less than 3 pS, suggesting that the channel conducts each of these ions with equal ease. A similar situation is seen in the relative permeability sequences measured from the reversal potentials of ATP-gated currents [30]. These very slight differences may explain the variability in relative permeability sequences reported in the literature. In contrast, both the relative conductance and relative permeability of Li^+ differs significantly from that of the other test ions. It has the smallest single channel conductance and is the most permeable [32], a pattern that suggests that the $P2X_2$ receptor has a site within the pore that binds Li^+ [37]. The binding site hypothesis is supported by the ability of Ca^{2+} and other divalent cations to reduce the single channel current carried by monovalent cations through the $P2X_2$ receptor in a manner consistent with a local blocking effect within the pore [35].

The location of the binding site is unknown. Ding and Sachs [37] measured single channel conductance from cells bathed in varying concentrations of extracellular Na^+. They found that the concentration–conductance curve saturated at high $[Na^+]_{out}$ and was well fit by a Michaelis–Menten model incorporating a single binding site located about 20% of the electrical distance from the extracellular surface of the channel. The equilibrium constant of Na^+ binding was 148 mM at 0 mV and was voltage dependent. The anionic site that binds Li^+, Na^+, and perhaps Ca^{2+} (see below) could arise from sites in TM1 and/or TM2. Migita et al. [30] addressed this issue by measuring the effect of site-directed mutagenesis of polar residues in TM2 on the permeability sequence of the $P2X_2$ receptor to Group 1 cations. They found that changing sidechain volume at two sites (Thr^{339} and Ser^{340}) transformed the permeability sequence of alkali metals to one that equaled the mobility sequence of the ions in water. These data suggest that Thr^{339}, Ser^{340}, and perhaps other polar amino acids [29, 30] contribute to a site that momentarily binds cations as they flow through the channel. The site may be a narrow part of the channel that forms an energy barrier to current flow, and Thr^{339} and Ser^{340} may provide the countercharge needed to help the permeating cations shed some, but not all [68], water molecules as they pass through the constriction [32]. However, there are limitations to this hypothesis. First, the polar residues identified in the $P2X_2$ receptor are not conserved in other family members, so the general applicability of the hypothesis is questionable. Although the relative permeabilities and conductances of other subtypes are less well characterized than the $P2X_2$ receptor, there is no reason to believe that any P2X receptor shows a strong preference for one monovalent cation over another, and it is therefore reasonable to assume that they share a common means of weakly discriminating among these ions. If so, then the lack of sequence conservation may indicate that the charge needed to dehydrate the ions is supplied by the carbonyl oxygens of the peptide backbone and not by the polar sidechains,

despite the fact that these sidechains face into the pore [32]. The second caveat is that relative Ca^{2+} and Cl^- permeabilities vary amongst family members, suggesting that a single mechanism is unlikely to explain the permeability and conductance properties of all the receptors. Future studies designed to study the role of other domains in cation and anion permeation are needed to provide a clear picture of the biophysics of the channel.

Permeability to group 2 divalent cations (alkaline earth metals) Perhaps the most important outcome of activating P2X receptors is a momentary increase in the free concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) [7, 69]. P2X receptors increase $[Ca^{2+}]_i$ in two distinct ways. First, P2X receptors mediate a membrane depolarization that opens voltage-dependent Ca^{2+} channels. Second, the P2X receptor itself is permeable to Ca^{2+}. When ATP activates the receptor, Ca^{2+} moves down its electrochemical gradient by traveling through the channel and into the cell. In many cases, the Ca^{2+} that enters the cell through the pore triggers a response in the absence of activation of voltage-dependent Ca^{2+} channels. For example, stimulation of sympathetic perivascular nerves causes vesicular release of ATP, initiating a $P2X_1$ receptor-mediated Ca^{2+} influx and vasoconstriction of postsynaptic smooth muscle cells [70–73].

A number of groups have measured the relative Ca^{2+} permeabilities (P_{Ca}/P_X, where X=Na^+, Cs^+, or, rarely, K^+) of native and recombinant P2X receptors and, in all cases, the Ca^{2+} permeability equals or exceeds that of the more abundant monovalent ions of the extracellular medium (i.e. $P_{Ca}/P_X \geq 1$). Specifically, P_{Ca}/P_X ranges from 1 to 71 for native receptors, and from 1 to 5 for recombinant receptors expressed in HEK293 cells or *Xenopus* oocytes (Table 1). The extremely high P_{Ca}/P_Xs recorded from macrophages [74] and lymphocytes [75] are an anomaly. The subunit compositions of these native receptors are unknown, although both cells are thought to express predominately the $P2X_7$ subtype [7]. It would be informative to compare the relative permeabilities of the

Table 1 $P_{Ca^{2+}}/P_{X^+}$ and $P_{f\%}$ of native and recombinant P2X receptors

Protein	Subtype	$P_{Ca^{2+}}/P_{X^+}$	$P_{f\%}$	Reference
Recombinant				
Rat $P2X_1$		4.8	12.4	[31, 105]
Human $P2X_1$		3.9	11	[31, 33]
Rat $P2X_{1/5}$		1.1	3.3	[31]
Rat $P2X_2$		2.8	5.7	[30, 31, 33]
Rat $P2X_{2/3}$		1.3	3.5	[31, 106]
Rat $P2X_{2/6}$			(7.7)	[31]
Rat $P2X_3$		1.2	2.7	[31, 106]
Rat $P2X_4$		4.2	11	[31, 107, 108]
Human $P2X_4$			15	[31]
Rat $P2X_{4/6}$			(11.3)	[31]
Rat $P2X_{5\ z}$			4.5	[31]
Human $P2X_5$		1.5		[42]
Rat $P2X_7$			4.6	[31]
Native				
Arterial smooth muscle	$P2X_1$	3.3		[109]
Guinea-pig bladder smooth muscle	$P2X_1$	1.5	5.8	[110]
Rat nodose ganglion	$P2X_2$	1.5		[106]
Rat superior cervical ganglion	$P2X_2$		6.5	[84]
Rat PC-12 cells	$P2X_2$	2.8		[52]
Rat/bullfrog dorsal root ganglion	$P2X_2$, $P2X_{2/3}$	0.3		[48]
Rat submandibular ganglion	$P2X_2$, $P2X_4$	1.0		[51]
Rat peritoneal macrophage	$P2X_7$	71		[74]
Human B-lymphocytes	$P2X_7$	35		[75]
Schistosoma mansoni	*Sch*P2X	3.8		[111]
Rabbit airway epithelial cells		9		[47]
Rat tuberomammillary neurons		2.6		[49]
Rat somatosensory neurons		12.3		[112]
Rat medial habenula neurons		>10		[113]
Rat retinal ganglion cells		2.2		[114]

The table presents a survey of Ca^{2+} permeabilities and fluxes for native and recombinant receptors. The $P_{f\%}$ measured for cells co-expressing $P2X_2$ or $P2X_4$ with $P2X_6$ may reflect a mix of currents through both homomeric and heteromeric receptors; these values are given in parentheses [31]. The subunit composition of the P2X receptors underlying the native responses are largely unknown and are the best guesses of the respective authors

native and recombinant $P2X_7$ receptors but, unfortunately, the P_{Ca}/P_X of the recombinant $P2X_7$ receptor is unknown because measuring it is problematic. This is because whole-cell current through the $P2X_7$ receptor is inhibited by the relatively high Ca^{2+} concentrations that are needed to accurately measure P_{Ca}/P_X (Alan North, personal communication). However, if one assumes that current through the $P2X_7$ receptor obeys the constant field assumptions, then it is possible to approximate P_{Ca}/P_X from an independent measure of Ca^{2+} flux called the fractional Ca^{2+} current or $P_{f\%}$ [76] (see below). Estimated in this way, the Ca^{2+} flux of recombinant $P2X_7$ receptors translates to a P_{Ca}/P_X of about 1.0, a value that greatly underestimates the values recorded from native cells. There is no easy answer to explain this difference. It seems unlikely that the native response reflects the action of a heteromeric receptor because the $P2X_7$ subtype is thought to form homomeric receptors only [9]. It is also unlikely that the high Ca^{2+} flux reflects the O_2 state of channel because it is hard to imagine a structure that would be wide enough to allow large cations to pass while at the same time being small enough to select between calcium and sodium ions of similar size.

Another way to estimate of the contribution of Ca^{2+} to ATP-gated current comes from measurements that use the fluorescence approach first described by the Neher laboratory in 1993 [76, 77]. In brief, the fraction of total ATP-gated current carried by Ca^{2+} is determined by simultaneously recording the total charge through the channel (Q_T, measured using electrophysiology) and the charge carried by Ca^{2+} (Q_{Ca}, measured using fluorescence microscopy and the Ca^{2+}-sensitive dye Fura-2). The fraction of the total current carried by Ca^{2+} (fractional Ca^{2+} current or $P_{f\%}$ for short) is calculated by dividing Q_{Ca} by Q_T and then multiplying the result by 100%. The $P_{f\%}$ method offers several advantages over estimating relative Ca^{2+} permeability from reversal potentials [78–80]. $P_{f\%}$ is a true measure of Ca^{2+} entry, and it allows the measurement to be made at a physiological $[Ca^{2+}]_o$ and at a range of membrane potentials. By contrast, reversal-based techniques do not measure Ca^{2+} flux directly; they often require abnormally high extracellular concentrations of Ca^{2+}, and they characterize Ca^{2+} permeability at a single membrane voltage equal to the reversal potential of the current. Furthermore, $P_{f\%}$ is model independent whereas reversal potential measurements assume that Ca^{2+} influx follows the constant field equations, an assumption that may be wrong [81, 82].

Two groups have used this technique to quantify the Ca^{2+} flux of P2X receptors. Rogers and Dani measured the native response of rat neurons to ATP, ACh, and *N*-methyl-D-aspartate (NMDA) [83, 84]. They found that 6.5% of the total ATP-gated current was carried by Ca^{2+}, a value higher than that of nicotinic currents (4.7%) but lower than that of NMDA currents (12.4%). In situ hybridization suggests that four of the seven P2X receptor subtypes are found in the sympathetic ganglion cells used in this study [85] and therefore, the identity of the P2X receptor responsible for the ATP-gated current is unknown. Egan and Khakh [31] overcame the problem of identifying the subunit composition of native receptors by measuring the $P_{f\%}$ of recombinant rat receptors expressed in HEK293 cells. They found that the $P_{f\%}$ ranged from a low of 2.7% for the $P2X_3$ receptor to a high of 12–13% for $P2X_1$ and $P2X_4$ receptors (Table 1). With the exception of the $P2X_3$ receptor, the $P_{f\%}$ of P2X receptors was equal to or greater than nicotinic α4β2 (3.1%), serotonergic $5HT_{3A}$ (4.7%), and protonergic VR1 channels (3.5%) Furthermore, both $P2X_1$ and $P2X_4$ channels display $P_{f\%}$s that equaled the glutamatergic NR1/NR2A channel (14.1%). The human orthologs of the rat $P2X_1$ and $P2X_4$ receptors also show large $P_{f\%}$s(11–15%), demonstrating that high Ca^{2+} flux is conserved across species [31]. In light of these findings, it is surprising that Ding and Sachs [35] report that Ca^{2+} and other divalent cations make a negligible contribution to the single channel current recorded in outside-out patches.

The molecular basis of the high Ca^{2+} flux is unknown. We previously discussed this issue in detail [32] and only briefly revisit it here. Some headway towards understanding how the $P2X_2$ receptor selects Ca^{2+} over monovalent ions has been made, but relatively little is known for other members of the family. For the $P2X_2$ receptor, the same mutations that affect the relative monovalent cation permeability also affect P_{Ca}/P_{Cs} and $P_{f\%}$. Changing the size and/or hydrophobicity of the polar residues Thr^{336}, Thr^{339}, and Ser^{340} specifically decreased Ca^{2+} permeability [30] and flux [31] in a manner consistent with disruption of a binding site within the pore. However, as discussed above, these residues are not conserved, and different family members may use diverse means to discriminate monovalent and divalent cations. The multiplicity would make sense when one considers the range of $P_{f\%}$s measured across family members. The $P_{f\%}$s of the $P2X_1$ and $P2X_4$ receptor are twice as large as those of the $P2X_2$ receptor, and it seems reasonable to assume that they use distinctive domains to regulate Ca^{2+} flux across the membrane. In other ligand-gated channels, acidic amino acids positioned in the mouth of the pore facilitate Ca^{2+} flux by simple electrostatic attraction [68, 86]. $P2X_1$ and $P2X_4$ receptors have a conserved glutamate and aspartate on the extracellular ends of TM1 and TM2, respectively. While it would make sense that the high Ca^{2+} fluxes recorded from these subunits could reflect the ability of –COO sidechains to attract Ca^{2+} into the pore, some of the less permeable P2X receptors (e.g., $P2X_3$ and $P2X_7$ receptors) have carboxylates at similar positions. More work is clearly needed to develop a unifying theory of Ca^{2+} flux through the P2X receptor family, and a reliable model of permeation and flux will ultimately depend on the availability of a picture of the atomic structure of channel.

Permeability to anions Most people think of P2X receptors as cation selective channels, but that is not entirely correct. Certain subtypes, most notably the $P2X_5$ receptors, show appreciable anion permeabilities. Hume and Thomas [40, 87, 88] recognized this first while investigating the ATP-gated biphasic depolarization of cultured chicken embry-

onic pectoral muscle evoked. The initial rapid phase of the depolarization showed many of the characteristics of a P2X response including rapid activation, an increase in conductance, and a reversal potential near −10 mV. Changing the concentration of small extracellular monovalent cations shifted the reversal potential in a manner consistent with a cation-permeable channel. The reversal potential also shifted when most of the extracellular Cl^- were replaced by larger anions [87], suggesting that the outward movement of Cl^- contributed to the ATP-evoked depolarization. Relative Cl^- permeability (P_{Cl}/P_{Cs}), estimated from reversal potentials obtained from voltage-clamped cells bathed in a range of solutions, was 0.39 [40]. A cDNA encoding a chicken purinergic receptor was cloned from embryonic skeletal muscle 10 years later [89]; originally thought to be a novel subtype, it is in fact a chick $P2X_5$ receptor that displays many of the features of the native receptor of chick muscle including an appreciable (P_{Cl}/P_{Cs}=0.53) permeability to Cl^- [41]. Orthologues of the chick $P2X_5$ receptor are found in rat [85, 90], mouse [91], bullfrog [92], zebrafish [93], and human [42]. Most of these are poorly characterized because they give relatively small currents in response to ATP, and their permeation properties are not described in detail. One exception is the full-length human $P2X_5$ receptor. Like its counterpart in chicken, human $P2X_5$ yields large ATP-evoked currents and shows significant Cl^- permeability (P_{Cl}/P_{Na}=0.52). The robust current and measurable Cl^- permeability make the human $P2X_5$ receptor a suitable model for the study of the structural basis of anion permeability. All $P2X_5$ receptors have a basic amino acid (Lys^{52}) in TM1 that is missing from the other subtypes that form functional homomeric channels, and Bo et al. [42] reasoned that the positive charge of the $–NH_3^+$ sidechain could increase Cl^- flux by concentrating anions in the mouth of the pore.[2] They tested this hypothesis by substituting glutamine for lysine (K52Q) and found that wild-type and mutant channels had the same P_{Cl}/P_{Na}s. Although the result was disappointing, history tells us that more than one mutation is needed to reverse the ion-charge selectivity of ligand gated channels. To be specific, both the charge/polarity of the interaction site and the relative position of this site in permeation pathway must change before an effect is evident [68].

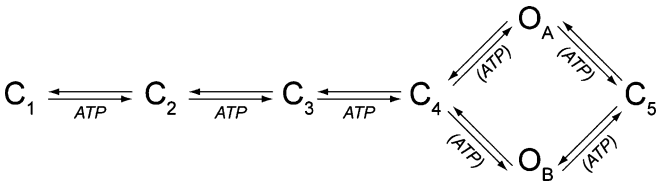
Do other subtypes of P2X receptors show anion permeability? The answer is unknown, but two cases are worth considering. First, the native receptor of cultured hippocampal neurons shows an appreciable Cl^- permeability (P_{Cl}/P_{Cs}=0.5) [94]. Other laboratories have yet to verify this finding and the molecular identity of the native receptor is unknown, so further study is warranted. Second, activation of native $P2X_7$ receptors allows large anions such as glutamate [3, 4] and lucifer yellow [44] to cross the membrane, which suggests that the $P2X_7$ pore shows a significant anion permeability in the O_2 state. By contrast, the Cl^- permeability of the recombinant $P2X_7$ receptor expressed in HEK293 cells remains low even as the pore dilates to a size that allows entry of the large cation $NMDG^+$ [36]. It is hard to reconcile these two observations except to say that the permeation pathway(s) that subserve the effects may be more complicated than originally envisioned [95], and these also deserve further study.

Permeability to larger cations and anions Some P2X receptors ($P2X_{2,4,5,7}$) display multiple conduction states defined by their relative permeability to large organic ions [7, 38, 42]. The smaller state, called O_1, has a pore diameter of about 8–20 Å and a P_{NMDG}/P_{Na} of 0.03 [33–36]. The second conduction state, called O_2, is at least 3 Å larger than O_1 when measured in $P2X_2$ receptors [34] and about 20–40 Å larger when measured in $P2X_7$ receptors [36]; it has a P_{NMDG}/P_{Na} of about 0.25–0.42 [34, 36]. The O_2 state is permeable to both monovalent and divalent cations ($NMDG^+$, $ethidium^+$, and YO-PRO-1^{2+}) [27, 28]. The relative permeability of large anions depends on the particular P2X subtype under consideration. For example, recombinant $P2X_7$ receptors are impermeable to Cl^- in both the O_1 and O_2 states [36]. In contrast, the recombinant human $P2X_5$ receptor shows a relatively high permeability to Cl^- but is impermeable to the $gluconate^-$ [42]. Finally, the native receptors of macrophages and astrocytes show significant permeabilities to $glutamate^-$ [3, 4] and $lucifer\ yellow^{2-}$ [44], despite the fact that they are probably homomeric $P2X_7$ receptors. The variability brings forth an argument worth considering: although many subtypes show the O_1 to O_2 transition, they may not reach O_2 through a common pathway. The structural basis of the transition to O_2 is unknown but could involve either: (1) dilation of an existing pore, (2) successive oligomerization of existing monomeric channels to form a new pore, or (3) activation of a separate conducting pathway. The second option is unlikely because a change in channel stoichiometry on a time scale of seconds is unlikely [96] and, besides, recent data using total internal reflection fluorescent microscopy show that $P2X_2$ channels do not cluster in response to ATP [97]. Some data point to activation of a new and separate pathway, while other experiments suggest that the existing pore slowly dilates [7, 27, 34, 36, 66, 95, 98, 99]. These studies are ongoing and should provide interesting findings in the future.

Gating So far, we have considered the simplest model of gating in which the channel moves between one closed and two open states of different minimum diameters. Comprehensive reports of the gating behavior of P2X receptors are missing, in part because an accurate portrayal of kinetics requires good recordings of single channel activity. As described above, P2X receptors open in flickery bursts of poorly defined events that are hard to quantify and, therefore, hard to model. Despite these problems, a number of more detailed models have been proposed [34, 36, 37, 59, 66, 100–102], although none are definitive.

[2] This is not to say that all $P2X_5$ receptors show anion permeability. Although Lys^{52} is positionally conserved, the Cl^- permeabilities of most $P2X_5$ orthologs are unknown.

The transition from closed to O_1 The most complete model of the transition from closed to initial opening comes from the work of Ding and Sachs [37] who found that the channel moves through five closed states (C_1 to C_5) and two open states (O_A and O_B)[3] as the channel binds three molecules of ATP. Each binding step makes the next faster, so the binding of ATP is cooperative, and partially liganded receptors fail to open. The last state, C_5, is bound to ATP but closed and represents a relatively stable conformation in the reaction pathway that is:



This model admirably describes the behavior seen in outside-out patches bathed in ATP. However, there are three caveats worth considering. First, in a separate paper, Ding and Sachs showed that the simultaneous opening of two or more channels occurs more frequently than expected of independent channels [100]. This suggests that the activity of one channel affects that of a second in a manner consistent with a direct protein–protein interaction between neighboring $P2X_2$ receptors. Therefore, a limit of the original Ding and Sachs model is that it describes the non-independent behavior of a single channel patch but does not consider the more realistic gating behavior that occurs when more than one channel is present. Second, caution must be used when applying the model to the behavior of even a single channel patch during the first few tens of seconds of an application of ATP. This is because the model is based on the residual and stable channel activity that remains during long applications of ATP, and this may or may not reflect channel activity during shorter applications. Almost all P2X receptors show some degree of desensitization and/or inactivation, a process that is accelerated in excised membrane patches bathed in physiological saline solutions [58, 103]. The presence of desensitization implies that additional kinetic state(s) are needed to complete the model. Third, Ding and Sachs did not observe the time-dependent increase in the permeability of $NMDG^+$ that is a hallmark of the transition to the larger O_2 state [35]. This should not be surprising because patch excision affects several P2X channel properties. To be specific, excision causes channels to: (1) rundown to a low level of activity that presumably results from the loss of an essential intracellular chemical [37] and (2) show an accelerated rate of desensitization during short applications of ATP. That is, the residual ATP-gated current that survives excision desensitizes at a rate that is faster (τ=0.1 s, [103]) than that of the O_1 to O_2 transition (τ=6 s, [101]). This means that the channel is fully desensitized before it enters O_2, making it difficult to observe the increase in P_{NMDG}/P_{Na}. We suggest that the kinetic model proposed by Ding and Sachs best describes the closed state to O_1 transition that occurs during the continued presence of an application of ATP and that O_A and O_B reflect different substates of the normal, $NMDG^+$-impermeable O_1 form of the pore.

The transition from O_1 to O_2 Some P2X receptors ($P2X_{2,4,7}$) show a time-dependent increase in P_{NMDG}/P_{Na} that is the hallmark of entry into the O_2 state [7, 96]. In these cases, $NMDG^+$ permeability is low at first and then gradually increases, suggesting that the channel moves in a linear fashion from the closed states through O_1 (i.e., $NMDG^+$-impermeable state) to O_2 (i.e., $NMDG^+$-permeable state). The kinetics of the O_1 to O_2 transition are not described at the level of the single channel but have been investigated using whole-cell electrophysiology, fluorescence microscopy, and $P2X_2$ [34, 101] and $P2X_7$ [36] receptors. The time constant of the transition from O_1 to O_2, measured from the change in P_{NMDG}/P_{Na}, is faster in $P2X_2$ (2–6 s) than in $P2X_7$ receptors (about 10 s), although both the minimum pore diameter of O_1 (8–11 Å, but see [35]) and the time constant of the closed to O_1 transition (<100 ms) are the same. Measurements of the movement of the C-terminal cytoplasmic domain of the $P2X_2$ receptor based on fluorescence resonance energy transfer mimicked the time course of the O_1 to O_2 transition in $P2X_2$ [101], thus supporting the hypothesis that this part of the protein plays a critical role in regulating the ionic permeability of larger cations [7, 104]. Priming $P2X_2$ receptors with a long (20 s) application of ATP leads to the appearance of a permissive state that lasts for several minutes and from which the channels enter O_2 with increased speed [101] (Fig. 3). The minimum pore diameter of the O_2 state of $P2X_2$ receptor is unknown but is at least 3 Å larger than O_1 [34]. Virginio et al. [36] estimated the minimum pore size of the O_2 state of the $P2X_7$ from the diameter of the largest polyethylene glycol that blocks the $NMDG^+$ current and YO-PRO-1 uptake and found it is about 30–50 Å. They also used a range of different-sized molecules to discriminate a sudden from a gradual change in the size of the $P2X_7$ pore, reasoning that a binary channel capable of instantly switching between small and large pores would show identical rates of permeability changes for all permeable molecules. By contrast, a gradual change in pore diameter would allow small molecules to enter first. Virginio et al. found a direct correlation between the size of the ion and the time constant of the permeability change, with larger ions entering slower than small ions [36]. This implies that the $P2X_7$ pore undergoes a gradual dilation on its way from O_1 to the O_2 state. The idea that all P2X receptors enter O_2 from O_1 may not be true. For example, the human $P2X_5$ receptors demonstrate a high P_{NMDG}/P_{Na} at the start of the ATP application that remains steady throughout [42]. This suggests that either the transition from O_1 to O_2 is too

[3] Ding and Sachs labelled the two open states "O_1" and "O_2". We call them "O_A" and "O_B" in this review to distinguish them from the two conductance states that describe the normal ($NMDG^+$ impermeable, O_1 state) and dilated ($NMDG^+$ permeable, O_2 state) forms of the pore.

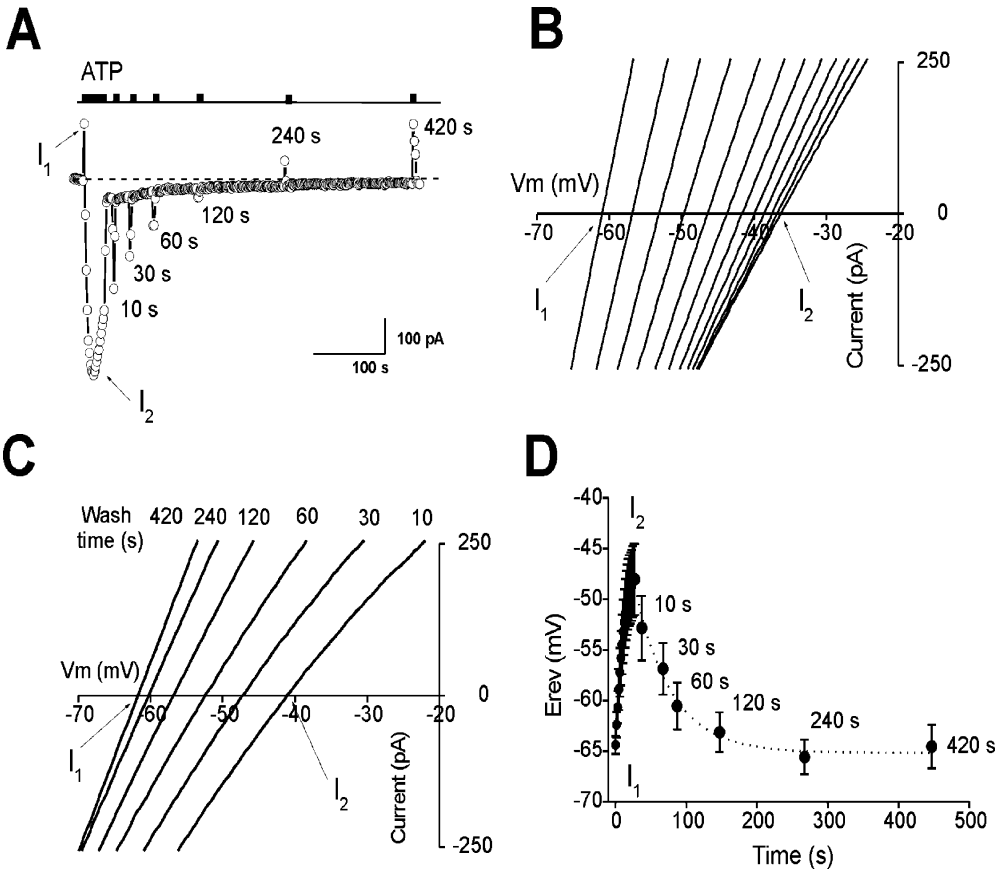


Fig. 3 Time-dependent changes in P_{NMDG}/P_{Na} in $P2X_2$ channels. **a** The trace is the steady-state current recorded during repeated applications of 100 μM ATP to an HEK293 cell expressing the P2X2 receptor. The only permeant cation in the extracellular solution is 154 $NMDG^+$, and the intracellular solution contains 154 Na^+. ATP was applied at the times indicated by the *solid bars* at the top of the graph. ATP was initially applied for 20 s, and this was followed by a series of shorter (<100 ms) applications. During the long application, ATP first causes an outward current carried by Na^+ leaving the cell as the channel opens to O_1. The current becomes inward as the channel enters O_2 and P_{NMDG}/P_{Na} increases, allowing $NMDG^+$ to enter the cell. The subsequent brief applications of ATP cause only inward currents suggesting that the cell has entered a permissive state that favors O_2. **b** The data are the voltage–current curves measured during the initial application of ATP. The value of the reversal potential of the *leftmost curve* indicates a low P_{NMDG}/P_{Na}. The curves then show a slow rightward shift as P_{NMDG}/P_{Na} increases. **c** Voltage–current curves evoked during short pulses of ATP. The time of the start of pulse in relation to the end of the 20-s application of ATP is indicated at the *top* of the graph. The curves show a slow shift back to the left, indicating that currents evoked soon after the long ATP application are more permeable to $NMDG^+$ than those applied later. **d** The time course of the shift in the reversal potential of the voltage–current curves shows the slow fade of the permissive state (reproduced from [101] with permission from the authors)

quick to measure or the channel bypasses O_1 and enters O_2 directly from the closed state.

A question of increasing importance is whether O_1 and O_2 represent different states of a common pore or two entirely different permeation pathways. The idea that the increase in P_{NMDG}/P_{Na} represents dilation of a single pore is generally well accepted. However, Surprenant and North have recently challenged the idea that $NMDG^+$ and YO-PRO^{2+} enter the cell through a common pathway [95]. They showed that the increase in P_{NMDG}/P_{Na} was greatly reduced in the presence of even a small amount (15 mM) of extracellular Na^+ whereas YO-PRO^{2+} uptake was not. Furthermore, deleting a cysteine-rich stretch of 18 amino acids just intracellular to TM2 prevented changes in P_{NMDG}/P_{Na} but had no direct effect on the uptake of YO-PRO^{2+}. The authors argue that the results are incompatible with the common pore hypothesis, an idea that is supported by the results of other laboratories (reviewed in [7, 95]). Other P2X receptors lack the cysteine-rich domain but still show a time-dependent increase in both $NMDG^+$ permeability and YO-PRO^{2+} uptake; this suggests that different P2X subtypes may use distinct pathways to mediate the transport of large cations.

Conclusions

The solution of the crystal structure of a P2X channel remains the single most important goal of future studies of P2X channel biophysics. Until then, site-directed mutagenesis provides the best clues to the relation of function to structure. A clear description of the biophysics may be difficult to obtain even with an atomic picture in hand because the unresolved channel openings make the kinetics

of gating difficult to measure. Nonetheless, these channels display a unique architecture and unusual properties, and they remain an interesting puzzle to solve.

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