J Physiol 587.22 (2009) pp 5317–5324

SYMPOSIUM REVIEW

Dynamic aspects of functional regulation of the ATP receptor channel $P2X_2$

Yoshihiro Kubo, Yuichiro Fujiwara, Batu Keceli and Koichi Nakajo

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

The $P2X_2$ channel is a ligand-gated channel activated by ATP. Functional features that reflect the dynamic flexibility of the channel include time-dependent pore dilatation following ATP application and direct inhibitory interaction with activated nicotinic acetylcholine receptors on the membrane. We have been studying the mechanisms by which $P2X_2$ channel functionality is dynamically regulated. Using a *Xenopus* oocyte expression system, we observed that the pore properties, including ion selectivity and rectification, depend on the open channel density on the membrane. Pore dilatation was apparent when the open channel density was high and inward rectification was modest. We also observed that $P2X_2$ channels show voltage dependence, despite the absence of a canonical voltage sensor. At a semi-steady state after ATP application, $P2X_2$ channels were activated upon membrane hyperpolarization. This voltage-dependent activation was also [ATP] dependent. With increases in [ATP], the speed of hyperpolarization-induced activation was increased and the conductance–voltage relationship was shifted towards depolarized potentials. Based on analyses of experimental data and various simulations, we propose that these phenomena can be explained by assuming a fast ATP binding step and a rate-limiting voltage-dependent gating step. Complete elucidation of these regulatory mechanisms awaits dynamic imaging of functioning $P2X_2$ channels.

(Received 25 July 2009; accepted after revision 12 September 2009; first published online 14 September 2009)

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

The $P2X_2$ channel is a member of the P2X family of ligand-gated ion channels activated by ATP. It is expressed in dorsal ganglia neurons, mesenteric ganglia neurons, taste buds and astrocytes, and plays a key role in synaptic transmission in various systems (Edwards *et al.* 1992; Evans *et al.* 1992; Khakh, 2001; North, 2002; Koizumi *et al.* 2005; Burnstock, 2007; Surprenant & North, 2008). Complementary DNA (cDNA) encoding $P2X_2$ was first isolated through expression cloning, and the encoded protein was shown to have two transmembrane (TM) segments (Brake *et al.* 1994; Valera *et al.* 1994). Various lines of evidence suggest that the functional unit is a trimer (Surprenant & North, 2008). Indeed, atomic force microscopic analysis (Barrera *et al.* 2005), single particle structure analyses (Mio *et al.* 2005, 2009) and crystal structure analysis (Kawate *et al.* 2009) have now confirmed that $P2X_2$ is trimeric.

The mechanism by which $P2X_2$ channel activity is regulated exhibits several unique features. One is the time-dependent dilatation of the pore following ATP application (Khakh & Lester, 1999; Virginio *et al.* 1999; Eickhorst *et al.* 2002). This was made manifest by the time-dependent increase in $P2X_2$ channel permeability to *N*-methyl-D-glucamine (NMDG), a large cation known to be generally impermeable to cation channels, following application of ATP. Another unique feature is the functional interaction of $P2X_2$ channels with nicotinic acetylcholine receptor channels on the membrane. When $P2X_2$ and the nicotinic acetylcholine receptor were co-expressed, simultaneous activation of both receptors under voltage clamp evoked a membrane current that was clearly smaller than the sum of the currents carried by the two channels individually, indicating direct mutual

This review was presented at *The Journal of Physiology* Symposium on *Dynamic aspects of functioning membrane proteins*, which took place at the 36th International Congress of Physiological Sciences in Kyoto, Japan on 31 July 2009. It was commissioned by the Editorial Board and reflects the views of the authors.

DOI: 10.1113/jphysiol.2009.179309

interaction among the activated channels (Nakazawa, 1994; Khakh *et al.* 2000). These phenomena are reflections of dynamic and flexible features of the $P2X_2$ channel. We have also been engaged in investigating some novel and dynamic regulation mechanisms of $P2X_2$ channels (Fujiwara & Kubo, 2004, 2006; Fujiwara *et al.* 2009). Our findings to date are summarized in this article.

Density-dependent changes in pore properties

While carrying out experiments in *Xenopus* oocytes, we noticed that the magnitude of the inward rectification of rat $P2X_2$ channel currents varied such that it appeared to be dependent on the current amplitude or the channel density on the membrane, i.e. inward rectification declined with increases in the channel density (Fujiwara & Kubo, 2004). We also observed that the aforementioned time-dependent pore dilatation seen after ATP application was also dependent on the channel density. In other words, pore dilatation, as reflected by the depolarizing shift in the reversal potential with NMG in the bath solution, was less apparent when the channel density was low than when it was high (Fujiwara & Kubo, 2004). Moreover, even when the expression level of the channel was high, if only a fraction of the channels was activated due to application of only a low concentration of ATP, the phenotype was similar to that seen when the expression level of the channel was low (Fujiwara & Kubo, 2004). Based on these results, we proposed that there is an interaction between open $P2X_2$ channels, whereby the channel pore becomes a weak rectifier with high NMG permeability (Fig. 1). We eventually identified a point mutant (I328C) whose phenotype was less affected by open channel density than wild-type $P2X_2$ (Fujiwara & Kubo, 2004).

The crystal structure of the zebrafish $P2X_4$ ($zP2X_4$) channel in the closed state was solved recently (Kawate *et al.* 2009). Although the subtype and the species are different from the rat $P2X_2$ ($rP2X_2$) which we used in the experiments, the overall structure is expected to be conserved between them. The residue Ile336 of $zP2X_4$ (Ile328 of $rP2X_2$) is located in the second transmembrane region and just above the residues which are reported to form the narrowest part of the pore (Leu340, Gly343 and Ala344 of $zP2X_4$; Fig. 2*A*). In the closed state, the side-chain of Ile336 does not point to the outside of the

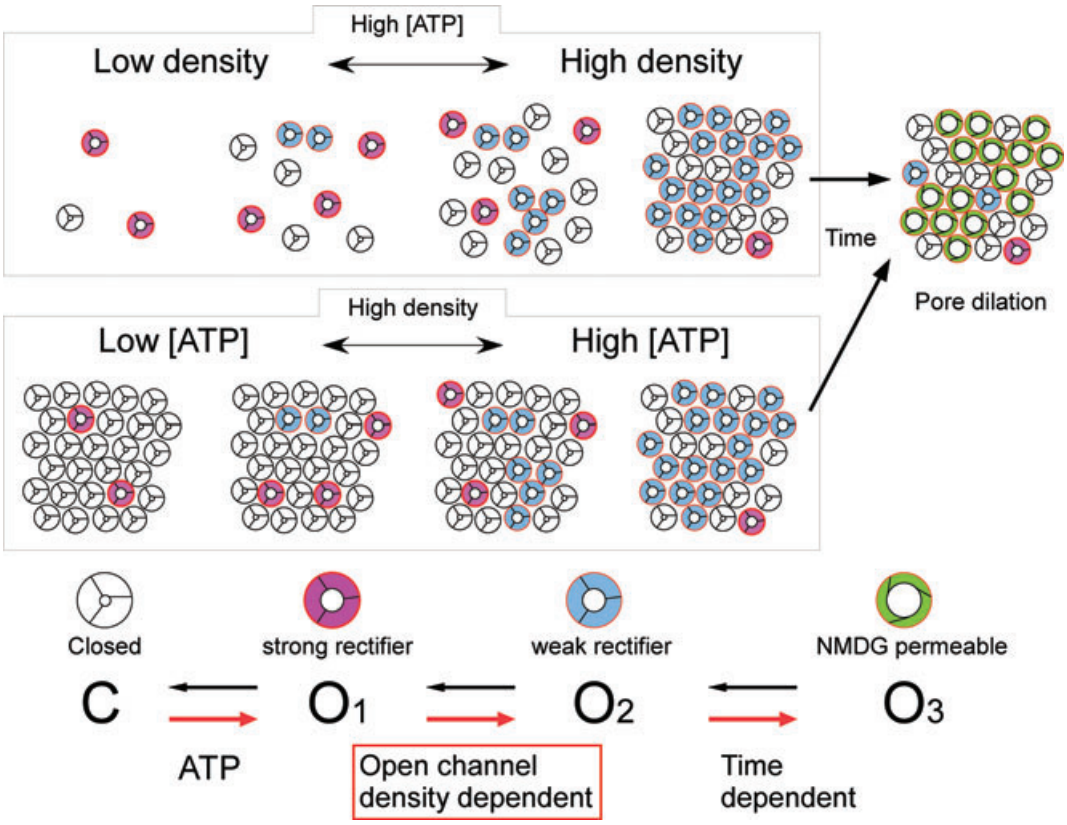

Figure 1. Schematic drawings explaining the dynamic variation of inward rectification intensity depending on the open channel density
The equilibrium is inclined toward the O2 state, depending on the open channel density. Definitions: O1, a state showing a strong inward rectification and a low permeability to NMDG; O2, a state showing no inward rectification; and O3, a state showing a high permeability to NMDG (from Fig. 7 of Fujiwara & Kubo, 2004).

trimer and it does not appear to serve as antennae for the inter-trimer interaction. It therefore remains to be elucidated how I328C mutation decreases the expression density-dependent changes.

Density-dependent changes in the pore properties have potential implications for physiological regulation, e.g. a change of the local density of the channel at a synapse due to the presence/absence or binding/unbinding of an anchoring molecule(s) could change the properties of the channel pore and, in turn, the properties of the synapse.

Regulation of desensitization and pore dilatation

The P2X channels show varying degrees of desensitization, and it is known that the C-terminal cytoplasmic region is the critical mediator of that desensitization (Koshimizu *et al.* 1999; Smith *et al.* 1999). Using wild-type and mutant $P2X_2$ channels, we observed that the binding of phosphoinositides to the proximal C-terminal cytoplasmic region, a region in which positively charged amino acids are clustered, is critical for preventing desensitization and thus maintaining $P2X_2$ channel activity (Fujiwara & Kubo, 2006). We also observed that channels enter the desensitized state not from the standard open state, but from the pore-dilated open state, suggesting that the channel–phosphoinositide interaction is weakened in the pore-dilated state (Fujiwara & Kubo, 2006).

Gating of the $P2X_2$ channel is voltage and [ATP] dependent, despite the absence of a canonical voltage sensor domain

The $P2X_2$ channel has only two TM regions, and it does not have a canonical voltage sensor. Nonetheless, $P2X_2$ channels exhibit voltage-dependent activation upon hyperpolarization (Nakazawa *et al.* 1997; Zhou & Hume, 1998; Nakazawa & Ohno, 2005). Similar voltage-dependent gating in the absence of a canonical voltage sensor has also been reported for the nicotinic acetylcholine receptor (Charnet *et al.* 1992; Figl *et al.* 1996). Prompted by our interest in the origins of voltage dependence, we used a *Xenopus* oocyte expression system to analyse the voltage dependence of the rat $P2X_2$ channel in detail (Fujiwara *et al.* 2009).

We applied voltage step pluses under two-electrode voltage clamp during the semi-steady state after ATP application and analysed the speed of the hyperpolarization-induced activation. We also analysed the conductance–voltage (G–V) relationship by measuring tail current amplitudes (Fig. 3*A*). With this approach, we made the novel and unexpected observation that the τ (activation time constant)–V relationship (Fig. 3*B*) and the normalized G–V relationship (Fig. 3*C*) were both clearly dependent on the applied [ATP]. When [ATP] was low, hyperpolarization-induced activation was slow, and the G–V relationship was shifted to hyperpolarized potentials. With increases in [ATP], the activation speed increased, and G–V was shifted to more depolarized potentials. The z value in the Boltzmann equation fitted to the G–V curve

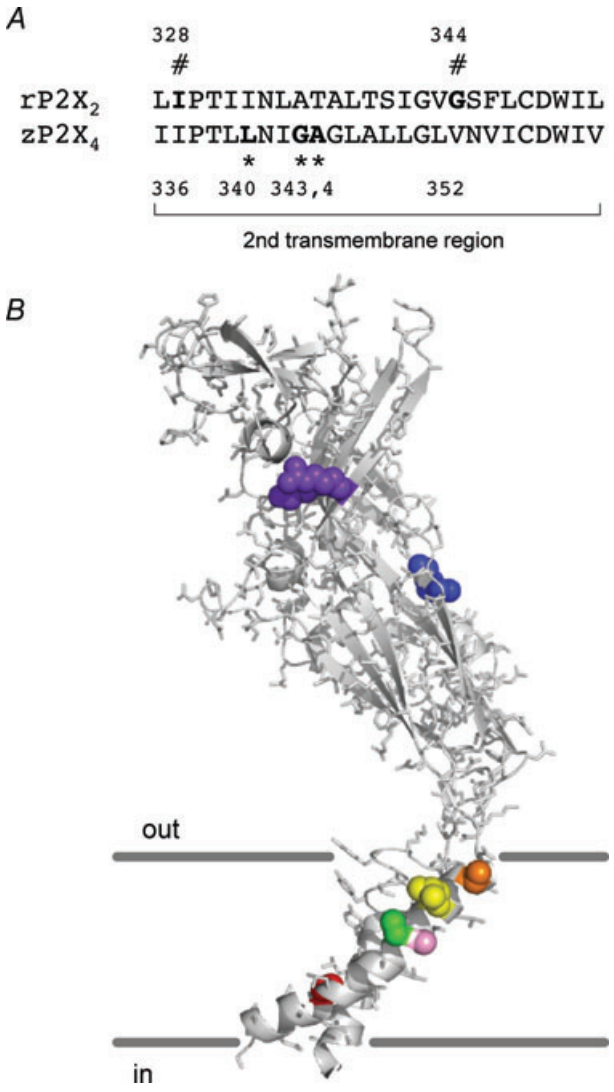

Figure 2. The crystal structure of zebrafish $P2X_4$ ($zP2X_4$) in the closed state and the location of the amino acid residues identified by the mutagenesis study
A, the alignment of the amino acid residues in the second transmembrane region of rat $P2X_2$ ($rP2X_2$) and $zP2X_4$. *B*, the crystal structure deposited in Protein Data Bank (accession number 3H9V) by Kawate *et al.* (2009) was used as a template, and the graphic presentation was made by PyMol software. Only one subunit of the trimer observed from the pore centre side is shown. The residue Ile336 (I328 of $rP2X_2$) is coloured in orange, while V352 (corresponding position to Gly344 of $rP2X_2$) is in red. The amino acid residues reported to form the gate (Leu340, Gly343 and Ala344) are shown in yellow, pink and green, respectively. The key amino acid residues of the ATP binding site, Lys70 and Lys72 (Lys69 and Lys71 of $rP2X_2$), are shown in blue, and Arg298 and Lys316 (Arg290 and Lys308 of $rP2X_2$) are shown in purple.

was approximately 0.5, which is much smaller than in typical voltage-dependent channels, and it did not indicate clear [ATP] dependence. The EC_{50} was also slightly voltage dependent, so that the value was reduced at more depolarized potentials (Fig. 3*D*). An obvious possibility is that the voltage-dependent activation is due to the block/unblock by a blocker extrinsic to the channel. However, when we carried out excised patch experiments, which enabled us to control both the internal and the external solutions, we still observed obvious voltage-dependent gating, confirming that the gating is truly intrinsic.

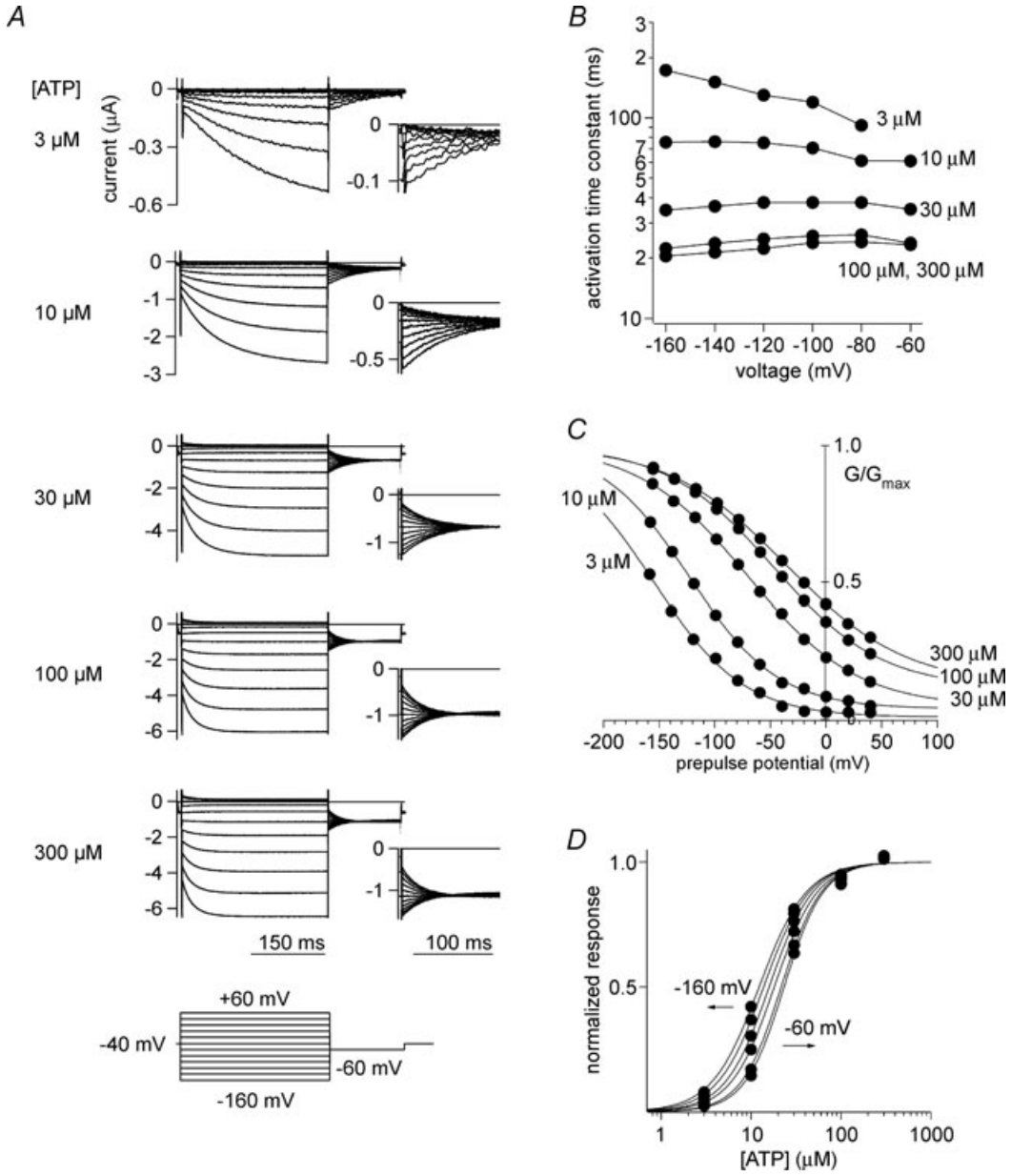

Figure 3. Macroscopic current recordings through $P2X_2$ evoked by step pulses during the steady state after application of various ATP concentrations, and analyses of the voltage-dependent gating
A, macroscopic currents through wild-type $P2X_2$ evoked by step pulses in the presence of various concentrations of ATP. The pulse protocol is indicated at the bottom. These current traces were recorded from a single oocyte and are shown after subtracting data obtained in the absence of ATP. *B*, dependence of the activation kinetics on voltage and [ATP]. The activation phases of the currents shown in *A* were fitted with a single exponential function, and the time constants of the fittings at each membrane potential are plotted. *C*, normalized *G–V* relationships derived from the recording in *A*. Tail current amplitudes at −60 mV were measured. Data were fitted with the two-state Boltzmann equation. *D*, normalized [ATP]–response relationships. From Figs 2 and 3*A*, *C* and *E* of ©Fujiwara *et al.* (2009), originally published in *J Gen Physiol* doi:10.1085/jgp.200810002.

What then is the origin of voltage-dependent gating that also depends on [ATP]? It is generally accepted that, during activation, ATP-gated channels proceed through three states: C, closed and ATP unbound; CA, closed with ATP bound; and OA, open with ATP bound. The transition between C and CA is the ATP binding step, while the transition between CA and OA is the gating step. Using this three-state, two-transition model, we analysed the gating properties of the $P2X_2$ channel (Fig. 4*A*). Given that the voltage-dependent gating is clearly dependent on [ATP], one simple and straightforward possibility is that the ATP binding step is the origin of the voltage dependence. The ATP binding could be voltage dependent if the binding site was located within an effective electric field, even if it was not embedded in the lipid bilayer. Another possibility is that the gating step following ATP binding is voltage dependent. In that case, the question is why the voltage-dependent gating also shows dependence on [ATP].

The rate constants β and α in the simple C–O model can be readily obtained, as $\tau = 1/(\beta + \alpha)$ and normalized $G = \beta/(\beta + \alpha)$. Using the calculated α and β values, we next calculated the on-rate (k_{on}) and the off-rate (k_{off}) values of the gating step in the C–CA–OA model (Fig. 4*A*). Two assumptions used were that the ATP binding step is fast and the gating step is rate limiting. We also used the reported K_d values (Ding & Sachs, 1999) to obtain the ratio of the unbinding-rate (k_{unbind}) and the binding-rate (k_{bind}) values. Although we had no concrete values for k_{bind} and k_{unbind}, we were able to calculate the k_{on} and k_{off} values as described in detail by Fujiwara *et al.* (2009). The calculated k_{on} and k_{off} values showed

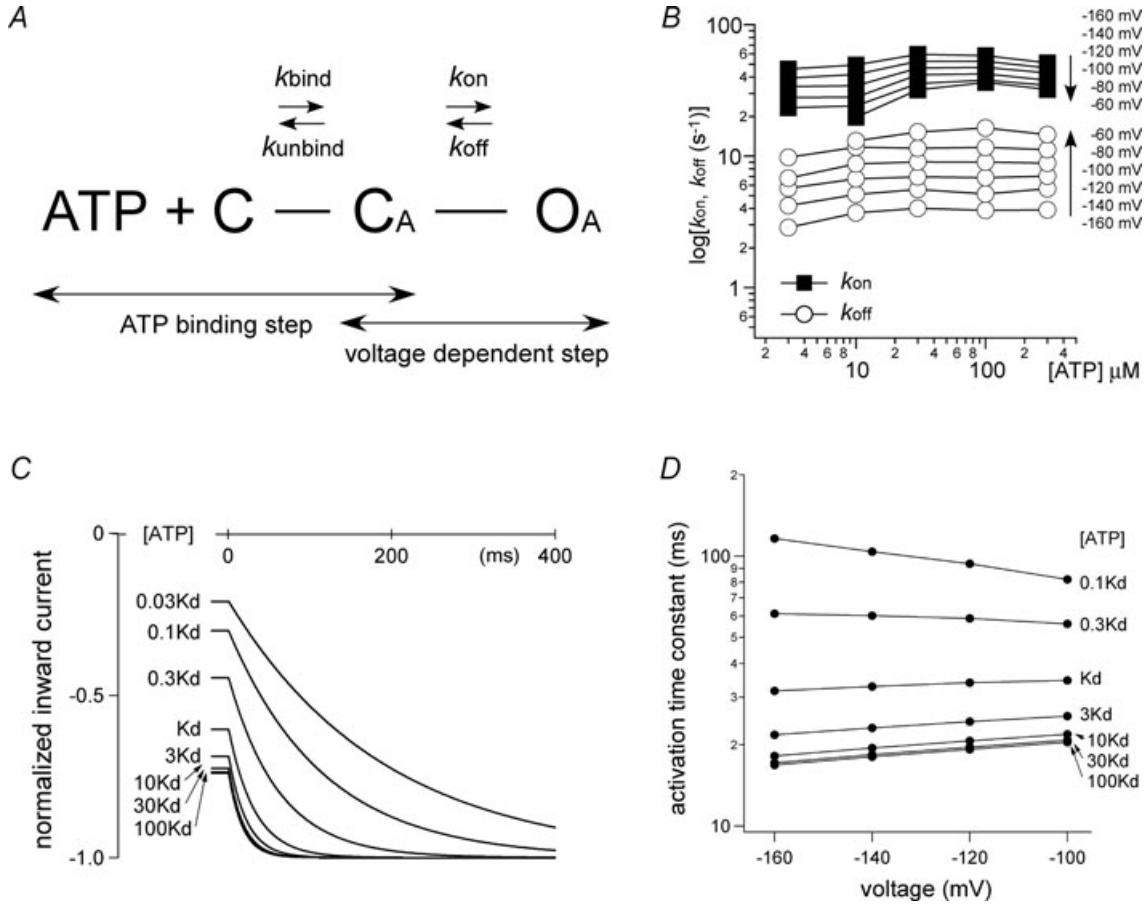

Figure 4. Three-state, two-transition model of voltage- and [ATP]-dependent gating, and simulation analyses of the activation phase evoked by a voltage step

A, simple three-state model consisting of an ATP binding step and a gating step. Definitions: C represents the closed state with no bound ATP, C_A represents the closed state after ATP is bound, and O_A represents the open state. *B*, k_{on} and k_{off} were calculated as described by Fujiwara *et al.* (2009). *C*, reproduction of the activation phase by simulation. The activation phases evoked by step pulses from −60 to −160 mV in the presence of various ATP concentrations were simulated. The applied [ATP] relative to K_d is indicated in the figure. *D*, summary of the simulation of the activation kinetics at various voltages and ATP concentrations. The activation phases of the simulated currents could be fitted satisfactorily with a single exponential function, and the time constants of the fittings at various concentrations of ATP relative to K_d were plotted *versus* membrane potential. From Figs 10*A* and *B* and 11*A* and *C* of ©Fujiwara *et al.* (2009), originally published in *J Gen Physiol* doi:10.1085/jgp.200810002.

clear voltage dependence with no [ATP] dependence; k_{on} was larger at hyperpolarized potentials, while k_{off} was larger at depolarized potentials (Fig. 4*B*). In addition, by using the calculated k_{on} and k_{off} values with the reported k_{bind} and k_{unbind} values (Ding & Sachs, 1999), we were able to reproduce the voltage-dependent activation as well as the ATP dependence (Fig. 4*C* and *D*). We also tried simulations using arbitrary k_{bind} parameters with voltage dependence. Although we managed to reproduce the ATP dependence of the rate of voltage-dependent gating, the EC_{50} value was steeply voltage dependent and clearly deviated from the experimental results. Thus, it was not possible to explain and reproduce the experimental results by simply assuming the ATP binding step is voltage dependent. Instead, our findings suggest that the gating step after the ATP binding step accounts for the voltage dependence. Notably, the fact that the gating step is rate limiting and much slower than the ATP binding step can also explain and reproduce the ATP dependence.

In the case of voltage-gated K^+ channels, a gating hinge is known to be present in the middle of the last (sixth) TM segment (Jiang *et al.* 2002; Magidovich & Yifrach, 2004; Ding *et al.* 2005). We identified Gly344 situated in the middle of the last (second) TM segment as the gating hinge in the rat $P2X_2$ channel (Fujiwara *et al.* 2009). By substituting an Ala for Gly344 (G344A), we were able to largely eliminate the voltage dependence of $P2X_2$ activation (a G344P mutant showed a slower voltage-dependent activation). Furthermore, by reintroducing a Gly at position 344 of the G344A mutant, voltage-dependent gating could be restored. Thus, in a manner analogous to voltage-gated K^+ channels, Gly344 serves as is the gating hinge involved in the voltage-gated activation of the $P2X_2$ channel (Fujiwara *et al.* 2009).

The amino acid residues of the second transmembrane region of $rP2X_2$ and $zP2X_4$ are aligned in Fig. 2*A*. As shown, Gly344 of $rP2X_2$ is not conserved in $zP2X_4$. The residues Leu340, Gly343 and Ala344 in $zP2X_4$ were shown to form the narrowest part of the pore (Kawate *et al.* 2009). The position in the crystal structure of these amino acid residues as well as Ile340 (Ile328 of $rP2X_2$) in the previous section is marked in Fig. 2*B*. The structure shown is that of only one subunit observed from the pore centre side. Based on these data, we speculate that the structure, especially the position of the gating hinge, differs significantly between the two clones. The difference might partly explain the difference in the extent of pore dilatation.

Future aspects

Expression density-dependent changes. Channel density-dependent modulation of pore properties is a novel mechanism for regulating $P2X_2$ channel activity, but a similar mechanism could account for the functional regulation of other membrane proteins. For example, the outward rectification of Transient Receptor Potential (TRP) channel currents in *Xenopus* oocytes shows similar amplitude-dependent changes (Nagatomo & Kubo, 2008).

Figure 1 illustrates a proposed mechanism whereby mutual interaction among activated channels determines density-dependent pore properties (Fujiwara & Kubo, 2004). In addition, there may be cases in which density-dependent changes in pore properties reflect the availability of one or more auxiliary molecules, such as a specific lipid. It would therefore be of interest to carry out analyses in other expression systems, including mammalian cultured cell lines.

We carried out single particle structure analyses of negatively stained electron microscopic images (Mio *et al.* 2005) and cryo-electron microscopic images (Mio *et al.* 2009), which enabled us to determine that the $P2X_2$ channel has a vase-like structure with lateral tunnels above the membrane. Although the resolution is much lower than that obtained with X-ray crystallography (Kawate *et al.* 2009), this approach may be better suited for analysis of density-dependent changes to the channel structure. To reproduce various densities, it might be effective to carry out single particle structure analyses using liposomes with embedded recombinant proteins (Wang & Sigworth, 2009). Since the density of recombinant proteins on liposome membranes can be manipulated, one would be able to analyse density-dependent structural changes, which are hard to analyse within a tightly packed crystal.

From a physiological point of view, it would be interesting to see the behavioural changes elicited in knock-in mice carrying the I328C mutation or a different $P2X_2$ mutant, whose density-dependent pore properties are altered with no obvious changes to other basic functions.

Voltage-dependent activation. We reported that the fast ATP binding step and the rate-limiting voltage-dependent gating step could reproduce the experimental results (Fujiwara *et al.* 2009). The most important and interesting questions that arise from those observations have to do with the structural background of the voltage dependence of the gating step. What happens after ATP binds? How is information about ATP binding transmitted to the main body of the channel to open the gate? Why is this step voltage dependent?

Through systematic mutagenic analysis, the ATP binding region in the extracellular loop was identified and shown to be rich in positive charges (Roberts *et al.* 2006). The contributions of amino acid residues in TM1 and TM2 to ATP-evoked gating and/or permeation have been studied extensively (Surprenant & North, 2008). It may be that a complex comprised of the negatively

charged ATP and the positively charged binding site interacts directly or indirectly with the extracellular end of the TM segment involved in the gating, and that the interaction with the TM segment is under the influence of the electric field. The mutagenesis studies cited above were carried out from the point of view of ATP-induced activation, but so far no studies have been done from the point of view of voltage dependence. It would therefore seem worthwhile to characterize the voltage dependence of $P2X_2$ channels with mutations in the ATP binding region, linker region and/or the upper TM regions, and to examine the effect of ATP analogues carrying four negative charges.

Voltage dependence has also been reported for metabotropic receptors such as muscarinic acetylcholine receptors (Ben-Chaim *et al.* 2003, 2006) and metabotropic glutamate receptors (Ohana *et al.* 2006). Although the biochemical analyses performed by these groups indicate that the ligand binding step itself is voltage dependent, our findings suggest that the voltage dependence of the binding step cannot explain the results (Fujiwara *et al.* 2009), though we cannot exclude the possibility that the binding step also has voltage dependence. Biochemical analysis of the binding would clarify this point.

The ATP binding site was clearly identified in the crystal structure of $zP2X_4$ (Kawate *et al.* 2009). It was shown to locate on the surface of the intersubunit boundary at a position distant from the transmembrane regions (Fig. 2*B*). Judging from the location of the binding site, it seems unlikely that the ATP binding site is in the electric field and that the ATP binding step is voltage dependent. Also, the interaction of the ATP–ATP binding site complex with the transmebrane regions mentioned above would be indirect and possibly mediated by the linker region.

Ben-Chaim *et al.* (2006) successfully recorded gating currents from muscarinic receptors, although the z value is relatively small. It would also be worth trying to record gating currents from cells expressing $P2X_2$ receptors. Of particular interest would be whether the gating current was observed only when ATP was bound or whether it could also be observed in the absence of ATP. If the origin of voltage dependence is the formation of a complex comprised of ATP and its binding site, movement of the gating charge would be recorded only when ATP is bound. Another powerful approach to characterizing the dynamic aspects of channel activity is real-time measurement of conformational changes using an optical technique. For instance, Fluorescent Resonance Energy Transfer (FRET)-based analyses have revealed conformational changes in the cytoplasmic region of $P2X_2$ channels during ATP-evoked activation and desensitization (Fisher *et al.* 2004). It would be interesting to apply this technique to analyse the conformational changes during the voltage-dependent gating.

References

Barrera NP, Ormond SJ, Henderson RM, Murrell-Lagnado RD & Edwardson JM (2005). Atomic force microscopy imaging demonstrates that $P2X_2$ receptors are trimers but that $P2X_6$ receptor subunits do not oligomerize. *J Biol Chem* **280**, 10759–10765.

Ben-Chaim Y, Chanda B, Dascal N, Bezanilla F, Parnas I & Parnas H (2006). Movement of 'gating charge' is coupled to ligand binding in a G-protein-coupled receptor. *Nature* **444**, 106–109.

Ben-Chaim Y, Tour O, Dascal N, Parnas I & Parnas H (2003). The M_2 muscarinic G-protein-coupled receptor is voltage-sensitive. *J Biol Chem* **278**, 22482–22491.

Brake AJ, Wagenbach MJ & Julius D (1994). New structural motif for ligand-gated ion channels defined by an ionotropic ATP receptor. *Nature* **371**, 519–523.

Burnstock G (2007). Physiology and pathophysiology of purinergic neurotransmission. *Physiol Rev* **87**, 659–797.

Charnet P, Labarca C, Cohen BN, Davidson N, Lester HA & Pilar G (1992). Pharmacological and kinetic properties of $\alpha 4\beta 2$ neuronal nicotinic acetylcholine receptors expressed in *Xenopus* oocytes. *J Physiol* **450**, 375–394.

Ding S, Ingleby L, Ahern CA & Horn R (2005). Investigating the putative glycine hinge in *Shaker* potassium channel. *J Gen Physiol* **126**, 213–226.

Ding S & Sachs F (1999). Single channel properties of $P2X_2$ purinoceptors. *J Gen Physiol* **113**, 695–720.

Edwards FA, Gibb AJ & Colquhoun D (1992). ATP receptor-mediated synaptic currents in the central nervous system. *Nature* **359**, 144–147.

Eickhorst AN, Berson A, Cockayne D, Lester HA & Khakh BS (2002). Control of $P2X_2$ channel permeability by the cytosolic domain. *J Gen Physiol* **120**, 119–131.

Evans RJ, Derkach V & Surprenant A (1992). ATP mediates fast synaptic transmission in mammalian neurons. *Nature* **357**, 503–505.

Figl A, Labarca C, Davidson N, Lester HA & Cohen BN (1996). Voltage-jump relaxation kinetics for wild-type and chimeric beta subunits of neuronal nicotinic receptors. *J Gen Physiol* **107**, 369–379.

Fisher JA, Girdler G & Khakh BS (2004). Time-resolved measurement of state-specific $P2X_2$ ion channel cytosolic gating motions. *J Neurosci* **24**, 10475–10487.

Fujiwara Y, Keceli B, Nakajo K & Kubo Y (2009). Voltage- and [ATP]-dependent gating of the $P2X_2$ ATP receptor channel. *J Gen Physiol* **133**, 93–109.

Fujiwara Y & Kubo Y (2004). Density-dependent changes of the pore properties of the $P2X_2$ receptor channel. *J Physiol* **558**, 31–43.

Fujiwara Y & Kubo Y (2006). Regulation of the desensitization and ion selectivity of ATP-gated $P2X_2$ channels by phosphoinositides. *J Physiol* **576**, 135–149.

Jiang Y, Lee A, Chen J, Cadene M, Chait BT & MacKinnon R (2002). Crystal structure and mechanism of a calcium-gated potassium channel. *Nature* **417**, 515–522.

Kawate T, Michel JC, Birdsong WT & Gouaux E (2009). Crystal structure of the ATP-gated $P2X_4$ ion channel in the closed state. *Nature* **460**, 592–598.

Khakh BS (2001). Molecular physiology of P2X receptors and ATP signalling at synapses. *Nat Rev Neurosci* **2**, 165–174.
Khakh BS & Lester HA (1999). Dynamic selectivity filters in ion channels. *Neuron* **23**, 653–658.
Khakh BS, Zhou X, Sydes J, Galligan JJ & Lester HA (2000). State-dependent cross-inhibition between transmitter-gated cation channels. *Nature* **406**, 405–410.
Koizumi S, Fujishita K & Inoue K (2005). Regulation of cell-to-cell communication mediated by astrocytic ATP in the CNS. *Purinergic Signal* **1**, 211–217.
Koshimizu T, Koshimizu M & Stojilkovic SS (1999). Contributions of the C-terminal domain to the control of P2X receptor desensitization. *J Biol Chem* **274**, 37651–37657.
Magidovich E & Yifrach O (2004). Conserved gating hinge in ligand- and voltage-dependent K^+ channels. *Biochemistry* **43**, 13242–13247.
Mio K, Kubo Y, Ogura T, Yamamoto T & Sato C (2005). Visualization of the trimeric $P2X_2$ receptor with a crown-capped extracellular domain. *Biochem Biophys Res Commun* **337**, 998–1005.
Mio K, Ogura T, Yamamoto T, Hiroaki Y, Fujiyoshi Y, Kubo Y & Sato C (2009). Reconstruction of the $P2X_2$ receptor reveals a vase-shaped structure with lateral tunnels above the membrane. *Structure* **17**, 266–275.
Nagatomo K & Kubo Y (2008). Caffeine activates mouse TRPA1 channels but suppresses human TRPA1 channels. *Proc Natl Acad Sci U S A* **105**, 17373–17378.
Nakazawa K (1994). ATP-activated current and its interaction with acetylcholine-activated current in rat sympathetic neurons. *J Neurosci* **14**, 740–750.
Nakazawa K, Liu M, Inoue K & Ohno Y (1997). Voltage-dependent gating of ATP-activated channels in PC12 cells. *J Neurophysiol* **78**, 884–890.
Nakazawa K & Ohno Y (2005). Characterization of voltage-dependent gating of $P2X_2$ receptor/channel. *Eur J Pharmacol* **508**, 23–30.
North RA (2002). Molecular physiology of P2X receptors. *Physiol Rev* **82**, 1013–1067.
Ohana L, Barchad O, Parnas I & Parnas H (2006). The metabotropic glutamate G-protein-coupled receptors mGluR3 and mGluR1a are voltage-sensitive. *J Biol Chem* **281**, 24204–24215.
Roberts JA, Vial C, Digby HR, Agboh KC, Wen H, Atterbury-Thomas A & Evans RJ (2006). Molecular properties of P2X receptors. *Pflugers Arch* **452**, 486–500.
Smith FM, Humphrey PP & Murrell-Lagnado RD (1999). Identification of amino acids within the $P2X_2$ receptor C-terminus that regulate desensitization. *J Physiol* **520**, 91–99.
Surprenant A & North RA (2008). Signalling at purinergic P2X receptors. *Annu Rev Physiol*. **71**, 333–359.
Valera S, Hussy N, Evans RJ, Adami N, North RA, Surprenant A & Buell G (1994). A new class of ligand-gated ion channel defined by P2X receptor for extracellular ATP. *Nature* **371**, 516–519.
Virginio C, MacKenzie A, Rassendren FA, North RA & Surprenant A (1999). Pore dilation of neuronal P2X receptor channels. *Nat Neurosci* **2**, 315–321.
Wang L & Sigworth FJ (2009). Structure of the BK potassium channel in a lipid membrane from electron cryomicroscopy. *Nature* **461**, 292–295.
Zhou Z & Hume RI (1998). Two mechanisms for inward rectification of current flow through the purinoceptor $P2X_2$ class of ATP-gated channels. *J Physiol* **507**, 353–364.

Author's present address

Y. Fujiwara: Department of Integrative Physiology, Graduate School and Faculty of Medicine, Osaka University, Osaka 565-0871, Japan.