Different Binding Properties and Affinities for ATP and ADP among Sulfonylurea Receptor Subtypes, SUR1, SUR2A, and SUR2B*

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ATP-sensitive potassium (K\textsubscript{ATP}) channels, composed of sulfonylurea receptor (SURx) and Kir6.x, play important roles by linking cellular metabolic state to membrane potential in various tissues. Pancreatic, cardiac, and vascular smooth muscle K\textsubscript{ATP} channels, which consist of different subtypes of SURx, differ in their responses to cellular metabolic state. To explore the possibility that different interactions of SURx with nucleotides cause differential regulation of K\textsubscript{ATP} channels, we analyzed the properties of nucleotide-binding folds (NBFs) of SUR1, SUR2A, and SUR2B. SURx in crude membrane fractions was incubated with 8-azido-[\textsuperscript{\gamma-\textsuperscript{32}P}]ATP or 8-azido-[\textsuperscript{\alpha-\textsuperscript{32}P}]ATP under various conditions and was photoaffinity-labeled. Then, SURx was digested mildly with trypsin, and partial tryptic fragments were immunoprecipitated with antibodies against NBF1 and NBF2. Some nucleotide-binding properties were different among SUR subtypes as follows. 1) Mg\textsuperscript{2+} dependence of nucleotide binding of NBF2 of SUR1 was high, whereas those of SUR2A and SUR2B were low. 2) The affinities of NBF1 of SUR1 for ATP and ADP, especially for ATP, were significantly higher than those of SUR2A and SUR2B. 3) The affinities of NBF2 of SUR2B for ATP and ADP were significantly higher than those of SUR2A. This is the first biochemical study to analyze and compare the nucleotide-binding properties of NBFs of three SUR subtypes, and our results suggest that their different properties may explain, in part, the differential regulation of K\textsubscript{ATP} channel subtypes. The high nucleotide-binding affinities of SUR1 may explain the high ability of SUR1 to stimulate pancreatic K\textsubscript{ATP} channels. It is also suggested that the C-terminal 42 amino acids affect the physiological roles of SUR2A and SUR2B by changing the nucleotide-binding properties of their NBFs.

ATP-sensitive potassium (K\textsubscript{ATP})\textsuperscript{3} channels are inwardly rectifying potassium channels, which are inhibited by ATP and stimulated by MgATP (1–3). They play important roles by linking cellular metabolic level to membrane potential by sensing intracellular ATP and ADP levels in various tissues such as pancreatic ß-cells, heart, brain, skeletal muscle, and vascular smooth muscle. The K\textsubscript{ATP} channel is a hetero-octamer composed of sulfonylurea receptor (SURx) and Kir6.x subunits in 4:4 stoichiometry (4–7). SURx is a member of the ATP-binding cassette (ABC) superfamily including P-glycoprotein (MDR1), multidrug resistance-associated protein (MRP1), and the cystic fibrosis transmembrane conductance regulator (CFTR) (8, 9), all of which have two nucleotide-binding folds (NBFs) per molecule; Kir6.x is a member of the inwardly rectifying potassium channel family (10–12).

Both SURx and Kir6.x have a number of subtypes as follows: SUR1, SUR2A, and SUR2B and Kir6.1 and Kir6.2. SUR1 has been cloned as a high affinity binding protein for sulfonylurea (9), the most commonly used drug for treatment of patients with type 2 diabetes. SUR2A shares 68% amino acid identity with SUR1, and SUR2B is a splicing variant of SUR2A differing only in its C-terminal 42 amino acids (13, 14). The terminal 42 amino acids of SUR2B are similar to those of SUR1. Kir6.1 and Kir6.2 share 71% amino acid identity with each other, both of which have two putative transmembrane domains and an ion pore-forming (H5) region.

Pancreatic ß-cell K\textsubscript{ATP} channels, composed of SUR1 and Kir6.2, regulate insulin secretion by altering the ß-cell membrane potential (1, 3, 15, 16). Coexpression of SUR2A/Kir6.2, SUR2B/Kir6.2, and SUR2B/Kir6.1 has been reported to reconstitute cardiac, smooth muscle, and vascular smooth muscle K\textsubscript{ATP} channels, respectively (2, 3). These channels have different sensitivities to ATP and show different responses to sulfonylurea drugs and potassium channel openers (10, 11, 14, 17, 18). The IC\textsubscript{50} (ATP) of SUR1/Kir6.2 K\textsubscript{ATP} channels is about 10 \(\mu\)M, whereas those of SUR2A/Kir6.2 and SUR2B/Kir6.2 are about 100 and 300 \(\mu\)M, respectively (10, 11, 14). SUR2B/Kir6.1 K\textsubscript{ATP} channels are not inhibited by ATP but are stimulated by ADP and ATP (17). SUR1/Kir6.2 K\textsubscript{ATP} channels are inhibited by glibenclamide at \(K_{\text{I}}\approx 10\) \(n\)M, whereas SUR2A/Kir6.2, SUR2B/Kir6.2, and SUR2B/Kir6.1 K\textsubscript{ATP} channels are inhibited with \(K_{\text{I}}\) values in the low micromolar range (10, 11, 14, 17). Both SUR1/Kir6.2 and SUR2B/Kir6.2 K\textsubscript{ATP} channels are stimulated by diazoxide, but SUR2A/Kir6.2 K\textsubscript{ATP} channels are not (10, 11, 14). The differences between these channels may be caused, at least in part, by differences in SUR subtype. However, it is not clear how SUR subtypes cause the different properties of K\textsubscript{ATP} channel subtypes.

We have already shown that NBF1 of SUR1 is a Mg\textsuperscript{2+}-independent high affinity ATP-binding site, that NBF2 is a Mg\textsuperscript{2+}-dependent low affinity ATP-binding site, and that MgADP binding at NBF2 stabilizes the 8-azido-ATP binding at NBF1 of SUR1 (19–21). In this study, to determine the reasons...
for differences among $K_{\text{ATP}}$ channel subtypes, we investigated the nucleotide-binding properties and the stabilization effect of three subtypes of SUR, SUR1, SUR2A, and SUR2B. Our results indicate that three subtypes of SUR have different nucleotide-binding properties, which may explain the differential regulation of $K_{\text{ATP}}$ channel subtypes.

**EXPERIMENTAL PROCEDURES**

**Materials**—8-Azido-$[\alpha-^{32}\text{P}]$ATP and 8-azido-γ-$[32\text{P}]$ATP were purchased from ICN Biomedicals. Hamster SUR1 (9), rat SUR2A (13), and mouse SUR2B (14) cDNAs were gifts from Dr. Joseph Bryan (Baylor College of Medicine), Dr. Susumu Seino (Chiba University), and Dr. Yoshikazu Kurachi (Osaka University), respectively. Antibodies against the C-terminal 21 amino acids of rat SUR1 (22) and of rat SUR2A were gifts from Dr. Susumu Seino.

**Photoaffinity Labeling of SURx with 8-Azido-$[\alpha-^{32}\text{P}]$ATP and 8-Azido-γ-$[32\text{P}]$ATP—Membranes (20 μg of proteins) from COS-7 cells expressing SUR1, SUR2A, or SUR2B, prepared as described previously (20), were incubated with 50 μM 8-azido-$[\alpha-^{32}\text{P}]$ATP in the presence or absence of 0.1–1000 μM ATP or ADP in 3 μl of TEM buffer (40 mM Tris-Cl (pH 7.5), 0.1 mM EGTA, 1 mM MgCl₂), TEE buffer (40 mM Tris-Cl (pH 7.5), 1 mM MgCl₂, 1 mM EDTA), or TEC buffer (40 mM Tris-Cl (pH 7.5), 0.1 mM EGTA, 1 mM CDTA) containing 2 mM ouabain, 250 mM sucrose to 10 mM 32P-ATP, were mildly digested with trypsin, 100- and 35-kDa fragments were immunoprecipitated with 8-Azido-γ-$[32\text{P}]$ATP, ice-cold TEM buffer was added to the sample, and the supernatant was removed after centrifugation (15,000 × g, 5 min, 2 °C). Pellets were resuspended in TEE buffer containing 5 μg/ml trypsin and 250 μM sucrose to 10 μg of membrane proteins/μl and incubated for 15 min at 37 °C. Then 100 μl of RIPA buffer (20 μM Tris-Cl (pH 7.5), 1% Triton X-100, 0.1% SDS, and 1% sodium deoxycholate) containing 100 μg/ml (p-amidinophenyl)methanesulfonylefluoride was added to the samples to terminate proteolysis, and membrane proteins were solubilized for 30 min at 4 °C. After centrifugation for 15 min at 15,000 × g, tryptic fragments were immunoprecipitated from the supernatant with the antibody raised against NBF1 or NBF2 of hamster SUR1 prepared as described (20). Samples were electrophoresed on a 12% SDS-polyacrylamide gel and autoradiographed. Bound 8-azido-$[\alpha-^{32}\text{P}]$ATP in NBF1 or NBF2 was measured by scanning with a radioimaging analyzer (BAS2000, Fuji Photo Film Co.).

**Cooperative Nucleotide Binding of Two NBFs—Membranes (20 μg of proteins) were incubated with 50 μM 8-azido-$[\alpha-^{32}\text{P}]$ATP in 3 μl of TEM buffer containing 2 mM ouabain for 3 min at 37 °C. The reactions were stopped by adding ice-cold TEM buffer, and free 8-azido-$[\alpha-^{32}\text{P}]$ATP was removed after centrifugation (15,000 × g, 5 min, 2 °C). Pellets were resuspended in 10 μl of TEM or TEE buffer containing 2 mM ouabain and 1 mM ATP or ADP. The mixture was incubated for 15 min at 37 °C and UV-irradiated on ice. Samples were electrophoresed on a 12% SDS-polyacrylamide gel and autoradiographed. Bound 8-azido-$[\alpha-^{32}\text{P}]$ATP in SURx was measured using the radioimaging analyzer as described above.

**RESULTS**

**Limited Tryptic Digestion of SURx**—We demonstrated previously that mild digestion of SUR1 with trypsin produces 35- and 65-kDa fragments containing NBF1 and NBF2, respectively, and that we can analyze the ATP-binding properties of NBF1 and NBF2 separately after immunoprecipitation (20). We examined whether NBFs of SUR2A and SUR2B can be also separated by mild trypsic digestion followed by immunoprecipitation. When SUR1, photoaffinity-labeled with 50 μM 8-azido-$[\alpha-^{32}\text{P}]$ATP, was mildly digested with trypsin, 100- and 35-kDa labeled fragments were immunoprecipitated with the anti-NBF1 antibody, and 100- and 65-kDa labeled fragments were immunoprecipitated with the anti-NBF2 antibody as previously reported (20) (Fig. 1A). The 100- and 65-kDa fragments were detected with the anti-C terminus antibody on Western blotting (Fig. 2A). These results suggest that SUR1 is first digested with trypsin at site 1 to produce the trypsic 100-kDa fragment, containing both NBF1 and NBF2, and that the 100-kDa fragment is further digested to the 35-kDa fragment, which contains NBF1, and the 65-kDa fragment, which contains NBF2, as shown in Fig. 2D.

**8-Azido-ATP Binding to NBF1 and NBF2 of SURx**—To investigate the ATP-binding properties of NBFs of SURx, SURx was photoaffinity-labeled with 50 μM 8-azido-$[\alpha-^{32}\text{P}]$ATP under various conditions and mildly digested with trypsin (Fig. 3). Tryptic fragments were immunoprecipitated with anti-NBF1 or anti-NBF2 antibody and separated by 12% polyacrylamide gel electrophoresis and autoradiographed. Undigested SURx, 100-, 65-, and 35-kDa tryptic fragments are indicated. Photoaffinity-labeled bands indicated as asterisks are endogenous proteins of COS-7 cells. Experiments were performed in duplicate.

When photoaffinity-labeled SUR2A and SUR2B were digested with trypsin under the same conditions, they produced 65- and 35-kDa fragments but very little 100-kDa fragment (Fig. 1, B and C). On Western blotting, 65-kDa fragments of SUR2A and SUR2B, but little 100-kDa fragment, were detected by antibodies against the C termini of SUR2A and SUR1, respectively (Fig. 2, B and C). The antibody raised against the C terminus of SUR1 recognized SUR2B as shown in Fig. 2C, because the C-terminal 42 amino acids of SUR2B are similar to those of SUR1 (14). These results suggest that SUR2A and SUR2B are digested with trypsin at site 1 and site 2 simultaneously to produce the trypsic 35-kDa fragment, which contains NBF1, and the 65-kDa fragment, which contains NBF2. It also indicated that antibodies raised against NBFs of SUR1 can be used to precipitate NBFs of SUR2A and SUR2B.
Affinities of NBFs of SURx for Nucleotides—We have suggested that NBF1 of SUR1 is a high affinity 8-azido-ATP-binding site and NBF2 is a low affinity 8-azido-ATP-binding site (19, 20). To know the affinity for ATP and ADP of NBFs of SURx, we examined inhibition of 8-azido-ATP binding by ATP-stable, whereas that to NBF2 is Mg$^{2+}$-dependent and unstable as reported previously (20).

In the case of SUR2A (Fig. 3B) and SUR2B (Fig. 3C), both NBF1 and NBF2 were labeled with 8-azido-[α-32P]ATP either in the presence of EDTA (lanes 1 and 5) or Mg$^{2+}$ (lanes 3 and 7). However, when Mg$^{2+}$ was completely depleted by chelating with CDTA, which has stronger Mg$^{2+}$-chelating ability than EDTA, NBF2 was not labeled with 8-azido-[α-32P]ATP (lane 6), whereas NBF1 was (lane 2). NBF1 was labeled with 8-azido-[α-32P]ATP by UV irradiation after removing free ligand with excess cold buffer (lane 4), whereas NBF2 was not (lane 8). These results suggest that 8-azido-ATP binding to NBF1 of SUR2A and SUR2B is Mg$^{2+}$-independent and very stable, whereas that to NBF2 is Mg$^{2+}$-dependent and unstable similar to SUR1. However, Mg$^{2+}$ dependence of 8-azido-ATP binding to NBF2 of SUR2A and SUR2B is much lower than that of SUR1.

Photoaffinity Labeling of SURx with 8-Azido-[α-32P]ATP and 8-Azido-[γ-32P]ATP—In our previous study, it was suggested that NBF2 of SUR1 may have ATPase activity, because NBF2 of SUR1 was photoaffinity-labeled with 8-azido-[α-32P]ATP but not with 8-azido-[γ-32P]ATP as shown in Fig. 4A (lanes 2 and 4). We examined whether NBFs of SUR2A and SUR2B could be labeled with 8-azido-[α-32P]ATP and 8-azido-[γ-32P]ATP (Fig. 4, B and C). NBF1s of SUR2A and SUR2B were photoaffinity-labeled with both 8-azido-[α-32P]ATP (lane 1) and 8-azido-[γ-32P]ATP (lane 3) in the presence of Mg$^{2+}$. However, NBF2s of SUR2A and SUR2B were labeled with 8-azido-[α-32P]ATP (lane 2) but not with 8-azido-[γ-32P]ATP (lane 4) in the presence of Mg$^{2+}$. NBF2s of SUR2A and SUR2B were labeled with both 8-azido-[α-32P]ATP and 8-azido-[γ-32P]ATP in the presence of EDTA but were not labeled in the presence of CDTA (data not shown). These results suggest that bound 8-azido-[α-32P]ATP is hydrolyzed and γ-phosphate dissociates from NBF2 of SURx in the presence of Mg$^{2+}$, although NBF1 of SURx have no or little ATPase activity.

FIG. 2. Limited digestion of SURx with trypsin. Membrane proteins (25 μg) from host COS-7 cells or COS-7 cells expressing SUR1 (A), SUR2A (B), or SUR2B (C) were digested with 5 μg/ml trypsin at 37 °C for the indicated periods and separated by 12% SDS-polyacrylamide gel electrophoresis. Limited trypptic fragments were detected with anti-C terminus of SUR1 (A and C) or SUR2A (B) antibody. Undigested SURx, 100-kDa, and 65-kDa trypsic fragments are indicated. Experiments were performed in duplicate. D, predicted diagram of limited trypsic digestion of SURx.

FIG. 3. 8-Azido-[α-32P]ATP binding of NBFs of SURx. Membrane proteins (20 μg) from COS-7 cells expressing SUR1 (A), SUR2A (B), or SUR2B (C), or host COS-7 cells (D) were incubated with 50 μM 8-azido-[α-32P]ATP in the presence of EDTA (lanes 1 and 5), CDTA (lanes 2 and 6), or Mg$^{2+}$ (lanes 3, 4, 7, and 8) for 10 min at 0 °C and UV-irradiated before (lanes 1–3 and 5–7) or after (lanes 4 and 8) removing free 8-azido-[α-32P]ATP by washing membranes with excess buffer. Photoaffinity-labeled proteins were digested with 5 μg/ml trypsin for 15 min at 37 °C and solubilized with RIPA buffer. The trypsic fragments were immunoprecipitated (IP) with anti-NBF1 (lanes 1–4) or anti-NBF2 (lanes 5–8) antibody and separated by 12% polyacrylamide gel electrophoresis. Undigested SURx, a 100-kDa tryptic fragment containing both NBF1 and NBF2, a 65-kDa fragment containing NBF2, and a 35-kDa fragment containing NBF1 are indicated. Photoaffinity-labeled bands indicated as asterisks are endogenous proteins of COS-7 cells. They are stronger in B and C than A and D due to longer exposure. Experiments were performed in triplicate.
and ADP. When SURx was photoaffinity-labeled with 50 μM 8-azido-[α-32P]ATP in the presence of cold ATP or ADP, photoaffinity labeling of both NBFs of SURx was inhibited by ATP and ADP in a concentration-dependent manner (Fig. 5), indicating that ADP as well as ATP binds to NBFs. The $K_i$ values for ATP and ADP of NBFs were calculated, and the values and nucleotide-binding properties are summarized in Table I. The $K_i$ values of NBF1 for ATP were 4.4 ± 3.7, 110 ± 41, and 51 ± 13 μM in SUR1, SUR2A, and SUR2B, respectively. Those of NBF1 for ADP were 26 ± 8.6, 86 ± 23, and 66 ± 7.5 μM in SUR1, SUR2A, and SUR2B, respectively. Thus, the affinities of NBF1 for both ATP and ADP are significantly higher in SUR1 than in SUR2A and SUR2B. This is consistent with the concentration dependence of photoaffinity labeling of NBF1 of SURx with 8-azido-[α-32P]ATP; the degree of photoaffinity labeling was saturated at about 5 μM in SUR1, but saturation was seen at about 50 μM in SUR2A and SUR2B (data not shown). The affinity of NBF1 of SUR1 for ATP is significantly higher than that for ADP, although the affinities of NBF1 of SUR2A and SUR2B are not significantly different between ATP and ADP. The $K_i$ values of NBF2 for ATP were 60 ± 26, 120 ± 39, and 38 ± 26 μM in SUR1, SUR2A, and SUR2B, respectively. Those of NBF2 for ADP were 100 ± 26, 170 ± 70, and 67 ± 40 μM in SUR1, SUR2A, and SUR2B, respectively. Thus, the affinities of NBF2 of SUR2A for ATP and ADP are significantly lower than those of SUR2B.

Cooperative Binding of Nucleotides to NBF1 and NBF2—We reported previously that ADP stabilizes the binding of pre-bound 8-azido-[α-32P]ATP at NBF1 on SUR1, either by direct binding to NBF2 or hydrolysis of bound ATP at NBF2 (21), and this effect was impaired in mutant SUR1 (R1420C) found in Japanese persistent hyperinsulinemic hypoglycemia of infancy patients (24). We examined whether two NBFs of SUR2A and SUR2B bind nucleotides cooperatively. When membranes from COS-7 cells expressing SUR2A or SUR2B were incubated with 50 μM 8-azido-[α-32P]ATP, both NBF1 and NBF2 bind 8-azido-[α-32P]ATP. Because 8-azido-[α-32P]ATP bound to NBF2 dissociates during washing of membranes with excess buffer at 0 °C but that bound to NBF1 does not, we can estimate the stabilization effect of nucleotides bound to NBF2 on 8-azido-[α-32P]ATP binding to NBF1 during postincubation at 37 °C as shown in Fig. 6. When membranes were postincubated in the absence of Mg2+, 8-azido-[α-32P]ATP quickly dissociated from NBF1 of SUR2A and SUR2B (Fig. 7). When membranes were postincubated in the presence of Mg2+ without nucleotide, 8-azido-[α-32P]ATP gradually dissociated from NBF1 in a time-dependent manner as in the case of SUR1 (21). However, 8-azido-[α-32P]ATP did not dissociate from NBF1 in the presence of both Mg2+ and nucleotide (Fig. 7), indicating that MgATP and MgADP stabilize 8-azido-[α-32P]ATP binding at NBF1 as in the case of SUR1 (21).

**DISCUSSION**

In the present study, we have compared the nucleotide-binding properties of each NBF of SURx to elucidate the molecular mechanisms responsible for the differential regulation of KATP channel subtypes in various organs. Some nucleotide-binding properties were found to be similar among all the SUR subtypes as follows: 1) NBF1 is a Mg2+-independent ATP- and ADP-binding site; 2) NBF2 is a Mg2+-dependent ATP- and ADP-binding site; 3) 8-azido-ATP binds to NBF1 very stably and does not dissociate at 0 °C; 4) MgATP or MgADP binding to NBF2 stabilizes 8-azido-ATP binding at NBF1; 5) NBF2 may have ATPase activity, whereas NBF1 has showed no or little ATPase activity. However, some properties are different among SUR subtypes as follows. 1) Nucleotide binding to NBF2 of SUR1 is highly Mg2+-dependent, whereas Mg2+-dependence of nucleotide binding to NBF2 of SUR2A and SUR2B is low. 2) The affinities of NBF1 of SUR1 for ATP and ADP, especially for
ATP, are significantly higher than those of SUR2A and SUR2B.
3) The affinity of NBF1 of SUR2B for ATP is relatively higher
than that of SUR2A, and the affinities of NBF2 of SUR2B for
ATP and ADP are significantly higher than those of SUR2A.

It has been reported that coexpression of SUR1/Kir6.2 and
SUR2A/Kir6.2 reconstitutes pancreatic β-cell and cardiac mus-
cle K_ATP channels, respectively (10, 11). They have different
physiological functions, with different sensitivity to ATP, and
have different responses to sulfonylurea drugs or potassium
channel openers. Under normal conditions, pancreatic β-cell
K_ATP channels stay open to maintain membrane potential, and
they close when elevation of blood glucose concentration results
in increased intracellular concentration of ATP to trigger insu-
lin secretion (1, 3, 15, 16). On the other hand, cardiac muscle
K_ATP channels remain closed under normal conditions, and
they open when the intracellular concentration of ATP de-
creases under ischemic stress to shorten action potential dura-
tion and protect the myocardium from lethal injury (2, 3, 25).
Because pancreatic and cardiac K ATP channels contain
Kir6.2 as the potassium channel pore components, the differ-
ences in their responses to cellular metabolic state are likely to
be due to SUR subtypes. ATP inhibits the channel by binding to
Kir6.2 (26–28), whereas MgATP and MgADP stimulate chan-
cel activity by binding to the NBFs of SUR1 (15, 20, 21, 29–31).

This study provides biochemical evidence that the affinities of
NBF1 of SUR1 for ATP and ADP are significantly higher than
those of SUR2A and that the affinities of NBF2 of SUR1 for
ATP and ADP are relatively (but not significantly) higher than
those of SUR2A (Table I). These results suggest that SUR1 has
a higher ability to open Kir6.2 subunit than SUR2A and that
the higher nucleotide binding affinities of SUR1 may explain
the higher ability of SUR1 than SUR2A to open the K_ATP
channels.

We proposed a model of nucleotide stimulation of K_ATP chan-
nels through the SUR1 subunit, in which channel opening is
induced by SUR1 binding ATP in NBF1 and ADP in NBF2 (21,
32). Our working model of the function of \( K_{ATP} \) channel is as follows. When intracellular ADP concentration is high enough, SUR1 binds ATP in NBF1 and ADP in NBF2 cooperatively and stimulates Kir6.2 to open the potassium channel. After eating, the plasma glucose concentration rises. The increase in glucose metabolism raises the cellular ATP concentration and concomitantly lowers the cellular ADP concentration in pancreatic \( \beta \)-cells. The decrease in cellular ADP concentration causes dissociation of ADP from NBF2 of SUR1. ATP may bind to NBF2 after ADP dissociation because of the cellular high ATP concentration, but SUR1 binding ATP at NBF2 cannot stimulate the channel. ATP bound to NBF2 would be hydrolyzed to ADP, and ADP would readily dissociate from NBF2. Therefore, SUR1 would not stay long in the active state, when the cellular ADP concentration is low. Recently, we demonstrated that ATP binds directly to Kir6.2 (27, 28), and the binding is speculated to be involved in stabilization of the long closed state of KATP channel (33). With the rise in the cellular ATP concentration, Kir6.2 would stay longer in ATP-binding form. Therefore, we assume that the activity of pancreatic \( \beta \)-cell KATP channels is determined by the balance of the action of ADP, which stimulates channel through SUR1, and the action of ATP, which stabilizes the long closed state by binding to Kir6.2.

It has been reported that mutations within the Walker A motif in either of the NBFs of SUR1 abolish MgADP-induced channel stimulation (29), suggesting that both NBFs are necessary for channel stimulation. If the binding of ATP to NBF1 is essential for SURx to stimulate \( K_{ATP} \) channels, we may assume that competition between ATP and ADP for the binding to NBF1 of SUR2A, of which NBF1 has similar affinities for ATP and ADP, reduces the ability for \( K_{ATP} \) channel opening and that SUR1 might have the high ability to open \( K_{ATP} \) channels, because NBF1 of SUR1 has about 6-fold higher affinity for ATP than for ADP.

Interestingly, the nucleotide-binding properties of NBFs are significantly different between SUR2A and SUR2B, i.e. the affinity of NBF1 of SUR2B for ATP is relatively higher than that of SUR2A, and the affinities of NBF2 of SUR2B for ATP and ADP are significantly higher than those of SUR2A. Because SUR2A and SUR2B share the same amino acid sequence except for their C-terminal 42 amino acids, their C-terminal region is thought to affect the nucleotide-binding properties of NBF1 and NBF2. SUR2A/Kir6.2, SUR2B/Kir6.2, and SUR2B/Kir6.1 are suggested to reconstitute cardiac, smooth muscle, and vascular smooth muscle \( K_{ATP} \) channels, respectively (11, 14, 17). Their C-terminal regions may affect the physiological roles of SUR2A and SUR2B by changing the nucleotide-binding properties of their NBFs. Recently, Babenko et al. (34) reported that the last 42 amino acids of SURx specify the effect of ATP on gating. The C-terminal regions may alter the sensitivity of \( K_{ATP} \) channel to inhibitory ATP through affecting affinities of NBFs of SURx.

SUR1/Kir6.2 \( K_{ATP} \) channels are stimulated by diazoxide but not by pinacidil and cromakalim (10, 35, 36); SUR2A/Kir6.2 \( K_{ATP} \) channels are stimulated by pinacidil and cromakalim but not by diazoxide (13, 35), and SUR2B/Kir6.2 \( K_{ATP} \) channels are stimulated by both of them (14, 37). Recently, it was reported that transmembrane domains 6–11 and NBF1 control responsiveness to diazoxide (35, 38) and that transmembrane domains 12–17 confer sensitivity to cromakalim and pinacidil (38, 39). Because nucleotide binding and/or hydrolysis at both NBFs of SURx are suggested to be essential for the specific binding and action of these potassium channel openers (29, 37, 40, 41) and for slowing the off-rate of pinacidil (42), the differences in response to the potassium channel openers could be partially due to the different nucleotide-binding properties of SURx.

Because NBF2s of all the SUR subtypes were photoaffinity-labeled with 8-azido-[\( \alpha^{-32}P \)]ATP but not with 8-azido-[\( \gamma^{-32}P \)]ATP, they are supposed to have ATPase activity. ATPase activities of some ABC proteins have been measured, and it was reported that MDR1 has relatively high ATPase activity (300–1900 nmol/min/mg) (43–46), whereas CFTR has low ATPase activity (50 nmol/min/mg) (47, 48). It has been suggested that changes in intracellular ADP concentration are responsible for metabolic regulation of \( K_{ATP} \) channel activity, and ADP is suggested to be involved in channel opening, either by direct binding to NBF2 or hydrolysis of bound ATP at NBF2. Therefore, if ATPase activities of NBF2 of SURx are different, this might account for their different physiological roles. Unfortunately, it was not possible to quantify the ATPase activity by the photoaffinity labeling methods used in this study. We are currently attempting to measure their ATPase activities using purified SURx.

The \( Mg^{2+} \) dependence of 8-azido-ATP binding to NBF2 is different among SUR subtypes. NBF2s of SUR2A and SUR2B can be photoaffinity-labeled even in the presence of EDTA, although NBF2 of SUR1 is labeled only when \( Mg^{2+} \) is added to the reaction mixture. Because \( Mg^{2+} \) has been reported to be involved in the regulation of various ion channels (49–51), the differences in \( Mg^{2+} \) dependence might be involved in the differential regulation of \( K_{ATP} \) channel subtypes.

Previously, we demonstrated that MgATP and MgADP stabilize 8-azido-ATP binding at NBF1 in SUR1 (21). This stabilization effect was also observed in SUR2A and SUR2B as shown in Fig. 7. Because a non-hydrolyzable ATP analog does not stabilize 8-azido-[\( \alpha^{-32}P \)]ATP binding at NBF1 of SUR1 (21) and because NBF2s of SUR2A and SUR2B have been suggested to have ATPase activity similar to that of SUR1, MgADP probably stabilizes 8-azido-[\( \alpha^{-32}P \)]ATP binding at NBF1 of SURx, either by direct binding or by hydrolysis of bound MgATP at NBF2.

ATPase activity of MDR1 is inhibited by orthovanadate, because MDR1 binds orthovanadate (Vi) to form a stable inhibitory complex, MDR1-MgADP-Vi, after ATP hydrolysis and release of phosphate ion (52). This vanadate-induced nucleotide trapping was also reported in MRP1 (53–56). Szabó et al. (57) has reported that the photoaffinity labeling of NBF2 of CFTR increases in the presence of orthovanadate, whereas NBF1 of CFTR binds 8-azido-[\( \alpha^{-32}P \)]ATP stably similar to SURx. However, we observed no vanadate-induced nucleotide trapping in any NBFs of SURx subtypes (data not shown), in agreement with the results of experiments demonstrating that orthovanadate does not influence \( K_{ATP} \) channel activity (41, 58). This suggests that the mechanism of ATP hydrolysis at NBF2 of SURx is different from other ABC proteins.

SURx was expressed transiently in COS-7 cells without Kir6.2 subunit in this study. However, SURx couples with Kir6.2 to form a hetero-octamer in vivo. We cannot exclude the possibility that the affinities of NBFs of SURx coupled with Kir6.2 are different from those obtained in this study. Further studies on nucleotide-binding properties and affinities of coexpressed SURx and Kir6.2 are to be examined.

In summary, we have analyzed the nucleotide-binding properties of two NBFs of SURx and demonstrated differences among SUR subtypes using biochemical methods for the first time. The results of this study will facilitate our understanding of the molecular mechanism of \( K_{ATP} \) channel regulation.

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