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Allosteric Regulation of the P2X4 Receptor Channel Pore Dilation

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Abstract

Allosteric modulators of ligand-gated receptor-channels induce conformational changes of the entire protein that alter potencies and efficacies for orthosteric ligands, expressed as the EC₅₀ and maximum current amplitude, respectively. Here we studied the influence of allostery on channel pore dilation, an issue not previously addressed. Experiments were done using the rat P2X4 receptor expressed in HEK-293T cells and gated by ATP in the presence and absence of ivermectin (IVM), an established positive allosteric regulator of this channel. In the absence of IVM, this channel activates and deactivates rapidly, does not show transition from open to dilated states, desensitizes completely with a moderate rate, and recovers only fractionally during washout. IVM treatment increases the efficacy of ATP to activate the channel and slows receptor desensitization during sustained ATP application and receptor deactivation after ATP washout. The rescue of the receptor from desensitization temporally coincides with pore dilation, and the

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dilated channel can be reactivated after washout of ATP. Experiments with vestibular and transmembrane domain receptor mutants further established that IVM has distinct effects on opening and dilation of the channel pore, the first accounting for increased peak current amplitude and the latter correlating with changes in the EC₅₀ and kinetics of receptor deactivation. The corresponding kinetic (Markov state) model indicates that the IVM-dependent transition from open to dilated state is coupled to receptor sensitization, which rescues the receptor from desensitization and subsequent internalization. Allosterically-induced sensitization of P2X4R thus provides sustained signaling during prolonged and repetitive ATP stimulation.

Keywords

Purinergic receptor channels; ATP; Ivermectin; Sensitization of receptors; Pore dilation; Markov state model

Introduction

The family of P2X receptors (P2XR) belongs to the superfamily of ligand-gated ion channels orthosterically activated by extracellular adenosine triphosphate (ATP) [17] and modulated by numerous allosteric ligands [9, 4]. These receptors are homo- or heterotrimeric proteins, with three intersubunit ATP binding sites located extracellularly, whereas allosteric binding sites are located within all segments of P2XR protein molecule. The occupancy of orthosteric binding sites of P2XR channels causes the rapid opening of a small cation-permeable pore [22]. Some P2XRs also display a time-dependent modulation of ion selectivity by switching to a new open state that permits permeability to larger cations [27]. The latter phenomenon is called pore dilation, with the dilated pore representing a second open state with higher ion conductance [16]. Among P2XRs, pore dilation was first identified in P2X7R-expressing cells [32,34], and subsequently in P2X2R [35], P2X2/X5R [5] and P2X4R [15].

The P2X4 subunit is the most widely distributed in the brain with expression frequently overlapping with P2X2 or P2X6 subunits [21]. Homomeric P2X4Rs desensitize with moderate rates [31], and desensitization is coupled to extensive internalization [1] and recycling of receptors to the plasma membrane [25]. P2X4R is allosterically modulated by ivermectin (IVM), which increases both the ATP potency and the peak amplitude of the current in response to supramaximal ATP concentrations ($I_{\max}$), reduces the desensitization rate, greatly prolongs deactivation of current after ATP removal, and alters the recycling process [18,24,11,33]. The most probable location of the IVM binding site is at the interface of the transmembrane 1 and 2 domains [30,10,12], leading to increased probability of channel opening [24].

Many aspects of P2X4R gating have not yet been clarified and there is no comprehensive mathematical model describing this process, in contrast to P2X2R and P2X7R [13,14]. Pore dilation of P2X4R was previously seen when the receptor was expressed in *Xenopus* oocytes and bathed in Ca²⁺-deficient medium [15,33], but when bathed in physiological buffer, P2X4R did not dilate [14]. The relevance of allosteric regulation for pore dilation has not yet been studied for P2XRs, or for ligand-gated receptor channels in general. No rationale has

been provided for how IVM rescues receptors from desensitization, why it slows receptor deactivation, or why it affects receptor recycling [31]. Using electrophysiological and mathematical approaches, we show here that IVM binding to P2X4R and subsequent ATP application causes a transition from open to dilated states, which rescues the receptor from desensitization and the subsequent internalization by placing it in the sensitized and slow deactivating state.

Material and Methods

Cell culture and transfection

Experiments were performed on human embryonic kidney 293T cells (hereafter HEK-293T cells; American Type Culture Collection, Rockville, MD, USA) grown in Dulbecco modified Eagle's medium supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 50 µg/ml streptomycin in a humidified 5% CO₂ atmosphere at 37 °C. These cells express endogenously several subtypes of metabotropic P2Y receptors [29,8,6], but not P2X4R [38]. Cells were cultured in 75 cm² plastic culture flasks (NUNC, Rochester, NY) for 36–72 h, until they reached 80–95% confluence. Before the day of transfection, ~150,000 cells were plated on 35 mm culture dishes (Sarstedt, Newton, NC) and incubated at 37 °C for at least 24 h. For each culture dish of HEK-293T cells, transfection of either wild type or mutant receptors was conducted using 2 µg of DNA with 2 µl of jetPRIME™ reagent in 2 ml of Dulbecco modified Eagle's medium, according to the manufacturer's instructions (PolyPlus-transfection, Illkirch, France). After 24–48 h of incubation, the transfected cells were mechanically dispersed and re-cultured on 35 mm dishes of Corning 3294 CellBIND Surface for 2 – 8 hours before recording. Transfected cells were identified by the fluorescence signal of GFP using an inverted research microscope with fluorescence illuminators (Model IX71; Olympus, Melville, NY).

DNA constructs

The rat P2X4-pIRES2-EGFP construct [7] was used for generation of alanine mutants. Oligonucleotides (synthesized by VBC-Genomics, Vienna, Austria and Sigma Chemical Company, USA) containing specific point mutations were introduced into the rP2X4-pIRES2-EGFP template using Pfu Ultra DNA polymerase (Fermentas International Inc, USA). A High-Speed Plasmid Mini Kit (Geneaid, Shijr City, Taipei County, Taiwan) was used to isolate plasmids for transfection. Dye terminator cycle sequencing (ABI PRISM 3100, Applied Biosystems, Foster City, CA) was used to identify and verify the presence of the mutations.

Patch clamp recordings

All electrophysiological experiments were performed with rat P2X4R wild type and mutant receptors expressed in HEK-293T cells 24 h after transfection. Whole-cell patch-clamp recordings of ATP-induced currents were done using an Axopatch 200B patch-clamp amplifier (Axon Instruments, Union City, CA). Patch electrodes, fabricated from borosilicate glass (type GB150F-8P; Science Products GmbH, Hofheim, Germany) using a Flaming Brown horizontal puller (P-97; Sutter Instruments, Novato, CA), were heat-polished to a final tip resistance of 3.5 to 5 MOhm. All current traces were programmed,

captured, and stored using the pClamp 9.0 software package in conjunction with the Digidata 1322A A/D converter (Axon Instruments). Experiments were done on single cells with an average capacitance of about 10 pF. Unless otherwise stated, membrane potential was held at -60 mV, and patch electrodes were filled with a solution containing: 154 mM CsCl, 11 mM EGTA and 10 mM HEPES, adjusted to pH 7.2 with 1.6 M CsOH. The osmolality of the intracellular solution was 293 mOsm. During the experiments, the cell culture was perfused with an extracellular solution containing: 142 mM NaCl, 3 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 10 mM HEPES and 10 mM D-glucose, adjusted to pH 7.3 with 10 M NaOH. The control, ATP-containing and IVM-containing solutions were applied via a rapid superfusion system (RSC-200, BIOLOGIC, Claix, France). To estimate the half maximal effective concentration (EC_{50}) value, ATP was applied for 2–10 s at different concentrations. One or two responses were recorded from each cell and the responses from different cells were pooled. Current-voltage relations were obtained by voltage ramps from -80 mV to $+80$ mV twice per second and used to estimate changes in reversal potential during 10–30 s of agonist application [37]. Under a ramp protocol, patch electrodes were filled with solution containing: 145 mM NaCl, 10 mM EGTA and 10 mM HEPES; the pH was adjusted with 10 M NaOH to 7.2, and cells were bathed in extracellular solution containing: 155 mM *N*-Methyl-D-glucamine (NMDG^+), 3 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 10 mM HEPES and 10 mM D-glucose, adjusted to pH 7.3 with HCl.

Mathematical model

We developed a Markov state model defined by the scheme in Fig. 1. The 12 states are organized into three rows, representing receptors that are naïve (not previously exposed to ATP), sensitized, or desensitized. For each state the number of ATP binding sites occupied is identified by black circles. Two states are naïve/closed (C_1 , C_2), two are naïve/open (Q_1 , Q_2) with conductance g_{12} , two are sensitized/closed (C_3 , C_4), two are sensitized/open (Q_3 , Q_4) with conductance g_{34} ($>g_{12}$), and four states are desensitized/closed (D_1 , D_2 , D_3 , D_4). The state N represents the fraction of receptors that are internalized into the cytosol; we assume that they have to first desensitize before being internalized to account for the slow recovery after ATP washout. The receptor sensitization is IVM-dependent as illustrated below. Forward and backward rates between states within the same row are given by k_2 , k_4 , k_6 and k_1 , k_3 , k_5 , respectively, and the transition rates between naïve (middle row) and sensitized (bottom row) states and between naïve and desensitized (top row) states are L_1 , L_2 , L_3 and H_1 , H_2 , respectively. The rate of internalization and recycling are given by H_3 and H_4 , respectively. Values for these constants are shown in Table 1.

This scheme inherits the assumption from the P2X7R and P2X2R models that naïve states require at least two occupied binding sites for the channel to open. The binding of ATP to naïve and desensitized P2X4R is likewise further assumed to exhibit negative cooperativity (i.e., the binding of one ATP to one binding site reduces the binding affinity of the remaining unoccupied sites). This implies that the ratios that describe the binding affinity of these sites satisfy $k_2/k_1 > k_4/k_3 > k_6/k_5$. This feature is lost when the receptors sensitize due to IVM allostery.

The differential equations associated with this Markov model are given by

$$\frac{dD_1}{dt} = k_1 D_2 - (3k_2 A + H_1) D_1 \quad (1)$$

$$\frac{dD_2}{dt} = 3k_2 A D_1 + 2k_3 D_3 + H_2 C_2 - (k_1 + 2k_4 A) D_2 \quad (2)$$

$$\frac{dD_3}{dt} = 2k_4 A D_2 + 3k_5 D_4 + H_2 Q_1 - (2k_3 + k_6 A) D_3 \quad (3)$$

$$\frac{dD_4}{dt} = k_6 A D_3 + H_2 Q_2 - (3k_5 + H_3) D_4 \quad (4)$$

$$\frac{dC_1}{dt} = H_1 D_1 + k_1 G C_2 + L_1 C_4 + H_4 N - 3k_2 A F C_1 \quad (5)$$

$$\frac{dC_2}{dt} = 3k_2 A F C_1 + 2k_3 G Q_1 - (k_1 G + 2k_4 A F + H_2) C_2 \quad (6)$$

$$\frac{dQ_1}{dt} = 2k_4 A F C_2 + 3k_5 G Q_2 - (2k_3 G + k_6 A F + H_2) Q_1 \quad (7)$$

$$\frac{dQ_2}{dt} = k_6 A F Q_1 + L_2 Q_3 - (3k_5 G + L_3 (1 - G) + H_2) Q_2 \quad (8)$$

$$\frac{dC_4}{dt} = k_1 G C_3 - (L_1 + 3k_2 A F) C_4 \quad (9)$$

$$\frac{dC_3}{dt} = 3k_2 A F C_4 + 2k_1 G Q_4 - (k_1 G + 2k_2 A F) C_3 \quad (10)$$

$$\frac{dQ_4}{dt} = 2k_2 A F C_3 + 3k_1 G Q_3 - (2k_1 G + k_2 A F) Q_4 \quad (11)$$

$$\frac{dQ_3}{dt} = k_2 A F Q_4 + L_3 (1 - G) Q_2 - (3k_1 G + L_2) Q_3 \quad (12)$$

$$\frac{dN}{dt} = H_3 D_4 - H_4 N \quad (13)$$

where A is the applied ATP-agonist concentration (represented as a square wave) and

$$\begin{aligned} F &= 1 + \alpha K ([IVM]) ; \\ G &= 1 - K ([IVM]) ; \\ K ([IVM]) &= \frac{[IVM]}{\beta + [IVM]} \end{aligned} \quad (14)$$

are functions describing the allosteric effect of IVM on receptor kinetics (with [IVM] denoting the bath IVM concentration). To account for the data in Fig. 2, we assume that IVM increases the forward (agonist-association) rates of naïve and sensitized receptors by a factor F , decreases their backward (agonist dissociation) rates by a factor G , and sensitizes the receptors (causing channel dilation) by a factor $L_3(1-G)$, where F and G both depend on an increasing Hill function K shown in Fig. S1. The value of $\beta = 1.4 \mu\text{M}$ is taken from the measured EC_{50} after 10 s exposure to IVM (Fig. 2e).

The whole-cell current is given by

$$I = g_{12} (Q_1 + Q_2) (V - E_{12}) + g_{34} (Q_3 + Q_4) (V - E_{34}) \quad (15)$$

where V is the holding potential and E_{12} , E_{34} are the reversal potentials of the open ($Q_1 + Q_2$) and dilated ($Q_3 + Q_4$) states, respectively.

Calculations

The concentration-response data points were fitted with the equation $y = I_{\text{max}}/[1 + (\text{EC}_{50}/x)^h]$, where y is the amplitude of the current evoked by ATP, I_{max} is the maximum current amplitude induced by $100 \mu\text{M}$ ATP, EC_{50} is the agonist concentration producing 50% of the maximal response, h is the Hill coefficient, and x is the concentration of ATP (SigmaPlot 2000 v9.01; SPSS Inc., Chicago, IL). The kinetics of desensitization or deactivation (current decay in the continuous presence of agonist or current decay evoked by washout of agonist, respectively) were fitted by a single exponential function ($y = A_1 \exp(-t/\tau)$) or by the sum of two exponentials ($y = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2)$) using the program Clampfit 10 (Axon Instruments), where A_1 and A_2 are the relative amplitudes of the first and second exponentials, and τ_1 and τ_2 are the time constants. The derived time constants for deactivation and desensitization were labeled as τ_{off} and τ_{des} , respectively. Data points are presented as mean $\pm$ SEM. Significant differences (** $p < 0.01$ and * $p < 0.05$) between means were determined using the Mann-Whitney Rank Sum Test. Differential equations for the mathematical model were solved using xppaut software (<http://www.math.pitt.edu/~bard/xpp/xpp.html>) and MATLAB (Mathworks).

Results

IVM affects P2X4R gating

In the absence of IVM, repetitive $1 \mu\text{M}$ ATP application twice per min for 2 s activated receptors, but the peak amplitude of currents progressively decreased with time, reaching $15.1 \pm 2.3\%$ of the initial value after 13 ATP pulses (Fig. 2a, top panel). IVM treatment increased the peak amplitude of current in a concentration-dependent manner. Furthermore, in contrast to controls, there was no decrease in the current amplitude during repetitive agonist application in the presence of IVM (Fig. 2a). In addition, IVM treatment decreased

the rates of decay of currents after washout of ATP, termed receptor deactivation, in a concentration-dependent manner (Fig. 2a and b). The washout of IVM led to a gradual recovery of deactivation rates and decay in the amplitude of currents. Two minutes (five ATP pulses) after washout of 3, 6, and 10 μM IVM, the peak current amplitude was still larger than the peak amplitude prior to IVM application (Fig. 2a).

To evaluate the concentration-dependent effect of IVM on the current amplitude and receptor deactivation, with the latter expressed as a time constant from single exponential fitting (τ_{off}), we used responses with the largest effect on current amplitude and τ_{off} during 3 min of IVM application, and these data are summarized in Fig. 2c and d. These two responses to IVM treatments differed. At 0.3 μM , IVM increased the current amplitude without affecting the rate of receptor deactivation, at 1 μM IVM both responses were affected, and at higher IVM concentrations the peak amplitudes of currents were not further enlarged, but the rates of receptor deactivation continued to decrease.

IVM sensitizes P2X4R

In further experiments, we examined how IVM treatment affects the dependence of peak current amplitude on ATP concentration. All experiments were done with naïve receptors (not previously exposed to ATP), and the data shown in Fig. 2e are mean $\pm$ SEM values from different cells stimulated with variable ATP concentrations for 2 s. In the absence of IVM, ATP was effective at concentrations ranging from 0.1 μM to 100 μM , with an EC_{50} of $2.3 \pm 0.4 \mu\text{M}$ and a maximum current response of $2.3 \pm 0.2 \text{ nA}$. In cells treated with 3 μM IVM for 10 s, the maximum peak amplitude of the currents increased to $3.4 \pm 0.3 \text{ nA}$, and the EC_{50} value was shifted leftward to $1.6 \pm 0.3 \mu\text{M}$. Longer exposure of P2X4R to 3 μM IVM (for 0.5 – 4 min) did not change the maximum current amplitudes but caused a further leftward shift in the EC_{50} to $0.5 \pm 0.1 \mu\text{M}$ value (Fig. 2e). These results indicate that the dual action of IVM on P2XR gating is time dependent, first increasing the peak amplitude of agonist-induced current independently of the number of channels activated, as observed in experiments at supramaximal (100 μM) agonist concentration, and then sensitizing receptors to ATP (or increasing the potency of ATP).

Model simulations of increased potency of ATP

Figure 3a shows a naïve model-cell stimulated with 1 μM ATP for 2 s twice per min for 7 min and IVM applied at 80 s and removed at 260 s (compare to Fig. 2). Increasing IVM concentration in the bath led to an increase in the current amplitude induced by ATP application. We also observed that deactivation became slower as IVM concentration increased. The reason for this behavior is illustrated in panel b. The left traces show the time series of the open states Q_1+Q_2 , whereas the right traces show the time series of the IVM-induced dilated states Q_3+Q_4 . In the absence of IVM, receptors cannot reach the sensitized row of Fig. 1. At increasing IVM concentrations, we see a gradual increase in the number of receptors that reached the open Q_1+Q_2 and dilated states Q_3+Q_4 . They eventually reach a steady state. Panel c shows the fraction of receptors that are internalized, reaching up to 50%. Because IVM increases the forward rates, we initially see an increase in the number of internalized receptors when IVM concentration is increased, but the fraction of internalized

receptors eventually plateaus because IVM-induced sensitization (shown in panel b) protects the receptors from internalization.

Figure S2 summarizes the results shown in the previous figure. In panel a, we plot τ_{off} (black) and I_{max} (gray) as a function of IVM concentration (compare to Fig. 2c and d). Both quantities increased with IVM concentration, following Hill functions with Hill coefficient $n = 1$ and $\text{EC}_{50} \approx 9.2 \mu\text{M}$ for τ_{off} and $\text{EC}_{50} \approx 0.35 \mu\text{M}$ for I_{max} . These plots were generated from Fig. 3a using the last current peak before IVM removal. Panel b shows the I_{max} produced by the model in the absence (black) and presence (gray) of $3 \mu\text{M}$ IVM in the medium when the model cell was stimulated with ATP for 30 s (from $t = 10$ s to $t = 40$ s). In both cases, the Hill-like concentration response curves were generated with Hill coefficient $n = 1$. In the absence of IVM, $\text{EC}_{50} \approx 1.4 \mu\text{M}$, whereas in the presence of IVM, $\text{EC}_{50} \approx 0.2 \mu\text{M}$. At low ATP concentration, there was a significant upward shift in the concentration response curve because of IVM, but at high ATP concentration, the response curves plateaued roughly at the same level. In these simulations IVM and ATP were applied simultaneously unlike in the experiments shown in Fig. 2e, where cells were pretreated with IVM. The model would not be able to capture the effect of 10 vs. 30 s pretreatment with IVM along with the gradual decrease in amplitude and slow recovery of the deactivation kinetics to pre-stimulation values after IVM removal, because this simplified model lacks states describing IVM binding.

IVM causes transition from open to dilated state

In further experiments, we examined the effects of IVM during prolonged ATP stimulation. Application of IVM for 7 s, when the decay of current had reached about 50%, increased the current rapidly, followed by a slow decay. After washout of IVM, the decay accelerated and became similar to the rate seen before IVM application (Fig. 4a). Experiments with prolonged application of supramaximal ATP concentrations showed that the receptor desensitized almost completely within 20 s and only partial recovery of the peak current response was observed when the ATP pulse was delivered after 4 min washout, reaching $24.3 \pm 0.5\%$ ($n = 6$) of that observed prior to ATP application (Fig. 4b, top panel). In the presence of $3 \mu\text{M}$ IVM, the initial rate of receptor desensitization was comparable to that in the absence of IVM, but desensitization was only partial and the plateau current amplitude was $43.0 \pm 4.9\%$ ($n = 8$) of the initial peak current amplitude (Fig. 4b, bottom panel). This indicates that a substantial fraction of channels escaped receptor desensitization. The non-desensitizing fraction of channels fully recovered after the 40 s ATP washout, indicating that open receptors do not internalize but return to the closed state. Thus, IVM treatment only partially rescues P2X4R from desensitization during initial agonist application but slows further transition from open to desensitized state when the allosteric action of this compound is present.

Furthermore, we applied a ramp protocol to cells bathed in Ca^{2+} -containing medium with NMDG^+ substituted for Na^+ , and using patch electrodes filled with sodium-based intracellular solution as stated in Methods and previously described for P2X7R [37], to examine whether IVM affects the permeability of the P2X4R pore to cations. These studies revealed that the reversal potential (E_{rev}) of the receptor does not change during sustained

ATP application in the absence of IVM (Fig. 4c, left), i.e. that the receptor pore does not dilate when bathed in Ca^{2+} -containing medium, a finding consistent with the literature [15]. However, in the presence of IVM, there was a rightward shift in E_{rev} , indicating that receptor dilates within the first 10 s of ATP application (Fig. 4c, right). On average, E_{rev} shifted by 10.8 ± 1.1 mV, from -38.2 ± 0.9 mV to -27.4 ± 1.5 mV ($n = 35$) in the presence of 3 μM IVM. At 0.3 μM IVM, the shift in E_{rev} was 5.6 ± 1.8 mV ($n = 3$) and at 10 μM it was 16.3 ± 0.8 ($n = 3$). In the absence of ATP, IVM did not affect E_{rev} during 60-s application (Fig. 4d, open circles). This observation agrees with previous findings that IVM potentiates ATP-induced responses but does not activate P2X4R channels in the absence of ATP [18]. The effect of IVM on ATP-induced changes in reversal potential was not instantaneous but required more than 10 s of IVM pre-application and was maximal after 20 s of pre-application (Fig. 4d, closed circles).

Model simulations of transition from open to dilated state

Figure 5 shows model simulations during prolonged 100 μM ATP applications (compare to Fig. 4). Figure 5a shows desensitization properties in the presence and absence of IVM and the top panel of Fig. 5b shows the effect of repetitive ATP application in the absence of IVM; ATP was applied twice for 50 s with a 4 min washout period in between. The receptor desensitized completely within ~ 10 s and the current amplitude in the second stimulation was significantly smaller than the first stimulation, indicating that a large portion of receptors had internalized during the first pulse. In the presence of 3 μM IVM (bottom panel), however, the application of 100 μM ATP twice for 30 s produced a much larger second pulse even though the washout period was only 30 s (compare to Fig. 4b, bottom panel). This was a consequence of the reduced level of desensitization during the first pulse caused by the IVM-induced sensitization of receptors, which protected them from internalization.

As in the experiments (Fig. 4c), application of voltage ramps from -80 to $+80$ mV twice per second during 10 s ATP stimulation had different effects depending on the absence or presence of 3 μM IVM. In the model cell, no shift in reversal potential was observed in the absence of IVM, but a shift from -26 mV to -13 mV was observed in the presence of IVM (Fig. 5c). The shift in reversal potential parallels the process of sensitization in the model, as the sensitized receptors are assumed to have a more positive reversal potential than the naïve receptors in the presence of NMDG^+ (see right column, Table 1).

Single residue mutations alter IVM effects on P2X4R gating

To determine whether changes in the effects of IVM on I_{max} and receptor sensitivity described above are accompanied by alterations in E_{rev} , we examined the effects of single residue mutagenesis in the Leu⁴⁰-Asp⁵⁸ and Lys³²⁶-Asn³³⁸-P2X4R sequences on receptor gating in the absence and presence of IVM. These sequences were selected because the IVM binding site is most likely located in transmembrane domains and nearby extracellular regions of P2X4R [30,10,11,23]. The V49A, Y54A, Q55A, F324A and G325A mutants generated low- or non-responsive receptors [28]. All the other mutants were functional, with the EC_{50} values for ATP ranging between 1.1 and 5.2 μM except Y42A and P334A, which exhibited lower EC_{50} values [10,11,28]. The A41L [11], A41C, I44C [10], K52A, A327C,

G328A and K329A generated receptors that were comparable to wild type receptor as far as the deactivation effect of IVM is concerned (data not shown), and therefore their dilation properties were not studied. In total, we analyzed the gating of 21 single residue mutants using alanine as a substitute residue. Table 2 summarizes these investigations as fold changes in the EC_{50} and I_{max} values, difference in the E_{rev} values in the presence and absence of IVM (E_{rev}), and deactivation time constant (τ_{off}) values estimated in the presence of IVM.

Pore dilation was significantly reduced or lost in L40A, V43A, W46A, V47A, W50A, G53A, F330A, D331A, I332A, I333A, and I337A mutants. The deactivation effect of IVM was reduced in all these mutants except I333A. Two mutants, G45A and E51A, showed enhanced pore dilation as well as deactivation effect. On the other hand, in the F48A, E56A, T57A, D58A, K326A, T335A, M336A, and N338A mutants IVM-induced receptor dilation was comparable to that observed in the wild type receptor, but IVM-induced deactivation was modified in F48A, D58A, T335A, M336A and N338A. These results indicate that changes in E_{rev} values could be used to identify IVM hits in addition to changes in I_{max} , τ_{off} , and EC_{50} values used previously [10,11,30], and also show that changes in those three parameters can be dissociated.

Figure 6, top row illustrates the responses of the Y42A mutant, not listed in Table 2. Three lines of evidence indicate that substitution of Tyr with Ala at this position mimics the allosteric action of IVM: 1. In the absence of IVM, this mutant deactivated slowly with an average τ_{off} of 29 ± 4.4 s (left, gray trace) a phenomenon described previously [10,12], compared to τ_{off} of 21 ± 1 s for the wild type receptor in the presence of IVM (Table 2). The addition of IVM further delayed receptor deactivation (left, black trace). 2. In the absence of IVM, the E_{rev} value for this receptor was -25 ± 4.5 mV, and did not change with addition of IVM (right). In other words, the receptor opened immediately with a dilated pore (middle), and the addition of IVM did not further dilate the pore. 3. The ATP- EC_{50} value for this mutant in the absence of IVM was 0.6 ± 0.2 μ M, compared to 0.6 ± 0.1 μ M in the presence of IVM and 0.5 ± 0.2 μ M for the wild type receptor in the presence of IVM. On the other hand, the I_{max} value for this receptor was low (0.6 ± 0.3 nA) and did not increase in the presence of IVM. Thus, the Y42A mutation replicates the action of IVM with respect to dilation, affinity for ATP and deactivation but not amplitude. These results further suggest that dilation and sensitization, the latter expressed as increased potency of ATP combined with slow deactivation, are coupled but independent of current amplitude. This distinction was already suggested by Fig. 2.

Figure 6, middle and bottom rows, illustrates the responses to IVM of two mutants, E51A and I337A, which exhibited enhanced and reduced shift in E_{rev} , respectively. The first mutant deactivated slowly (left) and showed a rightward shift in E_{rev} of 20 ± 2.4 mV (right), accompanied by an increase in I_{max} (Table 2). Thus, this mutation enhances all the allosteric actions of IVM on receptor gating. In contrast, the I337A mutant deactivated rapidly (left), showed no shift in E_{rev} (middle and right), and exhibited almost no leftward shift in EC_{50} , whereas the effect of IVM on I_{max} was still present (Table 2). These mutants provide additional evidence for a distinction between sensitization and current amplitude.

To better understand the different components of receptor gating affected by IVM, we used correlation analysis of the data in Table 2, with four variables giving six possible pairs to correlate. In three cases, EC_{50} vs. τ_{off} , EC_{50} vs. E_{rev} , and E_{rev} vs. τ_{off} there was significant linear correlation (Fig. 7a–c). This further supports the conclusion that P2X4R dilation is coupled to sensitization and a decrease in the rate of receptor deactivation. In contrast, there was no significant correlation between I_{max} values on one side and EC_{50} , τ_{off} , and E_{rev} values on the other side (Fig. 7d–f). These data are in agreement with the findings described above, indicating that channel opening is distinct from channel dilation caused by IVM binding.

Discussion

At the present time, there is a general agreement in the field that the P2XR pore dilates during sustained occupancy of orthosteric binding sites. Recent studies have also suggested that pore dilation is just one of the gating parameters influencing the pattern of current response during sustained receptor activation; the profile of P2XR current is also influenced by the balance between the rate of receptor desensitization on the one hand and the rate of pore dilation and sensitization on the other hand [14]. The time course of the current response is also influenced by numerous allosteric regulators [4]. However, the influence of allosteric modulators on pore dilation has not been studied systematically, not only for P2XRs but for ligand-gated receptor channels in general.

The current generated by naïve rat P2X7R expressed in HEK-293 cells [37,26] and by human P2X7R expressed in *Xenopus* oocytes [19] features a biphasic response in whole-cell recordings; the initial rapid rise in current (I_1) is accompanied by a secondary slow current growth (I_2), the rate of which increases with agonist concentration [36]. The substitution of 90% or 100% of the extracellular Na^+ with NMDG⁺ did not alter the biphasic response pattern of P2X7R generated by the prolonged stimulation of this receptor. Under a ramp protocol, a positive shift was also found in the reversal potential, with the rate of reversal potential shift closely aligned to the rate of I_2 current growth. Experiments with a series of mutants further confirmed that secondary current growth reflects pore dilation [37]. Rat P2X7R in the dilated state allows the passage of molecules as large as 1.4 nm [3]. This complex pattern of gating can be accounted for by a Markov state model that includes negative cooperativity of agonist binding to unsensitized receptors when one or two binding sites are occupied, opening of the channel pore to a low conductance state when two sites are bound, and sensitization with pore dilation to a high conductance state when three sites are occupied [36].

In contrast to P2X7R, the full size receptor (P2X2aR) and the splice variant missing 69 C-terminal amino acids (P2X2bR) generate desensitizing currents during sustained agonist application; notably, P2X2bR desensitized more rapidly than rP2X2aR [2,20]. Yet, each receptor still exhibited shifts in reversal potential when bathed in NMDG⁺-containing media, indicative of pore dilation in both rP2X2Rs [35,14]. The mathematical model showed that it was possible for I_2 growth to be absent even though receptors dilate, because the high rate of Ca^{2+} -independent desensitization reduces the number of open receptors and overcomes the increase in the conductance of each open receptor [14]. However,

desensitization, though present, plays a much smaller role in P2X7R. This means that the same core-gating scheme is applicable to both P2X7R and P2X2R with quantitative differences in rates. In P2X7R, dilation masks desensitization, except at low ATP, whereas in P2X2R, desensitization masks dilation, as only I_1 current was observed but dilation could nonetheless be detected by examining the reversal potential.

When bathed in normal physiological buffer, P2X4R expressed in HEK-293 cells desensitizes with rates comparable to P2X2bR desensitization. In contrast to P2X2Rs, there was no shift in the reversal potential when cells were bathed in normal physiological solution, indicating the lack of a transition from open to dilated states [14]. However, when expressed in *Xenopus* oocytes and bathed in low Ca^{2+} medium, activated P2X4R exhibited a biphasic current response, with I_2 developing slowly over several minutes. The biphasic current was also observed when using NMDG⁺-containing medium. The I_2 growth occurred simultaneously with a positive shift in reversal potential and an uptake of YO-PRO-1 [15,33].

Here we have shown that rat P2X4R also dilates when expressed in HEK-293T cells and bathed in normal physiological solution provided IVM is present, but secondary current growth is not generated, as in P2X2R. The binding of IVM also reduces the rates of receptor desensitization and deactivation. The time-dependence of pore dilation and the leftward shift in EC_{50} values further suggest that dilated channels are sensitized channels. The time courses of pore dilation and biphasic current responses in the continuous presence of ATP suggest that pore dilation contributes to formation of a plateau phase rather than an I_2 current. Thus, dilation is sufficient to limit the extent of desensitization and internalization but not to overwhelm it.

We postulated that rat P2X4R bathed in physiological medium in the absence of IVM undergoes closed $\rightarrow$ open $\rightarrow$ desensitized $\rightarrow$ internalized transitions, and the rate of return from the desensitized state to the closed state is slow. When the allosteric binding sites for IVM are occupied, however, channels go through the closed $\rightarrow$ open transition, open channels desensitize or dilate with comparable probabilities, and desensitized channels go through the cycle described above. Dilation is coupled to sensitization of receptors, sensitization is preserved during ATP washout if IVM is present, and sensitized channels are capable of repetitive transitions from closed $\rightarrow$ open $\rightarrow$ dilated $\rightarrow$ closed states without large loss of receptors through internalization. Correlation analysis also indicated that P2X4R dilation is coupled to sensitization and decreased rate of receptor deactivation, whereas the lack of correlation between I_{max} values and EC_{50} , τ_{off} , or E_{rev} values suggests that the IVM effect on channel opening is distinct from its effect on channel dilation.

To put the allosteric effects of IVM on P2X4R gating into a broader context and relate the properties of P2X4R to other members of the P2X family, we developed a Markov state model defined by the scheme in Fig. 1. This scheme resembles the scheme used recently to describe in more detail P2X7R gating [13]. In that study, we reported that P2X7R also desensitizes when stimulated with low agonist concentration. To account for this desensitization, an earlier model [36] of P2X7R kinetics was modified to incorporate transitions from the open state to either the closed/desensitized state or to an additional open

(dilated) state, which is also the sensitized/facilitated state. For P2X7R, the transition to this open sensitized state is kinetically favored over the transition to the desensitized state, leading to the generation of a biphasic current response during the initial agonist application [13].

We hypothesized that such a model could account for P2X4R gating as well by making just two qualitative changes. First, an additional state was included, termed internalized, to describe the transitions from naïve-open to desensitized to internalized states. The internalization of P2X4R is profound, reducing the number of operative channels to about 50% after a single prolonged ATP stimulation, and thus needs to be taken into consideration. Second, in the presence of IVM, receptors enter the sensitized/dilated and desensitized states with comparable probabilities. Those changes were sufficient to mimic most of the responses observed experimentally under different paradigms. Thus, rapid internalization of P2X4R limits signaling by these receptors. The experiments with IVM demonstrate that such gating could be altered pharmacologically by transferring channels to the sensitized state. This would amplify the response by allowing a significant number of channels to escape desensitization and be reused during subsequent stimulations. Consistent with this, it was shown that such effect of IVM was lost in internalization-deficient P2X4R mutants [33].

Conformational changes in the transmembrane domain presumably underlie the transition from open to dilated state because IVM binds to P2X4R at the plasma membrane from the extracellular side [11,10,30]. However, as IVM cannot activate P2X4R in the absence of ATP [18], we propose that it indirectly confers on ATP ability to gate the transition from open to dilated state rather than cause this transition directly. This is consistent with the demonstrated ability of ATP alone to sensitize P2X7R and P2X2R.

The model presented here was deliberately kept simple at the expense of perfect fidelity. A more complete model would include more states representing the binding and unbinding of IVM. Such a model should be more successful in capturing one important feature that the current model cannot, namely, the priming of P2X4R by pretreating cells with IVM and removing it before ATP stimulation. The current model is not able to capture that effect because the removal of IVM makes the rate of sensitization instantaneously zero. Future extensions of the model could address this and other features. For the present study we have focused on a simple scheme with a core that is shared with two other channels in the same family with very different kinetics of activation and desensitization, P2X2R and P2X7R. The success of the simplest version of the model in reproducing a wide range of experiments should encourage further theoretical work toward a universal Markov model that could account for gating of all homotrimeric and heterotrimeric mammalian P2XRs, as expected for a closely related family of proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Fig. 1.

Mathematical model of P2X4R gating. The 13-state Markov model describing the binding and unbinding of ATP to P2X4R. The top row represents the desensitized states, middle row represents the naïve states and bottom row represents the IVM-dependent sensitized states. The states C_i and D_i are closed ($i=1, 2, 3, 4$), Q_1, Q_2 are open, and Q_3, Q_4 are dilated. The state N represents the receptors that are internalized. The model allows for receptor recycling via the very small transition rate H_4 , an assumption consistent with the observed experimental results. Circles represent the unoccupied (white) and occupied (black) ATP binding sites. The functions F and G increase the forward rates (k_2, k_4, k_6) and decrease the backward rates (k_1, k_3, k_6).

Fig. 2.

Concentration-dependent effect of IVM on ATP-induced P2X4R current. **a**, Repetitive stimulation of P2X4R with ATP in the absence and presence of IVM, an allosteric regulator of these receptors. ATP (1 μ M) was applied twice per min for 2 s. The gray area indicates duration of IVM treatment and numbers below traces indicate concentration of IVM. **b–d**, The IVM-dependent dose-response changes in peak current amplitude and rate of receptor deactivation, the later expressed as τ_{off} derived from monoexponential fitting of decay of currents after washout of ATP. Representative traces (**b**) and mean $\pm$ SEM values ($n = 3 - 7$ measurements per dose) (**c** and **d**). Asterisks indicate significant difference, $p < 0.05$. **e**, Concentration-dependent effect of ATP on the peak amplitude of P2X4R current in the absence (open circles) and presence (closed symbols) of 3 μ M IVM. The cells were pretreated with IVM for 10 s (closed squares) or for 30 s - 4 min (closed circles). Data shown are mean $\pm$ SEM values from $n = 4 - 20$ cells per dose. For clarity, only single-direction SEM values are shown. The calculated EC_{50} values are indicated above traces. The Hill coefficient of the fitted curves to the open circles was 1.4, to the closed squares was 1.6, and to the closed circles was 1.0.

Fig. 3.

Model simulation of concentration-dependent effects of IVM on P2X4R activity. **a**, A naïve model-cell was stimulated with ATP for 2 s twice per min for 7 min. IVM was applied at 80 s and removed at 260 s. Compare with Fig. 2a. **b**, The time series of the open states Q_1+Q_2 (left) and dilated states Q_3+Q_4 (right). **c**, The predicted time course of receptor internalization.

Fig. 4.

Relation between the desensitization rate of ATP-induced current response and the permeability of P2X4R. **a**, Modulation of ATP-induced current by transient application of 3 μM IVM, indicated by gray area. **b**, Decline of response during repetitive ATP application in the absence (top) and presence (bottom) of 3 μM IVM. **c**, Positive shift in the reversal potential observed during the initial 10-s of 100 μM ATP application in cells bathed in medium with Na^+ replaced by NMDG $^+$ in the absence (left) and presence (right) of 3 μM IVM. In this and following figures, 0.485 s voltage ramps from -80 mV to $+80$ mV were delivered twice per second from a holding potential of -60 mV, and cells were treated with IVM for 30 – 60 s. Traces shown are representative of 30 similar experiments. **d**, Time course of IVM effect on reversal potential in the absence and presence of 100 μM ATP. Data shown are mean $\pm$ SEM values and the number of replicates is indicated below or above data points.

Fig. 5.

Model simulation of effects of IVM on the rate of receptor desensitization and the reversal potential. **a**, Effect of 80-s stimulation with ATP in the absence (black) and presence (gray) of 3 μ M IVM. **b**, Effect of repetitive ATP application on the pattern of current response in the absence (top) and presence (bottom) of IVM. **c**, Effect of IVM on I-V curves generated by the voltage ramp protocol. Voltage ramps from -80 to $+80$ mV were applied twice per second for 25 s during a 30-s ATP application. Compare with Fig. 4.

Fig. 6.

The relationship between pore dilation and receptor deactivation in mutant channels. **Top row:** The P2X₄-Y42A mutant deactivation after washout of 1 μ M ATP (2 s application) was similar in IVM-treated (left, black trace) and –non-treated cells (gray trace). The reversal potential was positively shifted from -32 ± 0.5 mV (the wild type receptor) to -25 ± 4.5 mV (Y42A mutant) in 100 μ M ATP-stimulated cells in the absence of IVM (middle), and the addition of 3 μ M IVM did not cause further shifts in the reversal potential (right). For this mutant, the ATP EC₅₀ values in the presence and absence of IVM are indicated below traces. **Middle row:** The P2X₄-E51A mutation enhanced pore dilation, as indicated by slowed receptor deactivation after washout of ATP (left) and more positive shifts in reversal potential in the presence of IVM (middle and right). **Bottom row:** The P2X₄-I337A mutation, in contrast, accelerated receptor deactivation (left) and abolished ATP-induced pore dilation in the presence of IVM (middle and right). Traces shown are representative of 3–14 similar experiments.

Fig. 7. Correlation analysis of gating parameters for the wild type (filled circle) and P2X4R mutant receptors from Leu⁴⁰-Asp⁵⁸ and Lys³²⁶-Asn³³⁸-P2X4R sequences (open circles). **a–c**, Statistically significant linear correlations were found between the EC_{50} and τ_{off} values (**a**), EC_{50} and E_{rev} values (**b**) and E_{rev} and τ_{off} values (**c**). **d–e**, No correlation was found between I_{max} and EC_{50} (**d**) E_{rev} (**e**) and τ_{off} (**f**) values. Circles represent mean values shown in Table 1. R, coefficient of correlation.

Table 1

Parameter values and distributions used in the simulations of the model. Parameters with no values under NMDG⁺ column are identical to those listed under physiological solution. The NMDG⁺ column is used for the voltage ramps only. In the NMDG⁺-containing solution, bath Na⁺ was substituted with NMDG⁺.

Symbol	Values (physiological solution)	Values (NMDG ⁺ -containing solution)
k ₁	0.4 s ⁻¹	
k ₂	600000 (M.s) ⁻¹	
k ₃	0.5 s ⁻¹	
k ₄	500000 (M.s) ⁻¹	
k ₅	0.6 s ⁻¹	
k ₆	400000 (M.s) ⁻¹	
L ₁	0.01 s ⁻¹	
L ₂	0.04 s ⁻¹	
L ₃	0.5 s ⁻¹	
H ₁	0.013 s ⁻¹	
H ₂	0.35 s ⁻¹	
H ₃	1.4 s ⁻¹	
H ₄	0.001 s ⁻¹	
α	100 (unitless)	
β	1.4 μM	
g ₁₂	3×10 ⁻⁸ S	
g ₃₄	8×10 ⁻⁸ S	
V	-0.06 V	
E ₁₂	0 V	-0.035 V
E ₃₄	0 V	-0.012 V

Table 2

Influence of single residue mutations of P2X4R on IVM effects on channel gating. Each receptor was examined in the presence and absence of 3 μM IVM. The EC_{50} values were derived from dose-response curves in the 0.1 to 100 μM ATP concentration range. Pore dilation was measured as a shift in the reversal potential in ramp protocol experiments with cells bathed in Ca^{2+} -containing extracellular solution, in which Na^{+} was substituted with NMDG^{+} . Deactivation kinetics were measured as a decay in the current amplitude after washout of 1 – 3 μM ATP in the continuous presence of IVM and expressed as τ_{off} . In cells expressing the wild type (WT) receptor, IVM causes a leftward shift in the EC_{50} value (expressed as fold change), a rightward shift in the reversal potential (expressed as a difference in E_{rev} values at the beginning and the end of ramp protocol), and an increase of I_{max} (expressed as fold change).

Receptor	EC_{50} (Fold Change)	E_{rev} (mV)	I_{max} (Fold change)	τ_{off} (s)
WT	$-4.33 \pm .11$	10.8 ± 1.1	$1.48 \pm .13$	21 ± 1
L40A	$-2.45 \pm .03$	$4.5 \pm 1.7^{*}$	$1.84 \pm .15$	$3.8 \pm 0.7^{**}$
V43A	$-1.57 \pm .05$	$5 \pm 1^{*}$	$1.12 \pm .20$	$5.2 \pm 0.6^{**}$
G45A	$-8.00 \pm .06$	$16 \pm 5.4^{*}$	$1.37 \pm .16$	$71 \pm 15^{**}$
W46A	$-1.22 \pm .18$	$1 \pm 1.8^{**}$	$2.47 \pm .64$	$2.3 \pm 0.4^{**}$
V47A	$-1.90 \pm .13$	$3.8 \pm 2.5^{*}$	$1.85 \pm .38$	$1.9 \pm 0.4^{**}$
F48A	$-3.50 \pm .10$	9 ± 0.9	$1.56 \pm .09$	$32 \pm 5.4^{**}$
W50A	$-2.20 \pm .22$	$4.9 \pm 1.6^{*}$	$1.59 \pm .23$	$5.4 \pm 0.4^{**}$
E51A	$-5.75 \pm .13$	$20 \pm 2.4^{**}$	$3.66 \pm .33$	$52 \pm 7.4^{**}$
G53A	$-1.90 \pm .09$	$3.5 \pm 2^{*}$	$1.10 \pm .07$	$5.2 \pm 1^{**}$
E56A	$-5.57 \pm .05$	5.9 ± 2.1	$2.31 \pm .37$	21 ± 1.7
T57A	$-4.90 \pm .10$	11.7 ± 3.4	$2.35 \pm .32$	20 ± 1.5
D58A	$-4.69 \pm .09$	6.7 ± 2.4	$4.83 \pm .83$	$7.5 \pm 0.8^{**}$
K326A	$-4.24 \pm .13$	5.9 ± 1.8	$1.89 \pm .37$	$28 \pm 2.7^{*}$
F330A	$-4.00 \pm .15$	$4.8 \pm 1.5^{*}$	$1.40 \pm .26$	$14.3 \pm 2.2^{**}$
D331A	$-2.66 \pm .22$	$3.2 \pm 0.9^{*}$	$1.72 \pm .33$	$15 \pm 1.2^{**}$
I332A	$-3.20 \pm .15$	$0.3 \pm 1.9^{**}$	$2.20 \pm .40$	$47 \pm 5.6^{**}$
I333A	$-2.50 \pm .10$	$0.3 \pm 1.5^{**}$	$1.69 \pm .31$	23 ± 10
T335A	$-5.00 \pm .04$	13 ± 2.2	$1.68 \pm .27$	$37 \pm 5.1^{**}$
M336A	$-8.30 \pm .04$	13.8 ± 1.7	$1.93 \pm .63$	$52 \pm 7.8^{**}$
I337A	$-1.45 \pm .31$	$1.3 \pm 2.1^{**}$	$1.90 \pm .45$	$2.8 \pm 0.6^{**}$
N338A	$-1.33 \pm .10$	7.3 ± 1.7	$1.11 \pm .11$	$2.9 \pm 0.9^{**}$

Statistical significance was calculated using Mann-Whitney Rank Sum Test.

* $p < 0.05$ between WT and mutants,

** $p < 0.01$ between WT and mutants.