

Mutation in Nucleotide-Binding Domains of Sulfonylurea Receptor 2 Evokes Na-ATP-Dependent Activation of ATP-Sensitive K⁺ Channels: Implication for Dimerization of Nucleotide-Binding Domains to Induce Channel Opening

Mitsuhiko Yamada,¹ Masaru Ishii, Hiroshi Hibino, and Yoshihisa Kurachi

Department of Pharmacology II, Graduate School of Medicine Osaka University, Suita, Osaka, Japan

Received May 13, 2004; accepted July 13, 2004

ABSTRACT

The ATP-sensitive K⁺ (K_{ATP}) channel is composed of a sulfonylurea receptor (SUR) and a pore-forming subunit, Kir6.2. SUR is an ATP-binding cassette (ABC) protein with two nucleotide-binding domains (NBD1 and NBD2). Intracellular ATP inhibits K_{ATP} channels through Kir6.2 and activates them through NBDs. However, it is still unknown how ATP-bound NBDs activate K_{ATP} channels. A prokaryotic ABC protein, MJ0796, which is entirely NBD, forms a dimer in the presence of Na-ATP when its glutamate at position 171 is substituted with glutamine. Mg²⁺ or K⁺ destabilizes the dimer. We made the corresponding mutation in the NBD1 (D834N) and/or NBD2 (E1471Q) of SUR2A and SUR2B. As measured in the inside-out configuration of the patch-clamp method, SUR2x(D834N, E1471Q)/Kir6.2 channels mediated significantly larger currents in the presence of internal 1 mM Na-ATP than K-ATP alone or

Mg-ATP. The response to Na-ATP resulted from an increase in the open probability but not single-channel amplitude of the channels and was abolished by glibenclamide (10⁻⁵ M). In the presence of 1 mM Mg²⁺-free ATP, Na⁺ increased the activity of the channels in a concentration-dependent manner. The Na-ATP-dependent activation was never observed with K_{ATP} channels including either the wild-type SUR2x, SUR2x(D834N), or SUR2x(E1471Q). Nicorandil activated SUR2x(D834N, E1471Q)/Kir6.2 channels more strongly in the presence of Na-ATP than K-ATP alone, whereas the reverse was true for wild-type SUR2x/Kir6.2 channels. Therefore, it is likely that NBDs of SUR2x dimerize in response to ATP and nicorandil. The dimerization induces the opening of the K_{ATP} channel, probably by causing a conformational change of SUR2x.

The ATP-sensitive K⁺ (K_{ATP}) channel is an inwardly rectifying K⁺ channel that is inhibited by intracellular ATP (Terzic et al., 1995). This channel is a heterooctamer composed of four Kir6.2 subunits and four sulfonylurea receptors (SURs) (Fig. 8A) (Clement et al., 1997; Shyng et al., 1997; Babenko et al., 1998; Seino, 1999). Kir6.2 is a K⁺ channel subunit with two transmembrane α -helices and forms the channel pore (Inagaki et al., 1995). SUR is a member of the multidrug resistance-associated protein/cystic fibrosis transmembrane conductance regulator subfamily of ABC proteins

with 17 transmembrane α -helices that are subdivided into TMD0–2 (Babenko et al., 1998; Klein et al., 1999). SUR has two cytoplasmic nucleotide-binding domains between TMD1 and TMD2 (NBD1) and at the C terminus following TMD2 (NBD2). SUR interacts with Kir6.2 through TMD0 and L0, cytoplasmic linker connecting TMD0 and TMD1 (Babenko and Bryan, 2003; Chan et al., 2003). There are two genes of SUR encoding SUR1 and SUR2, and two isoforms of SUR2 (SUR2A and SUR2B), which differ only in the C-terminal 42 amino acids (C42) (Aguilar-Bryan et al., 1995; Inagaki et al., 1995; Isomoto et al., 1996). Intracellular ATP inhibits K_{ATP} channels through Kir6.2 and activates them through NBDs of SUR (Tucker et al., 1997; Gribble et al., 1998). K⁺ channel openers such as nicorandil [*N*-(2-hydroxyethyl) nicotinamide nitrate] activate K_{ATP} channels by binding to the 17th transmembrane α -helix of SUR (Moreau et al., 2000; Reimann et al., 2001).

We previously proposed that the nucleotide- and drug-induced activation of K_{ATP} channels arises from a conforma-

This work was supported by a grant-in-aid for scientific research on priority areas B from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to Y.K.) and a grant-in-aid for scientific research C from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to M.Y.).

¹ Current address: Department of Molecular Pharmacology, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto, Nagano 390-8621, Japan.

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.
doi:10.1124/mol.104.002717.

ABBREVIATIONS: K_{ATP} channel, ATP-sensitive K⁺ channel; SUR, sulfonylurea receptor; ABC, ATP-binding cassette; TMD, transmembrane domain; NBD, nucleotide-binding domain; HEK, human embryonic kidney.

tion change of SUR (Yamada and Kurachi, 2004). That is, SUR2x (SUR2A or SUR2B) has at least two distinct conformations that are able (R conformation) and unable (T conformation) to open the channel pore (Monod et al., 1965). The two conformations are intrinsically in equilibrium. NBDs and the drug receptor in the R conformation have higher affinity for respective ligands than those in the T conformation. Therefore, nucleotides or nicorandil shift the equilibrium toward the R conformation to open the channel pore. Based on this model, we analyzed the response of SUR2x/Kir6.2 channels to nucleotides and nicorandil and concluded that both nucleotide-bound NBD1 and NBD2 have a higher ability to cause the T-R transition in SUR2B than SUR2A. Thus, C42 modulates the function of both NBD1 and NBD2 (Matsuoka et al., 2000; Matsushita et al., 2002; Yamada and Kurachi, 2004). C42 is on only five amino acids C-terminal to NBD2 but more than 630 amino acids C-terminal to NBD1 (Inagaki et al., 1995; Isomoto et al., 1996). Therefore, in the tertiary structure, NBD1 seems to be in the vicinity of NBD2-C42. This raises the possibility that NBD1 and NBD2 dimerize as is the case for the NBD of some ABC proteins (Hopfner et al., 2000; Chang and Roth, 2001; Locher et al., 2002; Smith et al., 2002; Chang, 2003).

In this study, we sought to obtain further evidence in favor of the dimerization of NBDs with a different method and identify the functional significance of the dimerization. MJ0796, a prokaryotic ABC protein that is entirely NBD, forms an ATP-sandwich dimer in the presence of Na-ATP when the glutamate residue directly adjacent to the C terminus of the Walker B motif is substituted with glutamine (E171Q) (Fig. 1) (Moody et al., 2002; Smith et al., 2002). K^+ or Mg^{2+} weakens this dimer. This unusual dependence on cation cofactors seems to arise from the altered electrostatic charge balance of the active site caused by the mutation. The residue corresponding to E171 of MJ0796 is aspartate in NBD1 (D834) and glutamate in NBD2 (E1471) of SUR2x. We replaced these residues with asparagine and glutamine, respectively (D834N and E1471Q). SUR2x carrying both but not either of the mutations formed with Kir6.2 a K_{ATP} channel with significantly higher activity in the presence of Na-ATP than K-ATP alone or Mg-ATP. Nicorandil activated SUR2x(D834N, E1471)/Kir6.2 more strongly in the presence of Na-ATP than K-ATP alone, whereas the reverse was true for wild-type SUR2x/Kir6.2 channels. These results support our previous hypothesis that NBD1 and NBD2 of SUR2x dimerize. The results further indicate 1) that the dimerization leads to the opening of K_{ATP} channels, and 2) that this

reaction is also critically involved in the drug-induced activation of K_{ATP} channels.

Materials and Methods

Site-Directed Mutagenesis and Expression of Recombinant ATP-Sensitive K^+ Channels. Site-directed mutagenesis was carried out on cDNAs of mouse SUR2A and SUR2B subcloned into the expression vector pcDNA3 with QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Human embryonic kidney (HEK293T) cells were cotransfected with either of these plasmids, pcDNA3 containing mouse Kir6.2, and pd2EGFP (BD Biosciences Clontech, Palo Alto, CA) by using LipofectAMINE (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction as described previously (Yamada and Kurachi, 2004). The cells that expressed green fluorescent protein were identified by fluorescence microscopy and used for electrophysiology.

Electrophysiology. ATP-sensitive K^+ channels expressed in HEK293T cells were analyzed with the inside-out configuration of the patch-clamp method as described previously (Yamada and Kurachi, 2004). The pipette solution contained 140 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM HEPES (pH 7.3 with KOH). The internal side of the inside-out patch membranes was perfused with either of the three types of the internal solutions (the internal solutions A–C) whose compositions are summarized in Table 1. Internal solution A contained free Mg^{2+} concentration of 1.4 mM. When 1 mM ATP was added to the internal solution A, 1 mM $MgCl_2$ was added to the internal solution. Nicorandil was a gift from Chugai Pharmaceutical Co. Ltd. (Tokyo, Japan). It was dissolved at 2 M in glacial acetic acid and diluted before use to the desired concentration in the internal solution B or C. When nicorandil was used at final concentration of 3 and 10 mM in the internal solution B, ~25 and ~70 mM KOH was required to adjust pH to 7.3, respectively. In these experiments, the appropriate amount of KCl was removed from the internal solution, and ATP was added to these solutions after adjustment of pH to prevent hydrolysis of the nucleotide at acidic pH. Then, pH was readjusted. Similarly, the pH of the internal solution C containing 80 mM KCl, 60 mM NaCl, and 3 or 10 mM nicorandil was adjusted with the solution containing 2.9 mM KOH and 2.1 mM NaOH.

Statistical Analysis. All statistical values are indicated as mean \pm S.E. The statistical difference was evaluated by Student's *t* test. A value of $p < 0.05$ was considered statistically significant.

Results

Effect of Na-ATP on K_{ATP} Channel Containing SUR2x with or without D834N and/or E1471Q Mutations. The glutamate residue directly adjacent to the aspartate residue terminating the Walker B motif (Fig. 1, arrow) is well conserved among different types of ABC proteins (Yoshida and Amano, 1995; Geourjon et al., 2001). From the

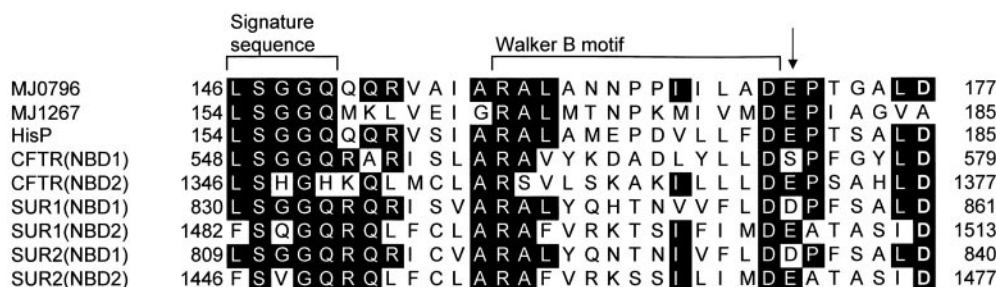


Fig. 1. Alignment of the signature sequence and Walker B motif of different ABC proteins. The signature sequence and Walker B motif of different ABC proteins are aligned. The glutamate residue directly adjacent to an aspartate residue terminating the Walker B motif (arrow) is predicted to serve as the catalytic base in MJ0796 and HisP (Hung et al., 1998; Smith et al., 2002). In this study, aspartate at this position in NBD1 of SUR2 (D834) was substituted with asparagine (D1N), and/or glutamate at this position in NBD2 of SUR2 (E1471) was replaced with glutamine (E2Q).

crystal structure of ABC proteins, this residue is predicted to serve as a catalytic base (Hung et al., 1998; Smith et al., 2002). Substitution of this residue with glutamine impairs the ATPase activity of different types of ABC proteins (Geourjon et al., 2001). A prokaryotic ABC protein, MJ0796 carrying this mutation [MJ0796(E171Q)] forms a stable ATP sandwich dimer in the presence of Na-ATP (Smith et al., 2002). K^+ or Mg^{2+} destabilizes the dimer (Moody et al., 2002). In SUR, NBD1 and NBD2 have an aspartate and glutamate residue at this position, respectively. We made the mutation to remove a negative charge from these residues in NBD1 (D834N) (D1N) and/or NBD2 (E1471Q) (E2Q) of SUR2A and SUR2B. The mutant SUR2x was coexpressed with Kir6.2 in HEK293T cells. The formed K_{ATP} channels were analyzed at -60 mV with the pipette solution containing 140 mM K^+ in the inside-out configuration of the patch-clamp method.

TABLE 1

Internal solutions used in this study

The concentration of each substance is indicated in millimolar concentration. The pH of all solutions was adjusted to 7.3.

Internal Solution	KCl	NaCl	MgCl ₂	EGTA	EDTA	HEPES
A	140	0	2 ^a	5	0	5
B	140	0	0	0	5	5
C	140- x^b	x^b	0	0	5	5

^a When 1 mM ATP was added to the internal solution A, 1 mM $MgCl_2$ was further added to maintain the free Mg^{2+} concentration.

^b Value of x was 30, 60, 90, or 120.

Figure 2A shows the currents mediated by SUR2A(D1N,

E2Q)/Kir6.2 channels. Upon excision of the patch in the internal solution A, which contained 140 mM KCl, 0 mM NaCl, and 2 mM $MgCl_2$ (green line) (Table 1), the spontaneous opening of the channels occurred. Addition of 1 mM ATP strongly inhibited the channel current. After washout of ATP, the stable spontaneous opening recovered. Under this condition, the internal solution A was switched to the internal solution B, which contained 140 mM KCl, 0 mM NaCl, and 0 mM $MgCl_2$ (blue line) (Table 1). Addition of 1 mM ATP again strongly inhibited the channel activity. In the continuous presence of ATP, the internal solution B was switched to the internal solution C, which contained 20 mM KCl, 120 mM NaCl, and 0 mM $MgCl_2$ (red line) (Table 1). Then, the channel current increased over seconds. This current was almost completely inhibited by 10 μ M glibenclamide, a specific inhibitor of K_{ATP} channels. The channel current was restored barely after washout of glibenclamide but promptly after removal of ATP. When the internal solution C was returned to the internal solution B, the spontaneous activity only slightly increased although the driving force for K^+ substantially increased on this occasion (for details, see below). Na-ATP did not significantly increase the activity of K_{ATP} channels composed of Kir6.2 and either SUR2A(D1N), SUR2A(E2Q), or the wild-type SUR2A [SUR2A(Wt)] (Fig. 2, B–D). On average, SUR2A(D1N, E2Q)/Kir6.2 channels had significantly higher activity in the presence of Na-ATP than K-ATP alone or Mg-ATP (Fig. 2E). SUR2A(D1N, E2Q)/Kir6.2 channels also had significantly higher activity than

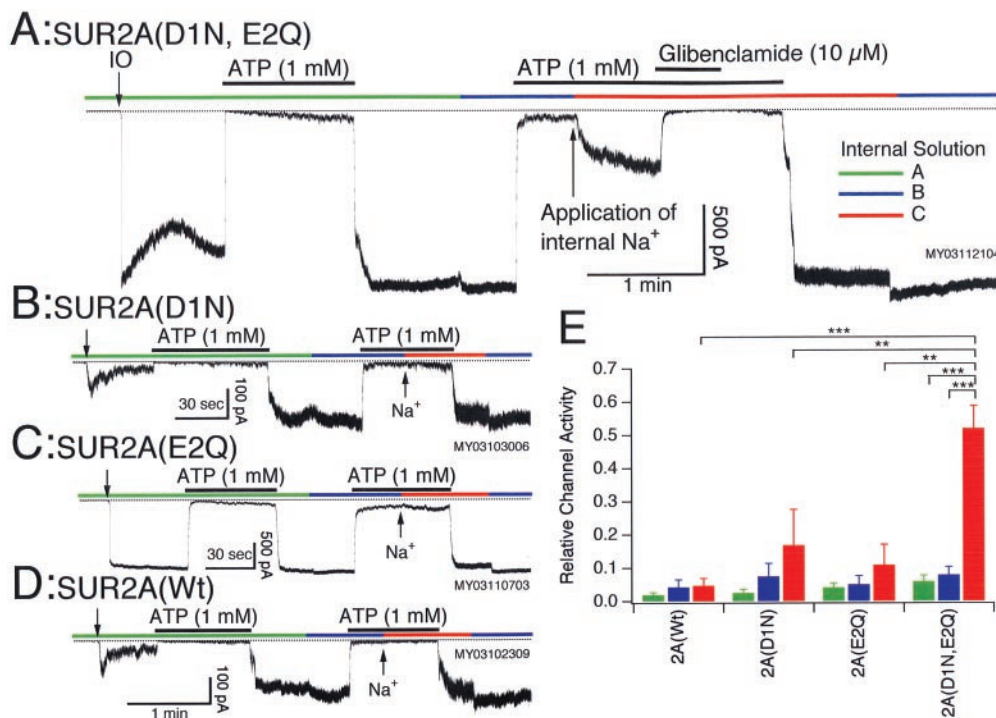


Fig. 2. Effect of Na-ATP on K_{ATP} channel containing SUR2A with or without D834N and/or E1471Q mutations. A to D, all the current traces were recorded at -60 mV in the cell-attached or inside-out configuration with 140 mM K^+ in pipettes. The patch membrane was excised from cells at the timing indicated by downward arrows. Dotted lines indicate the zero current level. Thick lines above the traces indicate the bath perfusion protocol. Green, blue, and red lines indicate the perfusion of the internal solution A, B and C, respectively (Table 1). In these experiments, the internal solution C contained 20 mM KCl and 120 mM NaCl. Upward arrows indicate the timing at which the internal solution B was switched to the internal solution C. The currents were measured from the membranes containing SUR2A(D1N, E2Q)/Kir6.2 (A), SUR2A(D1N)/Kir6.2 (B), SUR2A(E2Q)/Kir6.2 (C), or wild-type SUR2A/Kir6.2 (D) channels. E, activity of each type of the channels in the presence of 1 mM ATP normalized to that in the absence of ATP under the same ionic condition (relative channel activity). The activity was measured in the presence of either the internal solution A (green bars), B (blue bars), or C (red bars). The graph shows the mean and S.E.M. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

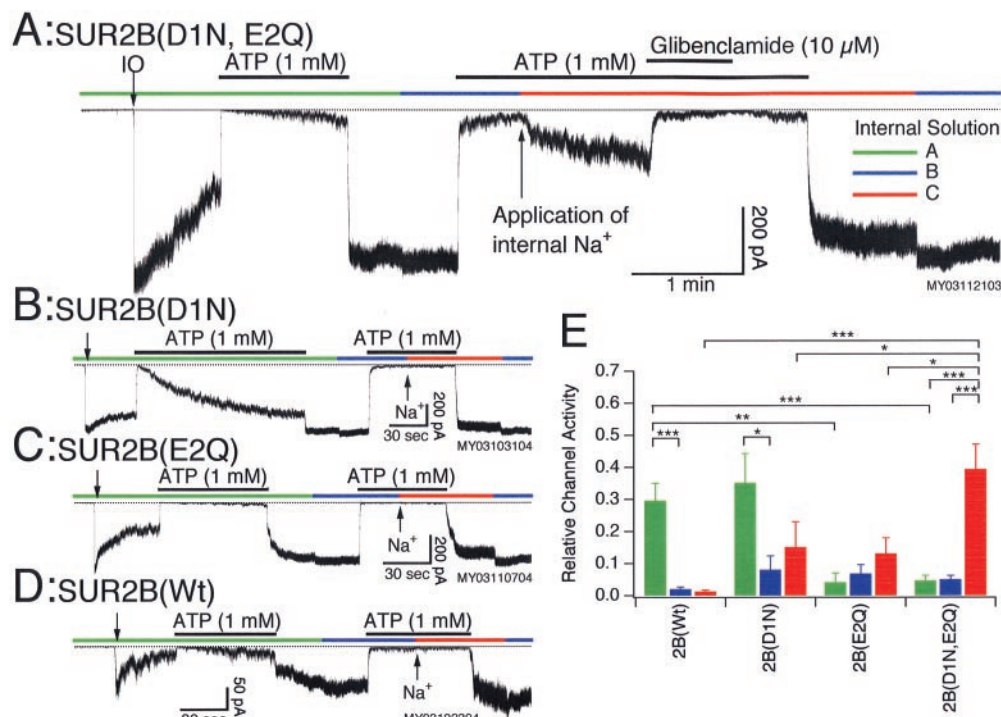


Fig. 3. Effect of Na-ATP on K_{ATP} channel containing SUR2B with or without D834N and/or E1471Q mutations. A to D, experimental protocol was the same as in Fig. 2. The currents were measured from the inside-out patch membranes containing SUR2B(D1N, E2Q)/Kir6.2 (A), SUR2B(D1N)/Kir6.2 (B), SUR2B(E2Q)/Kir6.2 (C), or wild-type SUR2B/Kir6.2 (D) channels. E, relative channel activity in the presence of either the internal solution A (green bars), B (blue bars), or C (red bars) containing 1 mM ATP. The graph shows the mean and S.E.M. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

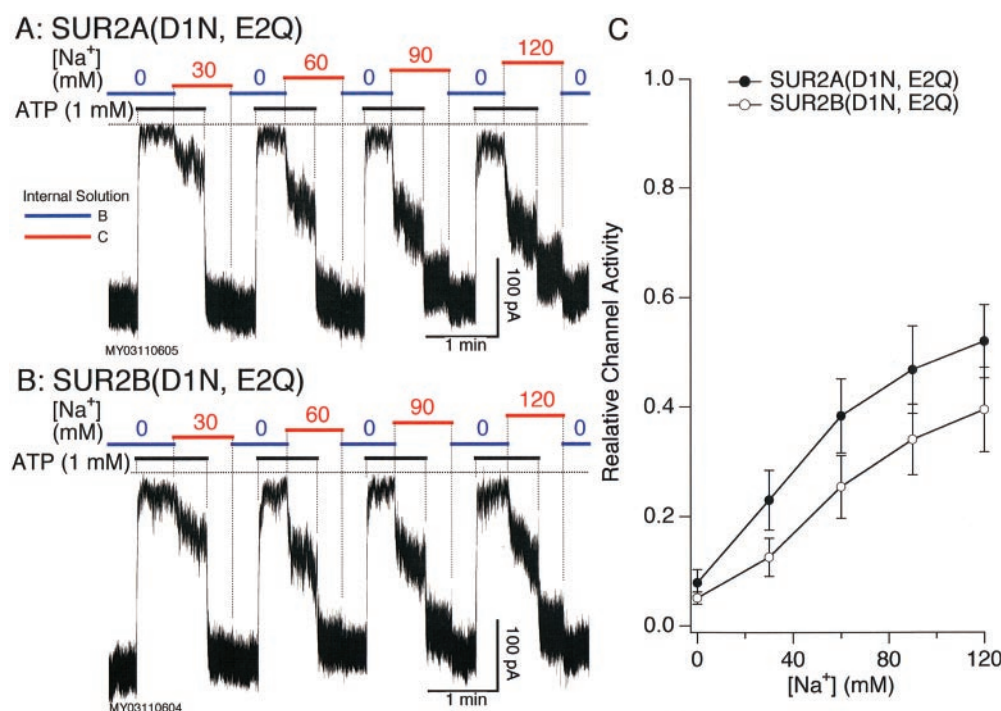


Fig. 4. Concentration-dependent effect of internal Na^+ on SUR2x(D834N, E1471Q)/Kir6.2 channels in the presence of ATP. A and B, current traces were recorded at -60 mV in the inside-out configurations with 140 mM K^+ in pipettes. Horizontal dotted lines indicate the zero current level. Thick lines above the traces indicate the bath perfusion protocol. Blue and red lines indicate the perfusion of the internal solution B or C, respectively. The concentration of internal Na^+ is indicated above the bars. Vertical dotted lines indicate the timing at which the solutions were switched. The currents were measured from the inside-out patch membranes containing SUR2A(D1N, E2Q)/Kir6.2 (A) or SUR2B(D1N, E2Q)/Kir6.2 (B) channels. C, relationships between internal Na^+ concentration and the relative channel activity of SUR2A(D1N, E2Q)/Kir6.2 (closed circles) or SUR2B(D1N, E2Q)/Kir6.2 (open circles) channels in the presence of 1 mM ATP. Symbols and bars indicate the mean and S.E.M., respectively. There was no statistical difference between the activities of the two channels at each concentration of internal Na^+ .

SUR2A(D1N)/Kir6.2, SUR2A(E2Q)/Kir6.2 or SUR2A(Wt)/Kir6.2 channels in the presence of Na-ATP (Fig. 2E).

Figure 3 shows the results obtained with K_{ATP} channels composed of Kir6.2 and either SUR2B(D1N, E2Q), SUR2B(D1N), SUR2B(E2Q), or the wild-type SUR2B [SUR2B(Wt)]. The current mediated by SUR2B(D1N, E2Q)/Kir6.2 channels again significantly increased after switching of the internal solution from B to C in the presence of 1 mM ATP (Fig. 3A). This current was also inhibited by 10 μ M glibenclamide. The activity of SUR2B(D1N)/Kir6.2 channels was only partly inhibited and gradually increased in the presence of the internal solution A containing 1 mM ATP (Fig. 3B). This is reminiscent of the behavior of SUR2B(Wt)/Kir6.2 channels (Fig. 3D). We previously ascribed this phenomenon to ATP hydrolysis by NBD2 of SUR2B (Yamada and Kurachi, 2004). Consistent with this notion, ATP (1 mM) more strongly suppressed the activity of these channels after removal of internal Mg^{2+} (Fig. 3, B, D, and E). In addition, SUR2B(E2Q)/Kir6.2 channels whose ATPase activity of NBD2 should be impaired had significantly lower activity than SUR2B(D1N)/Kir6.2 or SUR2B(Wt)/Kir6.2 channels in the presence of the internal solution A containing 1 mM ATP (Fig. 3, C and E). Neither SUR2B(D1N)/Kir6.2, SUR2B(E2Q)/Kir6.2, nor SUR2B(Wt)/Kir6.2 channels significantly increased their activity when the internal solution B was switched to the internal solution C in the presence of 1 mM ATP (Fig. 3, B–D). On average, SUR2B(D1N, E2Q)/Kir6.2 channels had significantly higher activity in the presence of Na-ATP than K-ATP alone or Mg-ATP (Fig. 3E). SUR2B(D1N, E2Q)/Kir6.2 channels also had significantly higher activity than the other

SUR2B-containing K_{ATP} channels in the presence of Na-ATP (Fig. 3E).

From these results and the finding with MJ0796 (Moody et al., 2002; Smith et al., 2002), it is likely that NBD1 and NBD2 of SUR2x dimerize in the presence of ATP and thereby induce the opening of K_{ATP} channels.

Concentration-Dependent Effect of Internal Na^+ on the Activity of K_{ATP} Channels Containing SUR2x with D834N plus E1471Q Mutations in the Presence of ATP. We examined the relationship between internal Na^+ concentration and the activity of SUR2A(D1N, E2Q)/Kir6.2 or SUR2B(D1N, E2Q)/Kir6.2 channels in the presence of 1 mM Mg^{2+} -free ATP (Fig. 4). The experiments were done as follows. First, in the presence of the internal solution B, 1 mM ATP was added to the internal side of a patch membrane to inhibit the channel activity (Fig. 4, A and B). In the continuous presence of ATP, the internal solution was switched to the internal solution C containing 30 mM NaCl. Thereafter, ATP was washed out in the presence of the internal solution C with the same composition to measure the maximum channel activity under this ionic condition. Then, the internal solution was switched back to the internal solution B, and the next round of the experiment was started with the internal solution C containing 60 mM NaCl. In this way, we sequentially raised the concentration of internal Na^+ up to 120 mM. The channel currents measured in the presence of a given concentration of Na^+ and 1 mM ATP were normalized to that in the presence of the same concentration of

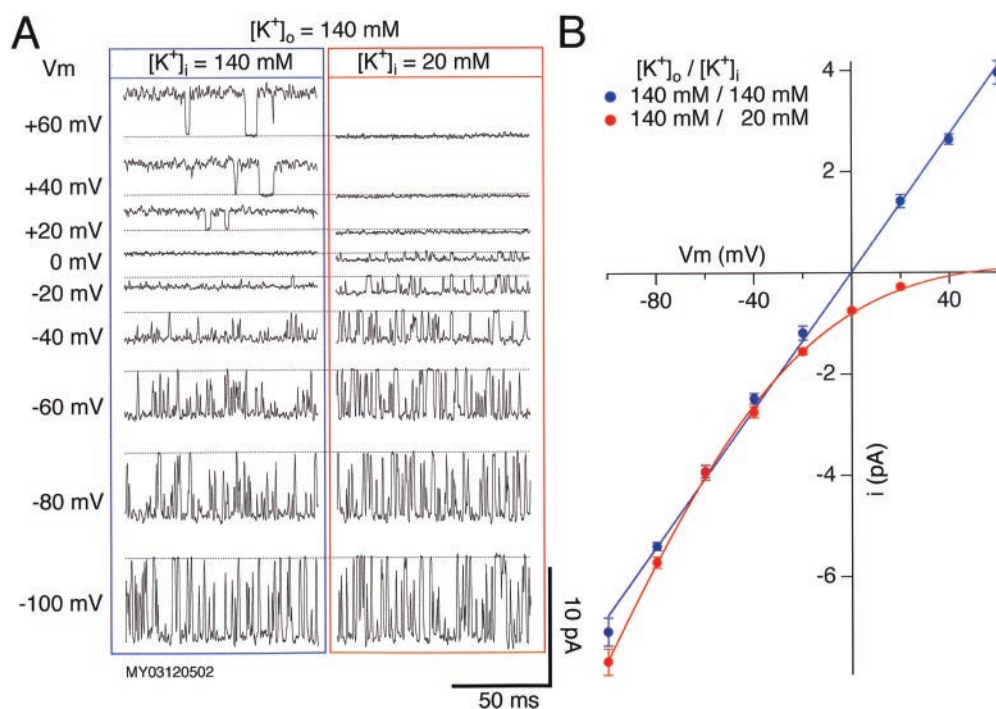


Fig. 5. Single-channel current-voltage relationship of SUR2x-containing K_{ATP} channels in the internal solution B or C. A, recordings obtained from an inside-out patch membrane containing a single SUR2A(D1N, E2Q)/Kir6.2 channel. The pipette solution contained 140 mM K^+ , whereas the internal side of the membrane was perfused with the internal solution B (left) or the internal solution C containing 20 mM K^+ and 120 mM Na^+ (right). These solutions did not contain ATP. The membrane was voltage-clamped at different membrane potentials as indicated at the left of the panels. Dotted lines indicate the zero current level at each potential. B, averaged single-channel current-voltage relationships measured with the pipette solution containing 140 mM K^+ and either the internal solution B (blue circles) or C with the same composition as in A (red circles). In the former case, the data were obtained from three membrane patches containing SUR2A(D1N, E2Q)/Kir6.2 channels, one containing SUR2B(E2Q)/Kir6.2 channels and three containing SUR2B(Wt)/Kir6.2 channels. In the latter case, the data were obtained from three membrane patches containing SUR2A(D1N, E2Q)/Kir6.2 channels, one containing SUR2A(Wt)/Kir6.2 channels, one containing SUR2B(E2Q)/Kir6.2 channels, and three containing SUR2B(Wt)/Kir6.2 channels. The blue and red lines are the fit of each set of the data with eqs. 1 and 2, respectively.

Na⁺ and no ATP (relative channel activity). Figure 4C shows the averaged data. Although the response was variable, internal Na⁺ increased the activity of SUR2A(D1N, E2Q)/Kir6.2 and SUR2B(D1N, E2Q)/Kir6.2 channels in a concentration-dependent manner. The relative channel activities of these two types of K_{ATP} channels were not significantly different at any concentrations of Na⁺.

Single-Channel Conductance of SUR2x-containing ATP-Sensitive K⁺ Channels in the Presence of Internal Na-ATP and K-ATP Alone. K_{ATP} channels are highly selective for K⁺ (Ashcroft, 1988). Nevertheless, a substitution of internal K⁺ with Na⁺ only slightly altered the magnitude of the spontaneous activity (Figs. 2–4). Thus, we analyzed the single-channel conductance of the SUR2x-containing K_{ATP}

channels in the presence of 140 mM K⁺ plus 0 mM Na⁺ and that in the presence of 20 mM K⁺ and 120 mM Na⁺ in the internal side of the patch membranes (Fig. 5). With the symmetrical 140 mM K⁺, the single SUR2A(D1N, E2Q)/Kir6.2 channel current reversed in direction at ~0 mV as expected (Fig. 5A, left) (Ashcroft, 1988). This current-voltage relationship was fit with the following function (Fig. 5B, blue circles and line):

$$i = a(V_m - b) \tag{1}$$

where *i* is the single-channel amplitude, *a* is the single-channel conductance, *V_m* is the membrane potential, and *b* is the reversal potential. The values of *a* and *b* were estimated as 67.8 pS and −0.24 mV, respectively.

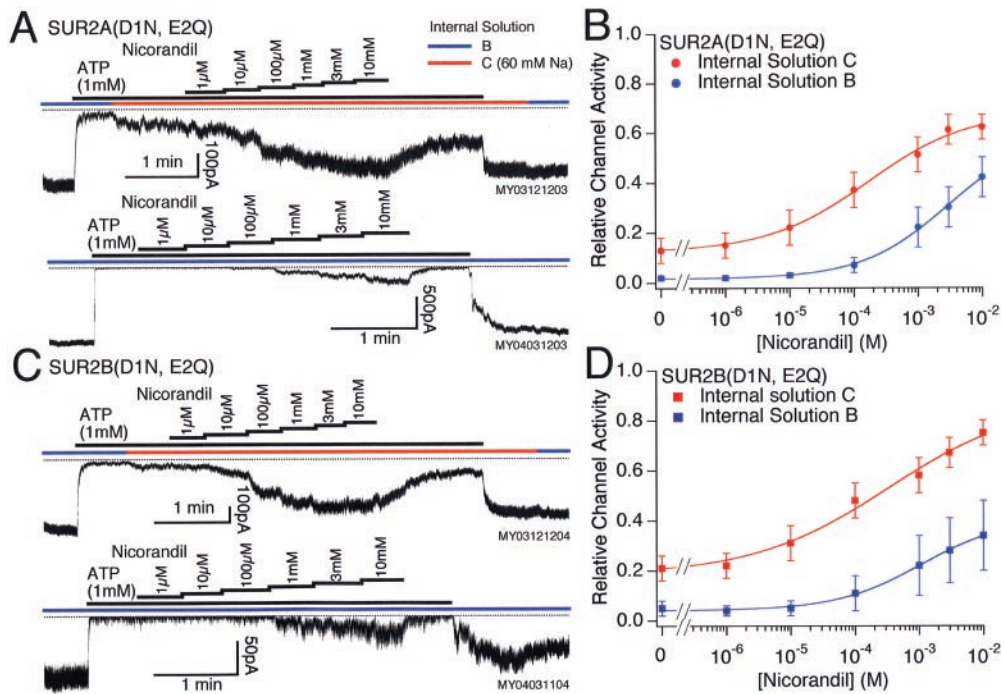


Fig. 6. Effect of nicorandil on SUR2x(D834N, E1471Q)/Kir6.2 channels in the presence of internal Na-ATP and K-ATP alone. A, current traces were recorded at −60 mV from the inside-out membrane patches containing SUR2A(D1N, E2Q)/Kir6.2 channels. Dotted lines indicate the zero current level. Thick lines above the traces indicate the bath perfusion protocol. Blue and red lines indicate the perfusion with the internal solution B or the internal solution C containing 80 mM K⁺ and 60 mM Na⁺, respectively. B, concentration-response relationships of the nicorandil-induced activation of SUR2A(D1N, E2Q)/Kir6.2 channels in the presence of Na-ATP (red circles) and K-ATP alone (blue circles). The ordinate indicates the channel activity normalized to that in the absence of ATP under the same internal ionic condition. Symbols and bars indicate the mean and S.E.M., respectively. Lines are the fit of each set of the data with the Hill equation (eq. 3). C, current traces recorded from the inside-out patch membranes containing SUR2B(D1N, E2Q)/Kir6.2 channels under the same condition as in A. D, concentration-response relationships of the nicorandil-induced activation of SUR2B(D1N, E2Q)/Kir6.2 channels in the presence of Na-ATP (red squares) and K-ATP alone (blue squares).

TABLE 2

Parameters used for the fitting of the concentration-response relationships of nicorandil-induced activation of SUR2x/Kir6.2 channels

The effect of nicorandil on the K_{ATP} channels containing SUR2x with or without D834N (D1N) plus E1471Q (E2Q) mutations was measured in the presence of the internal solution B or C containing 1 mM ATP. The concentration-response relationships were fit with eq. 3 with the following parameters.

	Type of Internal Solution			SUR Type	<i>a</i>	<i>b</i>	<i>K</i> _{1/2}	<i>h</i>
	[K ⁺] ^a	[Na ⁺] ^a	[Mg ²⁺] ^a					
C	80	60	0	SUR2A(D1N,E2Q)	0.12	0.56	0.17	0.55
				SUR2A(Wt)	0.01	0.14	0.98	1.52
				SUR2B(D1N,E2Q)	0.19	0.69	0.34	0.41
				SUR2B(Wt)	0.03	0.11	0.57	0.78
				SUR2A(D1N,E2Q)	0.02	0.50	2.95	0.65
B	140	0	0	SUR2A(Wt)	0.02	0.73	8.64	0.45
				SUR2B(D1N,E2Q)	0.04	0.37	1.09	0.65
				SUR2B(Wt)	0.04	0.40	1.12	0.49

In the presence of 20 mM K⁺ and 120 mM Na⁺ in the internal side of the patch membrane, a single SUR2A(D1N, E2Q)/Kir6.2 channel current was always inward at potentials negative to +20 mV (Fig. 5A, right). At +40 and +60 mV, the single-channel current amplitude was too small to be clearly identified. The current-voltage relationship under this condition was fit with the following function (Fig. 5B, red circles and line):

$$i = a(1/(1 + \exp((V_m - V_{0.5})/k)))(V_m - b) \quad (2)$$

where $V_{0.5}$ is V_m at which the single-channel conductance is half-maximum, and k is the slope factor. The values of a , $V_{0.5}$, k , and b were estimated as 73.7 pS, and -59.5 , $+48.1$, and $+48.7$ mV, respectively. The estimated reversal potential (i.e., $+48.7$ mV) was slightly negative to the theoretical K⁺ equilibrium potential under this condition (i.e., $+50.0$ mV). Thus, the channel current measured in the presence of 120 mM internal Na⁺ was indeed the K_{ATP} channel current. Because of the inward rectification of the single K_{ATP} channel current, the single-channel amplitude at -60 mV was almost identical in the presence of 140 mM K⁺ plus 0 mM Na⁺ and in the presence of 20 mM K⁺ plus 120 mM Na⁺ in the internal side of the patch membrane, accounting for the similar magnitude of the spontaneous activity under the two distinct ionic conditions. Therefore, Na-ATP increased the currents of SUR2x(D1N, E2Q)/Kir6.2 channels by increasing their NP_o (N is the number of the channels in a patch membrane, and P_o is the open

probability of each channel) but not the single-channel amplitude.

The Effect of Nicorandil on K_{ATP} Channels Containing SUR2x with or without D834N plus E1471Q Mutations in the Presence of Na-ATP and K-ATP Alone. Nicorandil, a K⁺ channel opener, activates SUR2x-containing K_{ATP} channels by interacting with the 17th transmembrane α -helix of SUR2x (Reimann et al., 2001). We previously proposed that this drug-receptor allosterically interacts with NBDs (Yamada and Kurachi, 2004). Therefore, we examined the effect of nicorandil on SUR2x(D1N, E2Q)/Kir6.2 channels in the presence of Na-ATP or K-ATP alone.

Nicorandil activated SUR2x(D1N, E2Q)/Kir6.2 channels more potently and efficiently in the presence of the internal solution C containing 60 mM NaCl, 80 mM KCl, and 1 mM ATP than the internal solution B containing 1 mM ATP (Fig. 6). The concentration-response relationships were fit with the following Hill equation:

$$y = a + b/(1 + (K_{1/2}/[\text{Nico}])^h) \quad (3)$$

where y is the relative channel activity, a is the relative channel activity in the absence of nicorandil, b is the fraction of the maximum relative channel activity induced by nicorandil, $K_{1/2}$ is the half-maximum effective concentration of nicorandil, $[\text{Nico}]$ is the concentration of nicorandil, and h is the Hill coefficient. The values of a , b , $K_{1/2}$, and h are summarized in Table 2. On the other hand, nicorandil activated SUR2x(Wt)/Kir6.2 channels less efficiently in the presence of

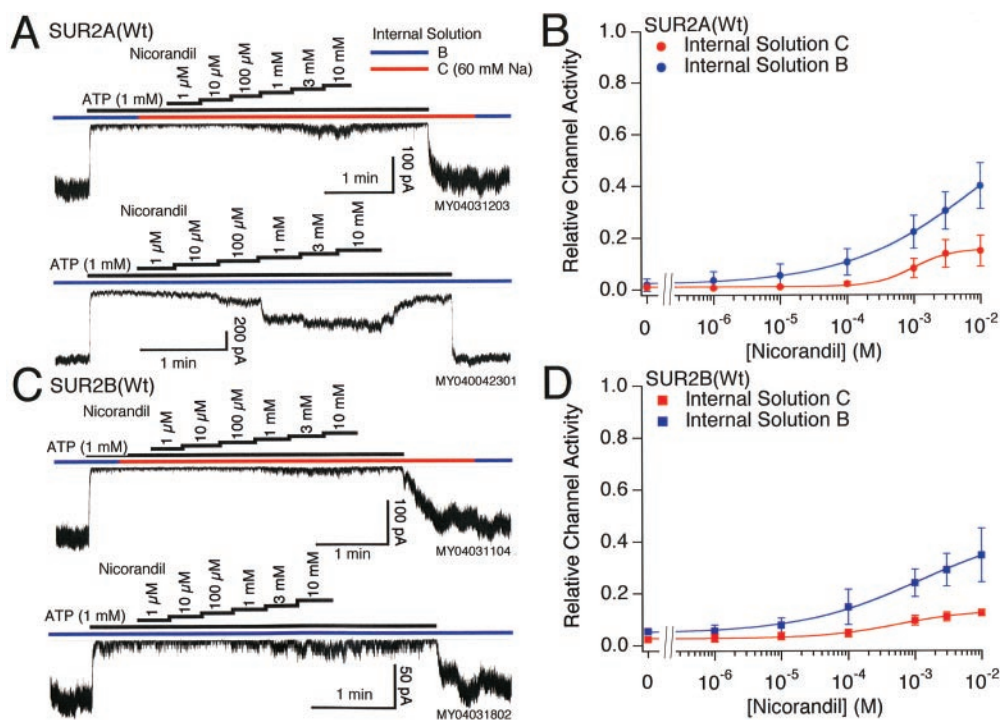


Fig. 7. Effect of nicorandil on wild-type SUR2x/Kir6.2 channels in the presence of internal Na-ATP and K-ATP alone. A, current traces were recorded at -60 mV from the inside-out patch membranes containing wild-type SUR2A/Kir6.2 channels. Dotted lines indicate the zero current level. Thick lines above the traces indicate the bath perfusion protocol. Blue and red lines indicate the perfusion with the internal solution B or the internal solution C containing 80 mM K⁺ and 60 mM Na⁺, respectively. B, concentration-response relationships of the nicorandil-induced activation of wild-type SUR2A/Kir6.2 channels in the presence of Na-ATP (red circles) and K-ATP alone (blue circles). The ordinate indicates the channel activity normalized to that in the absence of ATP under the same internal ionic condition. Symbols and bars indicate the mean and S.E.M., respectively. Lines are the fit of each set of the data with the Hill equation (eq. 3). C, current traces recorded from the inside-out patch membranes containing wild-type SUR2B/Kir6.2 channels under the same condition as in A. D, concentration-response relationships of the nicorandil-induced activation of wild-type SUR2B/Kir6.2 channels in the presence of Na-ATP (red squares) and K-ATP alone (blue squares).

the internal solution C containing 60 mM NaCl, 80 mM KCl, and 1 mM ATP than the internal solution B containing 1 mM ATP (Fig. 7) (Table 2). Together, these results suggest that dimerization of NBD1 and NBD2 of SUR2x also plays a critical role in the drug-induced activation of SUR2x/Kir6.2 channels.

Discussion

Mechanism Underlying Activation of ATP-Sensitive K⁺ Channels by ATP. SUR2x(D1N, E2Q)/Kir6.2 channels mediated larger currents in the presence of Na-ATP than K-ATP alone or Mg-ATP (Figs. 2–4). This phenomenon should be ascribed to an increase in the NP_o but not the single-channel amplitude (Fig. 5). ATP causes inhibitory and stimulatory effects on K_{ATP} channels through Kir6.2 and SUR, respectively (Tucker et al., 1997; Gribble et al., 1998). Na-ATP was not less efficient in inhibiting SUR2x(Wt)/Kir6.2 channels than K- or Mg-ATP (Figs. 2E and 3E). Therefore, Na-ATP exerted the stimulatory effect on SUR2x(D1N, E2Q)/Kir6.2 channels through the mutated NBDs.

A prokaryotic ABC protein, MJ0796, bearing the corresponding mutation (E171Q) forms an ATP-sandwich dimer in the presence of Na-ATP, which is destabilized by K⁺ or Mg²⁺ (Moody et al., 2002; Smith et al., 2002). There is a striking similarity in dependence on the cation cofactors between the dimerization of MJ0796(E171Q) and the activation of SUR2x(D1N, E2Q)/Kir6.2 channels. Therefore, the present results support our previous hypothesis that NBDs of SUR2x

dimerize. This study also indicates that the dimerization induces the opening of a K_{ATP} channel pore.

A K_{ATP} channel is a heterooctamer composed of four SUR and four Kir6.2 subunits (Clement et al., 1997; Shyng et al., 1997; Babenko et al., 1998; Seino, 1999). Na-ATP did not activate SUR2x(D1N)/Kir6.2 or SUR2x(E2Q)/Kir6.2 channels (Figs. 2E and 3E). Thus, it is unlikely that NBD1 or NBD2 derived from different SUR2x subunits within the same oligomer forms a homodimer to activate K_{ATP} channels. Cotransfection of SUR2x(D1N), SUR2x(E2Q), and Kir6.2 also failed to give rise to K_{ATP} channels responsive to Na-ATP (M. Yamada and Y. Kurachi, unpublished observation). Therefore, it is likely that NBD1 and NBD2 of the same SUR2x subunit dimerize in the presence of ATP.

How does the NBD dimerization induce the opening of the K_{ATP} channel pore? SUR possesses three groups of transmembrane α -helices, TMD0–2 (Fig. 8A) (Babenko et al., 1998; Klein et al., 1999). TMD0 and L0 mediate the SUR-Kir6.x interaction (Babenko and Bryan, 2003; Chan et al., 2003). Remaining TMD1-NBD1 plus TMD2-NBD2 satisfy the minimal structural requirement for an active ABC transporter (i.e., two TMDs and two NBDs) (Klein et al., 1999). A prokaryotic lipid flippase MsbA with a TMD-NBD structure forms a homodimer associating through the outer membrane leaflet half of its transmembrane domains (Chang and Roth, 2001). The formed cone-shaped dimer has an intermolecular chamber that opens to the inner membrane leaflet and the cytoplasm. Two distinct crystallographic structures of the

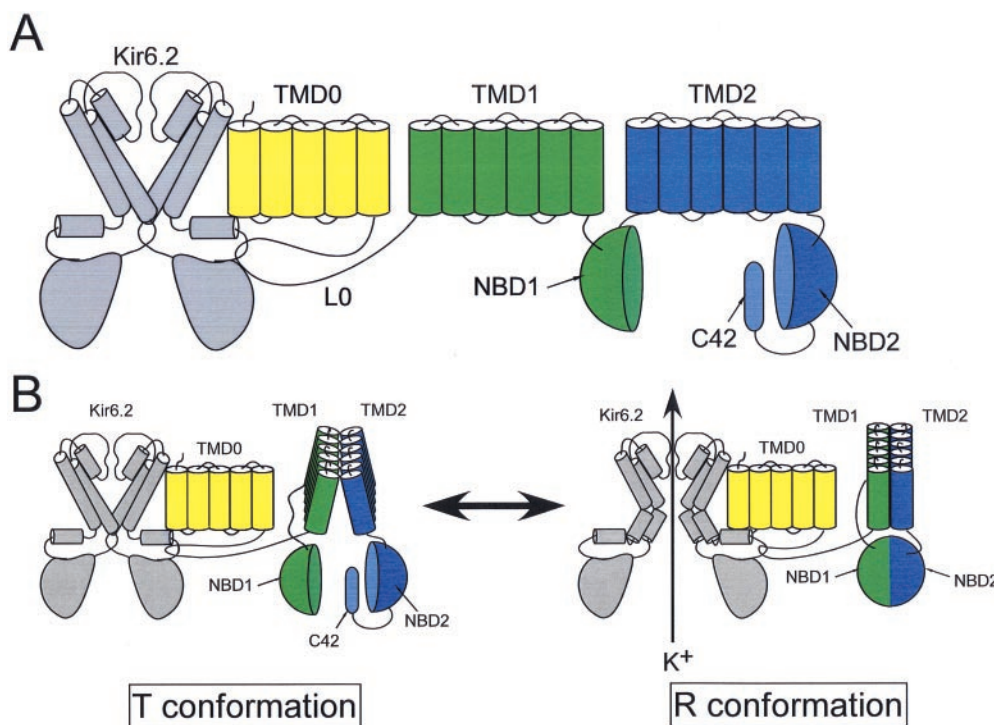


Fig. 8. Model of an SUR2x/Kir6.2 channel. A, an ATP-sensitive K⁺ channel is a heterooctamer comprising four pore-forming Kir6.2 subunits and four SURs. This scheme shows two opposing Kir6.2 subunits and one SUR2x interacting with one of the Kir6.2 subunits. The structure of Kir6.2 is depicted after Kuo et al. (2003). SUR has 17 transmembrane α -helices that are subdivided into TMD0–2. SUR has two nucleotide-binding domains between TMD1 and 2 (NBD1) and at the C terminus after TMD2 (NBD2), which are depicted as hemispheres. SUR2A and SUR2 differ only in the C-terminal 42 amino acids (C42). SUR interacts with Kir6.2 through TMD0 and L0, a cytoplasmic linker between TMD0 and 1 (Babenko and Bryan, 2003; Chan et al., 2003). B, hypothetical conformational change of SUR2x induced by dimerization of NBDs. In this model, TMD1-NBD1 and TMD2-NBD2 face each other. Intracellular ATP induces dimerization of NBDs and thereby a conformational change of TMD1 and TMD2. This conformational change is transferred to Kir6.2 through TMD0-L0, resulting in opening of the channel pore formed from Kir6.2. The conformation of SUR2x with and without the NBD dimer is referred to as R and T conformations, respectively.

MsbA dimer with “open” and “closed” chamber conformations have been identified (Chang and Roth, 2001; Chang, 2003). In the latter structure, the opposing NBDs associate upon ATP binding. Taking this information into account, we propose the following (Fig. 8B). The TMD1-NBD1 and TMD2-NBD2 of SUR2x face each other. Binding of ATP to the NBDs induces dimerization of the NBDs. This leads to a conformational change of TMD1 and TMD2, which is transferred to Kir6.2 through TMD0 and L0 to induce the opening of the K_{ATP} channel pore. We previously proposed that SUR2x has at least two distinct conformations that are able (R conformation) and unable (T conformation) to induce the opening of the K_{ATP} channel pore formed from Kir6.2 (Yamada and Kurachi, 2004). From the present results, the R and T conformations can be regarded as the conformation of SUR2x with and without the NBD dimer, respectively (Fig. 8B).

Mechanism Underlying Activation of ATP-Sensitive K^+ Channels by a Drug. SUR2x(D1N, E2Q)/Kir6.2 channels responded more strongly to nicorandil in the presence of Na-ATP than K-ATP alone (Fig. 6) (Table 2). In addition, SUR2x(D1N, E2Q)/Kir6.2 channels were more sensitive to the drug than SUR2x(Wt)/Kir6.2 channels in the presence of Na-ATP (Figs. 6 and 7; Table 2). Thus, dimerization of NBD1 and NBD2 seems to play a critical role also in the drug-induced activation of SUR2x/Kir6.2 channels.

We previously reasoned that nicorandil activates K_{ATP} channels because it has higher affinity for SUR2x in the R than T conformation (Yamada and Kurachi, 2004). From this aspect, the present results can be explained as follows: SUR2x(D1N, E2Q)/Kir6.2 channels are more sensitive to nicorandil in the presence of Na-ATP than K-ATP alone because NBDs more easily dimerize under the former than latter condition.

Difference between SUR2A and SUR2B. In the presence of Mg-ATP, SUR2B(Wt)/Kir6.2 channels have ~25 times higher basal activity in the absence of nicorandil and ~100 times greater sensitivity to nicorandil than SUR2A(Wt)/Kir6.2 channels (Yamada and Kurachi, 2004). However, we did not find such a large difference between SUR2A(D1N, E2Q)/Kir6.2 and SUR2B(D1N, E2Q)/Kir6.2 channels in their response to Na-ATP or nicorandil in the presence of Na-ATP (Figs. 2, 3, 4, and 6) (Table 2). SUR2B(D1N, E2Q)/Kir6.2 channels in the presence of Na-ATP were less/more sensitive to nicorandil ($K_{1/2} = 568 \mu\text{M}$) than SUR2B(Wt)/Kir6.2 ($K_{1/2} = 23.4 \mu\text{M}$) or SUR2A(Wt)/Kir6.2 channels ($K_{1/2} = 1.65 \text{ mM}$) in the presence of Mg-ATP. On the other hand, SUR2A(D1N, E2Q)/Kir6.2 channels in the presence of Na-ATP was slightly more sensitive to nicorandil ($K_{1/2} = 982 \mu\text{M}$) than SUR2A(Wt)/Kir6.2 channels in the presence of Mg-ATP ($K_{1/2} = 1.65 \text{ mM}$). That is, the D1N and E2Q mutations plus Na-ATP decreased the sensitivity to nicorandil of SUR2B/Kir6.2 channels and increased that of SUR2A/Kir6.2 channels, thereby diminishing the functional difference between the two channels.

The fact that the forced dimerization with the D1N plus E2Q mutations and Na-ATP made SUR2A and SUR2B behave similarly indicates that the transduction of the NBD dimerization to the channel activity is not significantly different between SUR2A and SUR2B. Thus, the difference between SUR2A(Wt)/Kir6.2 and SUR2B(Wt)/Kir6.2 channels in the presence of Mg-ATP should be ascribed to the difference in the function of NBDs between the two types of SUR.

We previously proposed that due to the difference in C42, SUR2B has 1) stronger effect of NBD1 and NBD2 to promote the T-R transition, and 2) higher ATPase activity of NBD2 than SUR2A in the presence of Mg-ATP (Yamada and Kurachi, 2004).

This study probably indicates that the dimerization occurs more easily in SUR2A(Wt) than SUR2B(Wt) in the presence of Mg-ATP. The residue corresponding to D834 or E1471 in MJ0796 or HisP interacts with hydrolytic water, which donates an H bond to the γ -phosphate of ATP (Hung et al., 1998; Smith et al., 2002). That in Rad50 binds to water interacting with Mg^{2+} , which in turn binds to the β - and γ -phosphate of ATP (Hopfner et al., 2000). Therefore, it is plausible that D834 and E1471 in SUR2x play a critical role in recognition of a cation cofactor and ATP. C42 may modulate these functions of D834 and E1471, thereby creating the different degree of the NBD dimerization in SUR2A(Wt) and SUR2B(Wt). The D1N and E2Q mutations and the presence of Na-ATP might directly or indirectly alter the interaction between these residues and C42, thereby diminishing the functional difference between SUR2A and SUR2B.

A loss of the ATPase activity caused by the D1N and E2Q mutations and the Mg^{2+} -free internal solution may also at least in part explain the small functional difference between SUR2A(D1N, E2Q) and SUR2B(D1N, E2Q). The activation of K_{ATP} channels by ATP hydrolysis would arise from ATP hydrolysis per se and/or the binding of produced ADP to NBDs. It is uncertain how the dimerization participates in these reactions. However, it is possible that the dimerization might facilitate ATP hydrolysis (Jones and George, 2002; Smith et al., 2002) and that NBDs might dimerize also in the presence of Mg-ADP. To verify these hypotheses, it is necessary to directly assess the physical association of NBD1 and NBD2 with biochemical or bioimaging techniques and correlate it with the ATPase and channel activity.

Acknowledgments

We are grateful to Kaori Iwai and Miki Fukumoto for technical assistance and to Rika Kitai and Junko Inoue for secretarial assistance.

References

- Aguilar-Bryan L, Nichols CG, Wechsler SW, Clement IVJP, Boyd IIIAE, González G, Herrera-Sosa H, Nguy K, Bryan J, and Nelson DA (1995) Cloning of the β cell high-affinity sulfonylurea receptor: a regulator of insulin secretion. *Science (Wash DC)* **268**:423–426.
- Ashcroft FM (1988) Adenosine 5'-triphosphate-sensitive potassium channels. *Annu Rev Neurosci* **11**:97–118.
- Babenko AP, Aguilar-Bryan L, and Bryan J (1998) A view of SUR/Kir6.X, K_{ATP} channels. *Annu Rev Physiol* **60**:667–687.
- Babenko AP and Bryan J (2003) SUR domains that associate with and gate K_{ATP} pores define a novel gatekeeper. *J Biol Chem* **278**:41577–41580.
- Chan KW, Zhang H, and Logothetis DE (2003) N-terminal transmembrane domain of the SUR controls trafficking and gating of Kir6 channel subunits. *EMBO (Eur Mol Biol Organ) J* **22**:3833–3843.
- Chang G (2003) Structure of MsbA from *Vibrio cholera*: a multidrug resistance ABC transporter homolog in a closed conformation. *J Mol Biol* **330**:419–430.
- Chang G and Roth CB (2001) Structure of MsbA from *E. coli*: a homolog of the multidrug resistance ATP binding cassette (ABC) transporters. *Science (Wash DC)* **293**:1793–1800.
- Clement JP IV, Kunjilwar K, Gonzalez G, Schwanstecher M, Panten U, Aguilar-Bryan L, and Bryan J (1997) Association and stoichiometry of K_{ATP} channel subunits. *Neuron* **18**:827–838.
- Georjon C, Orelle C, Steinfels E, Blanchet C, Deléage G, Pietro AD, and Jault J-M (2001) A common mechanism for ATP hydrolysis in ABC transporter and helicase superfamilies. *Trend Biochem Sci* **26**:539–544.
- Gribble FM, Tucker SJ, Haug T, and Ashcroft FM (1998) MgATP activates the β cell K_{ATP} channel by interaction with its SUR1 subunit. *Proc Natl Acad Sci USA* **95**:7185–7190.
- Hopfner K-P, Karcher A, Shin DS, Craig L, Arthur M, Carney JP, and Tainer JA

- (2000) Structural biology of Rad50 ATPase: ATP-driven conformational control in DNA double-strand break repair and the ABC-ATPase superfamily. *Cell* **101**:789–800.
- Hung L-W, Wang IX, Nikaido K, Liu P-Q, Ames GF-L, and Kim S-H (1998) Crystal structure of the ATP-binding subunit of an ABC transporter. *Nature (Lond)* **396**:703–707.
- Inagaki N, Gonoi T, Clement IVJP, Namba N, Inazawa J, Gonzalez G, Aguilar-Bryan L, Seino S, and Bryan J (1995) Reconstitution of I_{KATP} : an inward rectifier subunit plus the sulfonylurea receptor. *Science (Wash DC)* **270**:1166–1170.
- Isomoto S, Kondo C, Yamada M, Matsumoto S, Higashiguchi O, Horio Y, Matsuzawa Y, and Kurachi Y (1996) A novel sulfonylurea receptor forms with BIR (Kir6.2) a smooth muscle type ATP-sensitive K^+ channel. *J Biol Chem* **271**:24321–24324.
- Jones PM and George AM (2002) Mechanism of ABC transporters: a molecular dynamics simulation of a well characterized nucleotide-binding subunit. *Proc Natl Acad Sci USA* **99**:12639–12644.
- Klein I, Sarkadi B, and Váradi A (1999) An inventory of the human ABC proteins. *Biochem Biophys Acta* **1461**:237–262.
- Kuo A, Gulbis JM, Antcliff JF, Rahman T, Lowe ED, Zimmer J, Cuthbertson J, Ashcroft FM, Ezaki T, and Doyle DA (2003) Crystal structure of the potassium channel KirBac 1.1 in the closed state. *Science (Wash DC)* **300**:1922–1926.
- Locher KP, Lee AT, and Rees DC (2002) The E. coli BtuCD structure: a framework for ABC transporter architecture and mechanism. *Science (Wash DC)* **296**:1091–1098.
- Matsuoka T, Matsushita K, Katayama Y, Fujita A, Inageda K, Tanemoto M, Inanobe A, Yamashita S, Matsuzawa Y, and Kurachi Y (2000) C-Terminal tails of sulfonylurea receptors control ADP-induced activation and Diazoxide modulation of ATP-sensitive K^+ channels. *Circ Res* **87**:873–880.
- Matsushita K, Kinoshita K, Matsuoka T, Fujita A, Fujikado T, Tano Y, Nakamura H, and Kurachi Y (2002) Intramolecular interaction of SUR2 subtypes for intracellular ADP-induced differential control of K_{ATP} channels. *Circ Res* **90**:554–561.
- Monod J, Wyman J, and Changeux J-P (1965) On the nature of allosteric transitions: a plausible model. *J Mol Biol* **12**:88–118.
- Moody JE, Millen L, Binns D, Hunt JF, and Thomas PJ (2002) Cooperative, ATP-dependent association of the nucleotide binding cassette during the catalytic cycle of ATP-binding cassette transporters. *J Biol Chem* **277**:21111–21114.
- Moreau C, Jacquet H, Prost A-N, D'hahan N, and Vivaudou M (2000) The molecular basis of the specificity of action of K_{ATP} channel openers. *EMBO (Eur Mol Biol Organ) J* **24**:6644–6651.
- Reimann F, Ashcroft FM, and Gribble FM (2001) Structural basis for the interference between nicorandil and sulfonylurea action. *Diabetes* **50**:2253–2259.
- Seino S (1999) ATP-sensitive potassium channels: a model of heteromultimeric potassium channel/receptor assemblies. *Annu Rev Physiol* **61**:337–362.
- Shyng S-L, Ferrigni T, and Nichols CG (1997) Regulation of K_{ATP} channel activity by diazoxide and MgADP. Distinct functions of the two nucleotide binding folds of sulfonylurea receptor. *J Gen Physiol* **110**:643–654.
- Shyng S-L and Nichols CG (1997) Octameric stoichiometry of the K_{ATP} channel complex. *J Gen Physiology* **110**:655–664.
- Smith PC, Karpowich N, Millen L, Moody JE, Rosen J, Thomas PJ, and Hunt JF (2002) ATP binding to the motor domain from an ABC transporter drives formation of a nucleotide sandwich dimmer. *Mol Cell* **10**:139–149.
- Terzic A, Jahangir A, and Kurachi Y (1995) Cardiac ATP-sensitive K^+ channels: regulation by intracellular nucleotides and K^+ channel-opening drugs. *Am J Physiol* **263**:C525–C545.
- Tucker SJ, Gribble FM, Zhao C, Trapp S, and Ashcroft FM (1997) Truncation of Kir6.2 produces ATP-sensitive K^+ channels in the absence of the sulphonylurea receptor. *Nature (Lond)* **387**:179–183.
- Yamada M and Kurachi Y (2004) The nucleotide-binding domains of sulfonylurea receptor 2A and 2B play different functional roles in nicorandil-induced activation of ATP-sensitive K^+ channels. *Mol Pharmacol* **65**:1198–1207.
- Yoshida M and Amano T (1995) A common topology of proteins catalyzing ATP-triggered reactions. *FEBS Lett* **359**:1–5.

Address correspondence to: Dr. Yoshihisa Kurachi, Department of Pharmacology II, Graduate School of Medicine Osaka University, 2-2 Yamada-oka, Suita, Osaka 565-0871 Japan. E-mail: ykurachi@pharma2.med.osaka-u.ac.jp