Functional and structural identification of amino acid residues of the P2X\(_2\) receptor channel critical for the voltage- and [ATP]-dependent gating

Batu Keceli and Yoshihiro Kubo

Division of Biophysics and Neurobiology, Department of Molecular Physiology, National Institute for Physiological Sciences, Okazaki, Aichi, Japan

The extracellular ATP-gated cation channel P2X\(_2\) is known to show voltage-dependent gating in spite of the absence of a canonical voltage sensor domain. We previously observed that the hyperpolarization-evoked activation of P2X\(_2\) at the steady state in the presence of ATP varied depending on [ATP]. With increasing [ATP], the conductance–voltage (G–V) relationship shifted to more depolarized potentials and the activation kinetics were accelerated. Using a three-state model consisting of an ATP binding step and a rate limiting gating step, we successfully reproduced the voltage-dependent gating including its [ATP] dependence. In this study, in order to identify the structural basis of voltage and ATP dependence, we analysed various mutants in the Xenopus oocyte expression system under two-electrode voltage clamp. In the ATP binding region mutant K308R, the G–V relationship was shifted towards more hyperpolarized potentials, there was no clear [ATP] dependence, and activation was faster than in wild-type (WT). These results could be simulated by assuming an increase in the off rate of the gating step, in addition to changes in the ATP binding step. With F44C mutation in the 1st transmembrane (TM) region (TM1) or T339S in TM2, activation in low [ATP] was slow and the channel was constitutively active at all membrane potentials in high [ATP]. These results could be simulated by reducing the off rate of the gating step. Phenotypes of the double mutants, K308R/F44C and K308R/T339S, were similar to WT, suggesting that TM and ATP binding region mutants offset the effect of each other. Analysis of the effects on WT of two other agonists, ADP and AP\(_4\)A, revealed that the electrostatic charge is not the sole critical factor. Taking these results together with the recently reported crystal structure, it was suggested that upon binding of ATP, the occupied binding site indirectly interacts with the extracellular end of the TM regions to trigger conformational changes for gating in a voltage-dependent manner.

Introduction

P2X\(_2\) is a member of the ligand-gated cation channel P2X family which is activated by extracellular ATP. Seven subtypes of P2X receptor are now known to be distributed in various cells and tissues where they play significant physiological roles (Khakh, 2001; North, 2002; Koizumi et al. 2005; Burnstock, 2007; Surprenant & North, 2008). The isolation of cDNA encoding P2X\(_2\) receptors (Brake et al. 1994; Valera et al. 1994) showed that it has two transmembrane (TM) domains with a large extracellular loop and N- and C-terminal intracellular chains (Torres et al. 1998; Roberts et al. 2006). The stoichiometry of P2X\(_2\) was reported to be trimeric by biochemical, functional and structural analyses (Nicke et al. 1998; Stoop et al. 1999; Jiang et al. 2003; Aschrafi et al. 2004; Barrera et al. 2005; Mio et al. 2009). This was conclusively confirmed by the recently published crystal structure of zebra fish P2X\(_4\) (Kawate et al. 2009).

One interesting property of the P2X\(_2\) receptor is its voltage-dependent gating. Although there is no canonical voltage sensor domain at all, the inward current through the P2X\(_2\) receptor channel at the steady state in the presence of ATP has been shown to increase gradually upon further hyperpolarization of the membrane (Nakazawa et al. 1997; Zhou & Hume, 1998; Nakazawa & Ohno, 2005; Fujiwara et al. 2009). Typical voltage-gated channels harbour a voltage sensor domain comprising

Corresponding author Y. Kubo: Division of Biophysics and Neurobiology, Department of Molecular Physiology, National Institute for Physiological Sciences, Nishigoh-naka 38, Myodaiji, Okazaki, Aichi 444-8585, Japan. Email: ykubo@nips.ac.jp
several positively charged amino acid residues, and the charges are thought to cause the movement of the voltage sensor segment and the opening of the gate in response to a change in membrane potential. Therefore, it would be of great interest to know how P2X<sub>2</sub> channels, with no canonical voltage sensor, show voltage dependence. One obvious possibility is that it reflects voltage-dependent block–unblock of the channel by ions or extrinsic molecules, but we excluded this possibility and confirmed that the observed voltage-dependent gating is an intrinsic property of the channel by recording from excised patches (Fujiwara et al. 2009).

Through detailed analysis, we determined that, with increasing [ATP], the kinetics of hyperpolarization-evoked current activation are accelerated and the conductance–voltage (G–V) relationship is shifted toward more depolarized potentials. Apparently, the gating of the P2X<sub>2</sub> receptor is rather complex and dependent on both voltage and [ATP]. Assuming a simple three-state model for ligand-gated channels, the channel will be in one of three states: C (closed with no ATP), CA (closed with bound ATP), and OA (open with bound ATP). There are thus two transition steps: a ligand binding step and a gating step. To determine which step is voltage dependent, we carried out the following analysis.

Since the activation was fitted satisfactorily with a single exponential function and the ATP binding step is reportedly very fast (Ding & Sachs, 1999), we assumed that the gating step is rate limiting and calculated the k<sub>on</sub> and k<sub>off</sub> values at each voltage using the experimental data (Fujiwara et al. 2009) and previously reported K<sub>d</sub> values (Ding & Sachs, 1999). Using the calculated voltage-dependent k<sub>on</sub> and k<sub>off</sub> values, together with reported voltage-independent k<sub>bind</sub> and k<sub>unbind</sub> values (Ding & Sachs, 1999), we successfully simulated the [ATP]-dependent acceleration of the activation kinetics and the shift in the G–V relationship (Fujiwara et al. 2009). These results demonstrated that only in the rate limiting gating step does voltage dependence sufficiently explain the [ATP] dependence of the voltage-dependent gating. In the present study, we initially confirmed that the G–V relationship and the activation kinetics of wild-type P2X<sub>2</sub> (WT) changed substantially, depending upon [ATP] (see online Supplemental Material, Suppl. Fig. 1A–C). In addition, the results of simulation analyses done in our previous study (Fujiwara et al. 2009) are presented in a modified style (Suppl. Fig. 1D and E) that enables their direct comparison to the experimental data (Suppl. Fig. 1B and C).

The focus of the present study was on the structural determinants of the voltage- and [ATP]-dependent gating of the P2X<sub>2</sub> channel, which lacks a canonical voltage sensor. Previous mutagenesis analyses have enabled the functional identification of the ATP binding domain in the extracellular loop (Ennion et al. 2000; Jiang et al. 2000; Roberts et al. 2006; Marquez-Klaka et al. 2007), and these amino acid residues were confirmed in the crystal structure to be located at an intersubunit groove (Kawate et al. 2009). Functional roles of the amino acid residues in the TM1 and TM2 regions during ATP-evoked gating and permeation have also been investigated previously (Rassendren et al. 1997; Egan et al. 1998; Li et al. 2004; Khakh & Egan, 2005; Silberberg et al. 2005, 2007; Cao et al. 2007; Samways et al. 2008; Jindrichova et al. 2009). On the other hand, the voltage-dependent properties of the channel have not attracted much attention. In the present work, we used mutagenesis to identify amino acid residues in the ATP binding, TM1 and TM2 regions involved in the voltage- and [ATP]-dependent gating of P2X<sub>2</sub>. We also analysed their specific contributions to gating using the aforementioned three-state model. We also compared the effects of other agonists, ADP and diadenosine tetraphosphate ammonium (AP<sub>4</sub>A), with those of ATP, as they have different electrostatic charges, sizes and shapes.

Furthermore we generated a three dimensional structural model of rat P2X<sub>2</sub> based on the zebra fish P2X<sub>4</sub> crystal structure in the closed state (Kawate et al. 2009). We mapped the locations of amino acid residues that we identified as critical for the voltage-dependent gating. The experimental results are discussed in light of this structural model.

**Methods**

**In vitro mutagenesis and cRNA synthesis**

A BamH1–Not1 fragment of the original rat P2X<sub>2</sub> receptor cDNA (Brake et al. 1994) was subcloned into the Smal site of pGEMHE. Single- and double-point mutants were then made using a QuikChange site directed mutagenesis kit (Stratagene, La Jolla, CA, USA) and confirmed by DNA sequencing. cRNAs encoding WT and mutant receptors were then prepared from the plasmid cDNA linearized by PstI or NheI using a T7 RNA transcription kit (Ambion, Inc., Austin, TX, USA) as described previously (Fujiwara et al. 2009).

**Preparation of Xenopus oocytes**

*Xenopus* oocytes were collected from frogs anaesthetized in water containing 0.15% tricaine. After the final collection, the frogs were killed by decapitation. Isolated oocytes were treated with collagenase (2 mg ml<sup>−1</sup>, type 1; Sigma-Aldrich) for 6 h, after which oocytes of similar size at stage V were injected with 50 nl of cRNA solution as described previously. The injected oocytes were then incubated for 1 day at 17°C in frog Ringer solution. All experiments conformed to the guidelines of the Animal
Care Committees of the National Institute for Physiological Sciences (Okazaki, Japan).

**Two-electrode voltage clamp recordings in Xenopus oocytes**

Macroscopic currents were recorded from *Xenopus* oocytes using the two-electrode voltage clamp technique with a bath clamp amplifier (OC-725C; Warner Instruments, LLC, Hamden, CT, USA) as described previously (Fujiwara & Kubo, 2006; Fujiwara et al. 2009). Briefly, stimulation, data acquisition, and data analyses were performed on a Pentium-based computer using Digidata 1322A and pCLAMP 9.2 software (Axon Instruments/Molecular Devices, Sunnyvale, CA, USA). All recordings were obtained at room temperature. Intracellular glass microelectrodes were filled with 3 M potassium acetate containing 10 mM KCl (pH 7.2), and the resistances ranged from 0.1 to 0.2 MΩ. Two Ag–AgCl pellets (Warner Instruments) were used to pass the bath current and sense the bath voltage. The recording bath solution contained 95.6 mM NaCl, 1 mM MgCl₂, 5 mM Heps and 2.4 mM NaOH at pH 7.35–7.44. ATP disodium salt, ADP disodium salt and AP₁A-potassium salt (Sigma-Aldrich) were dissolved in potassium acetate containing 10 mM KCl (pH 7.2), and the bath volume of 5 mL was used to achieve sufficiently accurate voltage clamping and to avoid changes in the properties of the channel due to the high expression level (Fujiwara & Kubo, 2004; Fujiwara et al. 2009). The background leak current before ATP application was measured and subtracted to isolate the ATP-induced current. Oocytes having leak currents greater than 0.25 μA at −40 mV were discarded.

**Data analyses and simulation**

Data were analysed using Clampfit 9.2 (Axon Instruments) and Igor Pro (WaveMetrics, Inc., Lake Oswego, OR, USA) software as described previously (Fujiwara et al. 2009). For analysis of the G–V relationship, Clampfit 9.2 software was used to fit inward tail current amplitudes at −60 mV to a two-state Boltzmann equation:

\[
I = I_{\text{min}} + \frac{I_{\text{max}} - I_{\text{min}}}{1 + e^{-ZF(V-V_{1/2})/RT}}
\]

where \(I_{\text{min}}\) and \(I_{\text{max}}\) are the limits of the amplitudes in the fittings, \(Z\) is the effective charge, \(V_{1/2}\) is the half activation voltage, \(F\) is Faraday’s constant, \(R\) is the gas constant, and \(T\) is temperature in kelvins. ‘\(I_{\text{min}}\)’ here is the inward current with the largest amplitude. Normalized G–V relationships were fitted using the following equation:

\[
G/G_{\text{max}} = I/I_{\text{min}} = 1 - \left(1 + e^{-ZF(V-V_{1/2})/RT}\right)^{-1} (1 - I_{\text{max}}/I_{\text{min}})
\]

The activation time constants (τ) were obtained by fitting the activation phase of the inward currents elicited upon hyperpolarization with a single exponential function. The opening (β) and closing (α) rates in the two-state model (C–O) were calculated using two equations:

\[
G/G_{\text{max}} = \beta/(\alpha + \beta) \quad \text{and} \quad \tau = 1/(\alpha + \beta)
\]

A standard three-state model consisting of an ATP binding step and a following rate limiting gating step (C closed – CA (closed with ATP bound) – OA (open with ATP bound)) was used to simulate the experimental results from WT and mutant channels. The rate constants for each step are \(k_{\text{bind}}\) and \(k_{\text{unbind}}\) for the ATP-binding step and \(k_{\text{on}}\) and \(k_{\text{off}}\) for the gating step. \(k_{\text{on}}\) and \(k_{\text{off}}\) were calculated from the α and β values assuming the gating step is rate limiting, as described in detail elsewhere (Fujiwara et al. 2009).

\[
k_{\text{on}} = (1 + K_d/[\text{ATP}])\beta, \quad k_{\text{off}} = \alpha
\]

With the assumption that the \(K_d\) value is 42 μM and is not voltage dependent (Ding & Sachs, 1999), \(k_{\text{on}}\) and \(k_{\text{off}}\) were calculated at various voltages and [ATP] (Fujiwara et al. 2009).

Simulation of hyperpolarization-evoked current activation was performed using Igor Pro (WaveMetrics) software using the calculated \(k_{\text{on}}\) and \(k_{\text{off}}\) values, as described previously (Fujiwara et al. 2009). The rate constants for the ATP binding step were determined with reference to earlier single channel analyses: \(k_{\text{bind}} = 2.6 \times 10^7 \text{M}^{-1} \text{s}^{-1}\) and \(k_{\text{unbind}} = 1.1 \times 10^5 \text{s}^{-1}\) (Ding & Sachs, 1999). We used a \(K_d\) value of 1100, and the value of [ATP] \(\times k_{\text{bind}}\) was set depending on the [ATP] relative to \(K_d\). The fractions in the three states at the equilibrium shown in the table were calculated as follows.

\[
C = \frac{k_{\text{unbind}} k_{\text{off}}}{(k_{\text{unbind}} k_{\text{off}}) + (k_{\text{bind}}[\text{ATP}]k_{\text{off}}) + (k_{\text{bind}}[\text{ATP}]k_{\text{on}})}
\]

\[
CA = \frac{k_{\text{bind}}[\text{ATP}]k_{\text{off}}}{(k_{\text{unbind}} k_{\text{off}}) + (k_{\text{bind}}[\text{ATP}]k_{\text{off}}) + (k_{\text{bind}}[\text{ATP}]k_{\text{on}})}
\]

\[
OA = \frac{k_{\text{bind}}[\text{ATP}]k_{\text{on}}}{(k_{\text{unbind}} k_{\text{off}}) + (k_{\text{bind}}[\text{ATP}]k_{\text{off}}) + (k_{\text{bind}}[\text{ATP}]k_{\text{on}})}
\]

The modified rate constants used for the simulation of mutants and the fractions of C, CA and OA in the three states at the equilibrium are shown in Table 1.
Three dimensional structural modelling of rat P2X<sub>2</sub>

Homology modelling was performed by using DeepView/Swiss pdb viewer v.4.0.1 http://www.expasy.org/spdbv/ and SWISS-MODEL (Automated Protein Modelling Server) (Arnold et al. 2006; Bordoli et al. 2009) based upon sequence alignment of amino acid residues 34–351 of the rat P2X<sub>2</sub> and the crystal structure of the zebra fish P2X<sub>4</sub> (Protein Data Bank accession number 3I5D) (Kawate et al. 2009). Graphic presentation was made by PyMol (DeLano Scientific LLC, Palo Alto, CA, USA).

### Results

#### Effects of mutations in the ATP binding region on voltage- and [ATP]-dependent gating

To investigate the roles of positively charged amino acid residues in the putative ATP binding region (Roberts et al. 2006) during the voltage- and ATP-dependent gating of the P2X channel, we introduced charge neutralizing (A (alanine)) and charge maintaining (K (lysine) or R (arginine)) mutations into the region (K308A/R, K71A/R, R290A/K and K69A/R). We then recorded a series of macroscopic currents from identical oocytes in various [ATP] by applying a set of step pulses (bottom of Fig. 1) at the steady state in the presence of ATP. K69A and K308A could not be analysed because almost no currents (K69A) or only very small currents (K308A) were activated, even at high [ATP]. All other mutants showed voltage-dependent gating, and representative traces of K308R are shown in Fig. 1A.

Normalized G–V relationships for K308R at various [ATP] were constructed from tail current amplitudes and fitted with a two state Boltzmann function (Fig. 1B). The \( V_{1/2} \) and \( Z \) values were then plotted against [ATP] (Fig. 1C and D). The G–V relationship for K308R was shifted to more hyperpolarized potentials than that of WT. The [ATP]-dependent positive shift of \( G/G_{\text{max}} \) observed in WT (Suppl. Fig. 1B) was not apparent in K308R (Fig. 1B–D). The \( Z \) values for the mutants were approximately 0.5 with

---

<table>
<thead>
<tr>
<th>WT ( n \times K_d ) (( n = 0.1, 0.3, 1, 3, 10 ))</th>
<th>(-60 \text{ mV})</th>
<th>(-80 \text{ mV})</th>
<th>(-100 \text{ mV})</th>
<th>(-120 \text{ mV})</th>
<th>(-140 \text{ mV})</th>
<th>(-160 \text{ mV})</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k_{\text{bind}} \times \text{ATP} ) ( n \times 1100 )</td>
<td>( n \times 1100 )</td>
<td>( n \times 1100 )</td>
<td>( n \times 1100 )</td>
<td>( n \times 1100 )</td>
<td>( n \times 1100 )</td>
<td>( n \times 1100 )</td>
</tr>
<tr>
<td>( k_{\text{unbind}} )</td>
<td>1100</td>
<td>1100</td>
<td>1100</td>
<td>1100</td>
<td>1100</td>
<td>1100</td>
</tr>
<tr>
<td>( k_{\text{on}} )</td>
<td>33.5</td>
<td>36</td>
<td>40.5</td>
<td>45.7</td>
<td>50.7</td>
<td>56.3</td>
</tr>
<tr>
<td>( k_{\text{off}} )</td>
<td>14.8</td>
<td>11.5</td>
<td>8.9</td>
<td>6.9</td>
<td>5.4</td>
<td>3.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>((10 \times k_{\text{off}}) \times n \times K_d ) (( n = 0.1, 0.3, 1, 3, 10 ))</th>
<th>(-60 \text{ mV})</th>
<th>(-80 \text{ mV})</th>
<th>(-100 \text{ mV})</th>
<th>(-120 \text{ mV})</th>
<th>(-140 \text{ mV})</th>
<th>(-160 \text{ mV})</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k_{\text{bind}} \times \text{ATP} ) ( n \times 1100 )</td>
<td>( n \times 1100 )</td>
<td>( n \times 1100 )</td>
<td>( n \times 1100 )</td>
<td>( n \times 1100 )</td>
<td>( n \times 1100 )</td>
<td>( n \times 1100 )</td>
</tr>
<tr>
<td>( k_{\text{unbind}} )</td>
<td>1100</td>
<td>1100</td>
<td>1100</td>
<td>1100</td>
<td>1100</td>
<td>1100</td>
</tr>
<tr>
<td>( k_{\text{on}} )</td>
<td>33.5</td>
<td>36</td>
<td>40.5</td>
<td>45.7</td>
<td>50.7</td>
<td>56.3</td>
</tr>
<tr>
<td>( k_{\text{off}} )</td>
<td>148</td>
<td>115</td>
<td>89</td>
<td>69</td>
<td>54</td>
<td>39</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>((0.1 \times k_{\text{off}}) \times n \times K_d ) (( n = 0.1, 0.3, 1, 3, 10 ))</th>
<th>(-60 \text{ mV})</th>
<th>(-80 \text{ mV})</th>
<th>(-100 \text{ mV})</th>
<th>(-120 \text{ mV})</th>
<th>(-140 \text{ mV})</th>
<th>(-160 \text{ mV})</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k_{\text{bind}} \times \text{ATP} ) ( n \times 11000 )</td>
<td>( n \times 11000 )</td>
<td>( n \times 11000 )</td>
<td>( n \times 11000 )</td>
<td>( n \times 11000 )</td>
<td>( n \times 11000 )</td>
<td>( n \times 11000 )</td>
</tr>
<tr>
<td>( k_{\text{unbind}} )</td>
<td>11000</td>
<td>11000</td>
<td>11000</td>
<td>11000</td>
<td>11000</td>
<td>11000</td>
</tr>
<tr>
<td>( k_{\text{on}} )</td>
<td>33.5</td>
<td>36</td>
<td>40.5</td>
<td>45.7</td>
<td>50.7</td>
<td>56.3</td>
</tr>
<tr>
<td>( k_{\text{off}} )</td>
<td>148</td>
<td>115</td>
<td>89</td>
<td>69</td>
<td>54</td>
<td>39</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>((10 \times k_{\text{unbind}}) \times n \times K_d ) (( n = 0.1, 0.3, 1, 3, 10 ))</th>
<th>(-60 \text{ mV})</th>
<th>(-80 \text{ mV})</th>
<th>(-100 \text{ mV})</th>
<th>(-120 \text{ mV})</th>
<th>(-140 \text{ mV})</th>
<th>(-160 \text{ mV})</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k_{\text{bind}} \times \text{ATP} ) ( n \times 11000 )</td>
<td>( n \times 11000 )</td>
<td>( n \times 11000 )</td>
<td>( n \times 11000 )</td>
<td>( n \times 11000 )</td>
<td>( n \times 11000 )</td>
<td>( n \times 11000 )</td>
</tr>
<tr>
<td>( k_{\text{unbind}} )</td>
<td>11000</td>
<td>11000</td>
<td>11000</td>
<td>11000</td>
<td>11000</td>
<td>11000</td>
</tr>
<tr>
<td>( k_{\text{on}} )</td>
<td>33.5</td>
<td>36</td>
<td>40.5</td>
<td>45.7</td>
<td>50.7</td>
<td>56.3</td>
</tr>
<tr>
<td>( k_{\text{off}} )</td>
<td>148</td>
<td>115</td>
<td>89</td>
<td>69</td>
<td>54</td>
<td>39</td>
</tr>
</tbody>
</table>
no [ATP] dependence, as observed with WT (Fujiwara et al. 2009). The activation of inward currents through K308R was fitted satisfactorily with a single exponential function. The normalized currents at −160 mV and various [ATP] are shown in Fig. 1E, and the time constants are plotted against the membrane potential (Fig. 1F). The speed of K308R activation was faster than WT (Suppl. Fig. 1C), but the acceleration seen with the increases in [ATP] was less pronounced than with WT (Figs 1E and F and 2C).

The $V_{1/2}$ values for WT and the various ATP binding region mutants are shown in Fig. 2A. In Fig. 2B, $V_{1/2}$ values are plotted against [ATP] normalized by the EC$_{50}$ values, so comparisons could be made independently of the absolute [ATP]. All of the mutations reduced the sensitivity to [ATP], though the extent varied (Suppl. Fig. 2). Even after compensating for differences in EC$_{50}$, the $V_{1/2}$ values for all the mutants differed significantly from WT, irrespective of whether the mutation was charge maintaining or neutralizing. Moreover, $V_{1/2}$ values for the mutants accumulated at hyperpolarized potentials with no apparent [ATP]-dependent shift to depolarized potentials, which is in clear contrast to WT (Fig. 2B). This observation suggests that even subtle changes of the binding environment of ATP by charge maintaining mutations significantly alter the [ATP] dependence of the voltage dependent gating.

When we plotted the activation time constant at −160 mV for WT and various mutants against normalized [ATP] (Fig. 2C–H), we found that the activation speeds of K308R (Fig. 2C), R290K (Fig. 2E) and K69R (Fig. 2F) were faster than WT. On the other hand, the activation of K71R was relatively slow (Fig. 2D) and the data for K71A (Fig. 2H) could not cover a wide range of normalized [ATP] due to the mutant's very low sensitivity to [ATP]. In addition, acceleration of the activation speed with increases in [ATP] was less apparent with all of the mutants than with WT.

**Figure 1. Analysis of voltage- and [ATP]-dependent gating of the K308R mutant of P2X$_2$**

A, macroscopic currents through K308R evoked by step pulses were recorded during the steady state after ATP application. Recordings in the presence of various [ATP] were obtained from identical oocytes after subtracting data in the absence of ATP. The pulse protocol is shown at the bottom. Shown in the insets are expanded views of the tail currents recorded at −60 mV. B, normalized $G$–$V$ relationships for K308R derived from the maximum tail currents shown in A by fitting the data with a two-state Boltzmann equation (see Methods for details). C, $V_{1/2}$ (mV) and $D, Z$ values at various [ATP]. Representative data are shown in circles, others are in triangles. E, normalized traces showing the current activation phase for K308R at −160 mV. F, activation time constants calculated by fitting the activation phase with a single exponential function at various membrane potentials in the presence of various [ATP].

© 2009 The Authors. Journal compilation © 2009 The Physiological Society
Increasing $k_{\text{off}}$ reproduces the results for K308R in simulations

To determine which parameter within the three-state model is changed by the ATP binding site mutations, we carried out a simulation analysis. With K308R, the $G-V$ relationship was shifted toward hyperpolarized potentials, and the activation kinetics were faster than WT, i.e. the equilibrium between CA and OA was inclined toward CA, and equilibrium was reached more quickly due to faster kinetics. We therefore speculated that $k_{\text{off}}$ of the voltage-dependent gating step is increased in K308R and carried out a simulation using a 10 times larger $k_{\text{off}}$ value (Fig. 3A).

The OA fraction derived from the simulation was used to plot the relative conductance against membrane potential (Fig. 3B and C). The plots for the simulation data were shifted to more hyperpolarized potentials and, even at $10 \times K_d$, the relative conductance plot barely reached 50% of the maximum conductance at $-160 \text{ mV}$ (Fig. 3C), which is in clear contrast to WT (Suppl Fig. 1D). These results were similar to the experimental results for K308R (Fig. 1B), though the absence of an [ATP]-dependent $G-V$ shift in the experiment (Fig. 1B and C) could not be reproduced in the simulation (Fig. 3C). This may reflect uncertainty with the determination of $V_{1/2}$ values due to the marked shift to hyperpolarized potentials seen when fitting the $G-V$ relationship. The activation kinetics for the normalized traces at $-160 \text{ mV}$ (Fig. 3D) were faster than WT (Suppl. Fig. 1E), but the [ATP]-dependent change in the activation time constants at various potentials was minor (Fig. 3E), which is consistent with the experimental results (Figs 1F and 2C).

Needless to say, $k_{\text{bind}}$ and $k_{\text{unbind}}$ were also changed by ATP binding site mutations. This was expected from the change in the EC$_{50}$ value (Suppl. Fig. 2), but the change of $k_{\text{bind}}$ did not affect the result of simulation, which was always evaluated in terms of the $K_d$ value, i.e. an increase in [ATP] completely compensated for the decrease in $k_{\text{bind}}$. We also carried out a simulation in which $k_{\text{off}}$ varied, but it

Figure 2. [ATP]–$V_{1/2}$ and [ATP]–$\tau$ relationships for the ATP binding region mutants and WT

A, $V_{1/2}$ for WT and the mutants are plotted against [ATP]. B, the same set of data plotted against [ATP] normalized by the EC$_{50}$ values for each mutant and WT. C–H, the activation time constants at $-160 \text{ mV}$ for the indicated ATP binding region mutants and WT are plotted against [ATP] normalized by the EC$_{50}$ values for each mutant and WT. C, K308R; D, K71R; E, R290K; F, K69R; G, R290A; H, K71A.
did not remarkably affect the result (e.g. compare Fig. 3C, D and E and Suppl. Fig. 4A) when the ATP binding step was much faster than the gating step. In summary, the similarity of the experimental results for K308R (Fig. 1) and the simulation study (Fig. 3) suggests that mutations of the putative ATP binding site not only change the $k_{\text{bind}}/k_{\text{unbind}}$ values but also affect the $k_{\text{off}}$ value for the rate-limiting voltage-dependent gating step.

Effect of TM1 and TM2 mutations on the voltage- and [ATP]-dependent gating

Aromatic residues at the lipid–water interface are critical for the function of some ion channels (Domene et al. 2006; Zhou et al. 2007; Li et al. 2009), and the functional significance of aromatic amino acid residues situated close to the extracellular side of the TM1 region of P2X$_2$ has been

Figure 3. Simulation with high $k_{\text{off}}$ values

A, schematic drawing of the three-state model with fast ATP binding, a rate-determining voltage-dependent gating step, and high $k_{\text{off}}$ values. B, simulated traces of the voltage-induced activation phase with high $k_{\text{off}}$ values at various $K_d$ represented in different colours. From the simulation data, $G/G_{\text{max}}$ (C), normalized activation traces at $-160$ mV (D) and the $\tau$–$V$ relationship at various $K_d$ values (E) are plotted.
Figure 4. Macroscopic currents through representative TM1 (F44C) and TM2 (T339S) mutants
A and D, macroscopic currents through F44C (A) and T339S (D) evoked by step pulses were recorded during the steady state after the ATP application. Recordings in the presence of various (low and high) [ATP] were obtained from identical oocytes after subtracting data recorded in the absence of ATP. The pulse protocol is shown at the bottom of Fig. 1. Shown in the insets are expanded views of the tail currents at $-60\, \text{mV}$. B and C, data of TM1 mutants in the presence of $10\, \mu\text{M} \text{ATP}$. E and F, data of TM2 mutants in the presence of $30\, \mu\text{M} \text{ATP}$. B and E, normalized G–V relationships for Y43A, F44C, F44A and Y47C (B), and for T339S, I328C, I328S and I328L (E). C and F, normalized I–V relationship for F44C, Y47C, Y43A and F44A (C), and for T339S, I328C, I328S and I328L (F).
reported (Jiang et al. 2001; Li et al. 2004; Khakh & Egan, 2005; Jindrichova et al. 2009). We therefore analysed the effects of Y43A, F44C, F44A and Y47C mutations from the perspective of voltage-dependent gating.

The phenotypes of F44C (Fig. 4A) and Y47C (data not shown) were highly similar to one another but clearly different from WT (Suppl. Fig. 1A). Both mutants showed very slow gating upon application of low [ATP], and no activation phase was observed when high [ATP] was applied, i.e. the channel behaved as if it was constitutively active and voltage independent (Fig. 4A). Voltage independency was also apparent from the normalized G–V relationship in the presence of 10 μM ATP (Fig. 4B) and the I–V relationship (Fig. 4C). F44A showed a slight channel activity, even in the total absence of ATP (data not shown), but otherwise its phenotype was similar to F44C and Y47C (Fig. 4B and C). Y43A also did not show an activation phase in high [ATP] (data not shown), and its G–V relationship showed voltage-independent constitutive activity (Fig. 4B). However, the I–V relationship for Y43A shows that in the presence of 10 μM ATP there was strong inward rectification with no clear outward current (Fig. 4C). The other two aromatic residues in TM1, F49 and W46 were also analysed by introducing F49C and W46C mutations and their phenotypes were similar to WT (data not shown).

Functionally significant amino acid residues situated close to the extracellular side of TM2 have also been reported for P2X2 (Rassendren et al. 1997; Egan et al. 1998; Li et al. 2004; Khakh & Egan, 2005; Cao et al. 2007). We analysed I328 and T339 and found that the phenotypes of I328S (data not shown), I328C (data not shown) and I328L (data not shown) were similar to that of Y43A. In the presence of high [ATP], the G–V relationship indicated constitutive activity (Fig. 4E), but the I–V curves showed remarkable inward rectification (Fig. 4F). T339S phenotype (Fig. 4D–F) was very similar to that of F44C (Fig. 4A–C). The G–V relationship for T339S clearly showed that it acquired constitutive activity with increases in [ATP] (Fig. 5A), and the normalized currents show an activation phase only in low [ATP] (Fig. 5B and C). Based on a previous single channel analysis, T339S was reported to be constitutively active in the absence of ATP (Cao et al. 2007), but this finding could not be clearly reproduced in the present study. Consistent with previous reports (Li et al. 2004; Jindrichova et al. 2009), all of the TM mutants described here showed greater sensitivity to ATP than WT (Suppl. Fig. 3).

Reducing $k_{\text{off}}$ reproduces the results for T339S in simulations

The phenotype of T339S can be summarized as being the opposite of that of K308R – i.e. the equilibrium between CA and OA was markedly inclined toward OA and equilibrium was reached more slowly due to the slower kinetics. The experimental results suggest that with T339S, the $k_{\text{off}}$ value is reduced and we were able to reproduce the data by a simulation using a reduced $k_{\text{off}}$ value. As shown in Fig. 6, the slow activation in low ATP (Fig. 6B, D and E) and the constitutive or highly stable, voltage-independent activity (Fig. 6B and C) were reproduced by simulation.

As described in the methods, the $k_{\text{on}}$ and $k_{\text{off}}$ values used for the simulation were calculated from the G–V and τ–V data of WT at various [ATP] and could only be obtained with data in the rather hyperpolarized potential range. We therefore could not carry out simulations at more
depolarized potentials to highlight the difference between T339S and I328S. However, even if the simulation could be done at depolarized potentials, there would be no chance to reproduce the inward rectification observed with Y43A and I328C, S and L, as we assume the permeation property of the pore is linear and not voltage dependent.

Analysis of a double mutant with substitutions in the ATP binding and TM regions

Since K308R and T339S affected the same gating step oppositely, we next analysed the phenotype of the double mutant to determine whether the two mutations would mutually interact to offset one another.

Figure 6. Simulation results with low $k_{off}$ values

A, schematic drawing of the three-state model with fast ATP binding, a rate-determining voltage-dependent gating step and low $k_{off}$ values. B, simulated traces of the voltage-induced activation phase with low $k_{off}$ values, at various $K_d$ values represented in different colours. Using the simulation data, $G/G_{max}$ (C), normalized activation traces at $-160$ mV (D) and $\tau-V$ relationship at various $K_d$ values (E) were plotted.
Representative recordings of currents through K308R/T339S (Fig. 7A) are clearly different from K308R (Fig. 1A) and T339S (Fig. 4D), but very similar to WT (Suppl. Fig. 1A and Fujiwara et al. 2009). A shift in the G–V curve to depolarized potentials with the increase in [ATP] (Fig. 7B–D) and acceleration in the gating kinetics by ATP (Fig. 7E and F) were clearly observed. Similar results were also observed with the K308R/F44C double mutant (data not shown).

Effects of ADP and AP₄A on voltage-dependent gating

Given that mutations within the ATP binding site caused significant changes in voltage-dependent gating, it was of interest to examine the effect of agonists other than ATP. We selected ADP and AP₄A because both are known to activate P₂X₂ (Wildman et al. 1999; Gever et al. 2006). ADP has three negative charges on its two phosphate groups, one less than ATP, while AP₄A has four negative charges on its four free phosphate groups. Although the charge is the same as ATP, AP₄A has an extra adenine ring and phosphate group on the other end of the molecule.

Although WT P₂X₂ was significantly less sensitive to ADP than to ATP (Fig. 9A and B), the voltage dependence of the responses to ADP (Figs 8A–D and 9D and F) and ATP (Suppl. Fig. 1) were very similar. The voltage dependence of the response of WT to AP₄A (Figs 8E–H and 9D and F) was similar to the response of K308R to ATP (Fig. 1). The G–V relationship was shifted to more hyperpolarized potentials with no remarkable [ATP] dependence and the activation kinetics were relatively fast.

It is noteworthy that the Hill coefficient for AP₄A is clearly different from those of ATP and ADP. EC₅₀ values and the Hill coefficients were 5710 ± 454 μM and 1.88 ± 0.1 respectively for ADP (n = 8), and 740 ± 84 μM and 0.7 ± 0.2 for AP₄A (n = 10) (Fig. 9B). The Z values for the G–V curves constructed from the AP₄A and ADP responses were similar to the ATP response (Fig. 9E and F). These results suggest that the electrostatic charge is not

---

Figure 7. Analyses of the K308R/T339S double mutant

A, macroscopic currents through K308R/T339S evoked by step pulses were recorded during the steady state in the presence of the indicated [ATP]. Recordings at various [ATP] were obtained from identical oocytes after subtracting data obtained in the absence of ATP. The pulse protocol is shown at the bottom of Fig. 1. Shown in the insets are expanded views of the tail currents at −60 mV. B, normalized G–V relationship. C, V₁/₂ (mV) and D, Z values at various [ATP]. Representative data are shown in circles, others are in triangles. E, normalized current traces for the activation phase at −160 mV at various [ATP]. F, t–V relationship at various [ATP].
Figure 8. Effects of other agonists on the voltage-dependent gating of WT P2X2

A and E, responses were evoked by step pulses during the steady state after the application of the indicated [ADP] (A) and [AP4A] (E). Recordings at the indicated [ligand] were from identical oocytes after subtracting data obtained in the absence of ligand. The pulse protocol is shown at the bottom of Fig. 1. Shown in the insets at expanded views of the tail currents at −60 mV. B–D, data obtained with ADP; F–H, data obtained with AP4A. B and F, normalized G–V relationship. C and G, normalized current traces showing the activation phase at −160 mV in the presence of the indicated [ligand]. D and H, τ–V relationship at the indicated [ligand].

© 2009 The Authors. Journal compilation © 2009 The Physiological Society
the sole determinant of the voltage-dependent gating; the conformation of the ATP–ATP binding site complex and the manner of the binding are also critical.

Homology modelling of the rat P2X2 structure based on that of zebra fish P2X4 and the mapping of critical amino acid residues for voltage dependent gating

Based on the crystal structure of the zebra fish P2X4 in the closed state (Kawate et al. 2009), we performed a homology modelling of the rat P2X2 lacking the intracellular chains by using SWISS-MODEL.

The side view of the modelled structure is shown in Fig. 10A. Conserved residues critical for ATP binding, K69, K71, R290 and K308, are shown by spherical representation. It is obviously seen in Fig. 10B that the ATP binding site is located at a position very distant from the membrane and is linked to the TM1 and TM2 helices by two different β sheets and following chains of 11 or 5 amino acids. In the top view (Fig. 10D), it is seen that the binding site is located at the inter-subunit groove between closely associated charged residues shown by spherical representation.

In the views of TM domains from the bottom (Fig. 10C) and just above the extracellular margin of the membrane (Fig. 10E), the aromatic residues in TM1 (Y43, F44, Y47) critical for the voltage-dependent gating are shown in magenta colour. They appear to be surrounding and oriented towards the pore-forming, steeply angled TM2. I328 of TM2 and Y43 of TM1 from adjacent subunits are observed to be facing each other (Fig. 10E). On the other hand, T339 in TM2 is oriented towards the centre of the permeation pathway (Fig. 10C).

Discussion

Implications of the mutation analyses and the structural data

Previous mutagenesis and cys-scan accessibility analyses of P2X receptors showed that K69, K71, R290 and K308 (P2X2 numbering) contribute to ATP binding (Ennion et al. 2000; Jiang et al. 2000; Roberts et al. 2006). In the present study, we demonstrated by mutagenesis the involvement of these residues in the voltage-dependent gating. One straightforward possibility may be that the ATP binding step is voltage dependent and that it is affected by the mutations of the ATP binding site. However, this possibility is unlikely, because the structure of the rat P2X2 channel (Fig. 10), based on the crystal structure of the zebra fish P2X4 (Kawate et al. 2009), revealed that the ATP binding site is located at a position distant from the lipid bilayer apparently outside of the electric field of the membrane. Consistent with the structural data, our
Figure 10. Homology modelling of the rat P2X2 based on crystal structure data of the zebrafish P2X4
A, homotrimeric model structure of the P2X2 viewed parallel to membrane together with appropriately scaled ATP.
simulation analyses (Fujiwara et al. 2009) demonstrated that the voltage dependence of the ATP binding step is neither necessary nor sufficient, and that of the rate limiting gating step is necessary to reproduce the voltage-and [ATP]-dependent gating.

An alternate possibility is that these positively charged amino acid residues in the ATP binding pocket not only attract ATP but also induce, together with bound ATP, subsequent conformational changes in a voltage-dependent manner. It is noteworthy that mutation-induced changes in the $K_d$ and in the voltage-dependent gating properties did not go in parallel. The presence or absence of a positive charge on the substituted amino acid residue in the mutants had a critical effect on $EC_{50}$ (Suppl. Fig. 2), but the charge was not the sole determinant of the voltage- and [ATP]-dependent gating properties (Fig. 2). For example, activation of R290K and R290A was faster than WT, whereas activation of K71R and K71A was slightly slower than WT (Fig. 2). Thus subtle changes in the shape of the ATP–ATP binding site complex or the manner of the binding appear to be critical for the subsequent voltage-dependent gating.

If the ATP–ATP binding site complex is involved in the voltage-dependent gating, the obvious questions are how and to which region of the molecule the information is transmitted. The crystal structure data (Kawate et al. 2009) and P2X$_2$ structural model (Fig. 10) reveal that the ATP binding region is continuous with two β-sheets connected to the TM helices with two loops having several amino acid residues (Fig. 10A, B and D). The pore of the channel is composed of TM2 segments which are steeply angled to the membrane plane (Fig. 10A, B, C and E). Three TM1 helices from each subunit surround the pore-forming TM2 helices (Fig. 10A, B, C and E). Therefore it is possible that the ATP–ATP binding site complex indirectly interacts with the extracellular ends of TM1 and TM2 by way of the β-sheet and a linker chain to induce voltage-dependent conformational change.

The TM1 aromatic amino acid residues which lie close to the extracellular side were previously reported to have little contribution to the permeation pathway (Jiang et al. 2003; Khakh & Egan, 2005; Samways et al. 2008) but to play important roles in the ATP-evoked activation (Jiang et al. 2001; Khakh & Egan, 2005; Silberberg et al. 2005; Jindrichova et al. 2009). Amino acid residues at the extracellular end of TM2 (Rassendren et al. 1997; Egan et al. 1998; Li et al. 2004; Khakh & Egan, 2005) and the linker region between TM2 and the ligand binding domain (Yan et al. 2006; Roberts & Evans, 2007) have also been reported to contribute to ATP-evoked activation. We analysed the mutants of these amino acid residues from the aspect of the voltage-dependent gating (Figs 4 and 5). The phenotypes of F44C (TM1), Y47C (TM1) and T339S (TM2) were characterized by slow activation at low [ATP] and constitutive activity at all voltages at high [ATP]. Y43A (TM1) and I328S (TM2) also demonstrated constitutive activity in the $G$–$V$ relationships at high [ATP], although their $I$–$V$ relationship showed an inward rectification (Fig. 4) presumably due to a mutated pore property. These results demonstrate these amino acid residues are critically involved in the voltage-dependent gating.

According to the structural model of P2X$_2$ (Fig. 10), the aromatic residues Y43, F44 and Y47 in TM1 are oriented toward the pore-forming TM2. On the other hand, W46 and F49, which did not affect the voltage-dependent gating when mutated to cysteine (data not shown), are oriented away from the pore-forming TM2. Interestingly, Y47 and I328 on adjacent subunits face each other. Jiang et al. (2001) reported that a separation of the di-sulfide bond between V48C and I328C is necessary for channel activation, suggesting that those two components move away from each other upon activation (Jiang et al. 2001). The experimental data and the structural model together suggest that the interaction between the aromatic residues (Y43, F44, Y47) in TM1 and the hydrophobic residues in the extracellular edge of TM2 (I328) serve for stabilization of the closed state and that the interaction is released in a voltage-dependent manner toward activation upon arrival of a gating signal from the ATP–ATP binding site complex.

It is also noteworthy that all of the examined ATP binding site mutants had a lower sensitivity to ATP than WT (Suppl. Fig. 2) and all of the non-gating TM1 and TM2 mutants had a greater sensitivity to current activation (Suppl Fig. 3). It appears as if the structure of WT P2X$_2$ is tailored in such a way that it holds a high affinity for ATP and a strong barrier to gating at the same time, which could have the functional effect of strictly inhibiting spontaneous opening of the channel in the absence of ATP, while enabling reliable opening, even at low [ATP].

**Implications of the simulation analyses of the mutants**

We first carried out a simulation analysis to see whether changing only the ATP binding step could reproduce the experimental results obtained with K308R, an ATP binding site mutant. Instead of using an absolute [ATP] for this analysis, we determined [ATP] relative to $K_d$.
Consequently, mutation-induced changes in \( k_{\text{bind}} \) could be compensated for by increasing [ATP]. We then carried out a simulation with an increased \( k_{\text{bind}} \) value reflecting the increase in \( K_2 \) value (Suppl. Fig. 4C). The results were similar to the recordings of WT, but were clearly different from those of K308R. Furthermore, when we simulated K308R using a larger \( k_{\text{off}} \) value, the results obtained with \( 10 \times k_{\text{off}} \) (Fig. 3) and \( 10 \times k_{\text{off}} \) plus \( 10 \times k_{\text{bind}} \) (Suppl. Fig. 4A) did not significantly differ. These simulation results revealed that the K308R mutation affects both the ATP binding step and the gating step, but it is the effect on the gating step that is critical for producing a change in the voltage-dependent gating.

Some of the details apparent in the recordings, such as the limited shift in the G–V relationship with increasing [ATP] (Fig. 1B and C), could not be reproduced in the simulation (Fig. 3C). This may be due to uncertainty in the evaluation of \( V_{1/2} \) when fitting the experimental data or insufficient adjustment of the simulation parameters. At various membrane potentials, we used the same parameters used with WT and equal \( 10 \times \) increases in \( k_{\text{off}} \). It is important to emphasize that a very simple and uniform increase in \( k_{\text{off}} \) was sufficient to reproduce the overall experimental results and it is impossible to explain the phenotype without changing \( k_{\text{off}} \). Similarly, the overall phenotype of T339S (TM1) and F44C (TM2) (Figs 4 and 5) could be simulated quite accurately just by assuming a uniform decrease in \( k_{\text{off}} \) (Fig. 6), although there were differences such as the activation phase remaining even with \( 10 \times K_2 \) in the simulation.

Moreover, since K308R had a higher \( k_{\text{off}} \) and T339S had a lower \( k_{\text{off}} \), it seemed plausible that these two mutations would offset one another in the rate limiting voltage-dependent step. As expected, the phenotypes of K308R/T339S (Fig. 7) and K308R/F44C (data not shown) were very similar to WT (Fig. 7). Cao et al. (2007) also reported that the K308A mutation rescued the constitutive activity of T339S in the absence of ATP (Cao et al. 2007).

**Implications of the effect of ADP and AP4A on the voltage-dependent gating**

The effects of mutations in the ATP binding site suggest that the electrostatic charge is not the sole factor governing voltage-dependent gating. We therefore examined the effects of two other agonists, which differed in size, shape and electrostatic charge. ADP has three negative charges, one less than ATP, but the voltage-dependent properties (e.g. \( Z \) value; Fig. 9E and F) were very similar to those of ATP (Fig. 8), which is consistent with the idea that charge is not the critical factor. The AP4A response of WT (Fig. 8B, F, G and H) was similar to the ATP response of K308R (Fig. 1) including the Hill coefficient (Suppl. Fig. 2, Fig. 9A and B), which was clearly higher than the Hill coefficients of WT for ATP and ADP (Fig. 9A and B). It is, therefore, possible that the binding manner of AP4A to WT and that of ATP to K308R are similar to one another and different from that of ATP to WT.

**Comparison with other non-canonical voltage-dependent membrane proteins**

There are a number of other membrane proteins that show voltage dependence despite the absence of a canonical voltage sensor. One well-known example is the nicotinic ACh receptor channel (Charsnet et al. 1992; Figl et al. 1996), within which the information of ligand binding is transmitted to the main body of the channel through the interaction of V44 in the \( \beta 1–\beta 2 \) loop of the extracellular binding region and the M2–M3 linker located at the extracellular end of TM2 and TM3 (Miyazawa et al. 2003; Lester et al. 2004). It may be that the interaction that occurs at the edge of the electric field is voltage dependent and analogous to the scenario we suggested for the P2X2 channel, where the interaction would be between the ligand binding site and the extracellular ends of TM1 and TM2.

Voltage dependence of ligand binding to the metabotropic glutamate receptor, a G protein-coupled receptor, has also been reported (Ohana et al. 2006). The intracellular loops of this receptor are known to be critical to its voltage dependence, creating a possible localization for a voltage sensor, and voltage-dependent change in ligand affinity could be induced by a change in the structure of the intracellular loop. A correlation between the gating charge on the m2 muscarinic receptor and the voltage dependence of its affinity for its ligand has also been reported (Ben-Chaim et al. 2006). In the case of P2X2, we showed that the voltage dependence of the ATP binding step alone cannot explain the experimental results, though it is possible that the ATP binding step is also voltage dependent. The unexpected voltage dependence of membrane proteins with no canonical voltage sensor might significantly enhance our understanding of the physiological function of changes in membrane potential.

In summary, the present study demonstrates that the voltage dependence of the P2X2 receptor lies in the rate limiting gating step and that the ATP–ATP binding site complex and the extracellular end of TM1 and TM2 are indirectly interacting with each other in a voltage-dependent manner. Further information on the structure in the open state and the evaluation of the dynamic conformational changes will enhance our understanding of P2X2 channels.

**References**


Author contributions
B.K. and Y.K. designed the research, B.K. performed experiments and analyses, and B.K. and Y.K. wrote the manuscript.

Acknowledgements
We are grateful to Dr. D. Julius (University of California, San Francisco) for providing us with P2X2 cDNA. We are grateful to Drs. Y. Fujiwara (Osaka University) for discussion, K. Nakajo for discussion and comments, and A. Collins (Queen's University, Belfast) for comments and editing of the manuscript. We would also like to thank Ms. Y. Asai for technical assistance. This work was supported in part by research grants from the Ministry of Education, Science, Sports, Culture, and Technology of Japan (MEXT) to Y.K. and from the Japan Society for Promotion of Science to Y.K. B.K. was supported by a scholarship for foreign students from the MEXT.