MgADP Antagonism to Mg^{2+}-independent ATP Binding of the Sulfonylurea Receptor SUR1

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Pancreatic β-cell ATP-sensitive potassium (K_{ATP}) channels play an important role in the regulation of glucose-induced insulin secretion. The β-cell K_{ATP} channel comprises two subunits, the sulfonylurea receptor SUR1, a member of the ATP-binding cassette (ABC) superfamily, and Kir6.2, a member of the inward rectifier K^+ channel family. The activity of the K_{ATP} channel is under complex regulation by the intracellular ATP and ADP. To understand the roles of the two nucleotide-binding folds (NBFs) of SUR1 in the regulation of K_{ATP} channel activity, we introduced point mutations into the core consensus sequence of the Walker A or B motif of each NBF of SUR1 and characterized ATP binding and MgADP antagonism to it. SUR1 was efficiently photolabeled with 8-azido-[α-32P]ATP and 8-azido-[γ-32P]ATP in the presence or absence of Mg^{2+} or vanadate. NBF1 mutations impaired ATP binding, but NBF2 mutations did not. MgADP strongly antagonized ATP binding, and the NBF2 mutation reduced MgADP antagonism. These results show that SUR1, unlike other ABC proteins, strongly binds ATP at NBF1 even in the absence of Mg^{2+} and that MgADP, through binding at NBF2, antagonizes the Mg^{2+}-independent high affinity ATP binding at NBF1.

The sulfonylurea receptor (SUR) is a member of the ATP-binding cassette (ABC) superfamily (1, 2), which includes cystic fibrosis transmembrane conductance regulator (CFTR), P-glycoprotein (Pgp), and multidrug resistance-associated protein (MRP) (3). Recent studies have shown that a sulfonylurea receptor and Kir6.2, an inward rectifier K^+ channel member, constitute ATP-sensitive K^+ (K_{ATP}) channels (4, 5). K_{ATP} channels, as the ATP and ADP sensors in pancreatic β-cells, regulate glucose-induced insulin secretion by linking the cell’s metabolic state to its membrane potential (6). It has generally been accepted that an increase in the ATP/ADP ratio inhibits K_{ATP} channel activity, while a decrease in the ratio stimulates activity (7–10). ADP, in the absence of Mg^{2+}, however, may cause channel inhibition as a weaker analog of ATP. MgADP has been shown to antagonize channel inhibition by ATP (11, 12). ATP inhibition of K_{ATP} channel activity may require binding at two sites, and a MgADP antagonism might occur through competitive binding at one of the sites (10). The activity of the β-cell K_{ATP} channel thus is regulated by a complex mechanism of intracellular ATP and ADP.

SUR1 has the highly conserved Walker A and B motifs and the SGGQ ABC signature in each putative nucleotide-binding fold (NBF), both of which are thought to be critical for nucleotide regulation of the functional activity of ABC proteins (3, 13). Pgp has been suggested to have no high affinity binding site for MgATP, and the ATP hydrolysis step generates a Pgp-MgADP-P, conformation of high chemical potential that may be coupled to drug transport (14). Both NBFs of Pgp bind and hydrolyze MgATP. Thus, the roles of the two NBFs of Pgp in drug transport may be equivalent (15). On the other hand, in CFTR, a Cl^- channel, the roles of the two NBFs are thought to be different: ATP hydrolysis at one site controls channel opening and ATP hydrolysis at the other site regulates channel closing (16, 17). Although our previous studies have suggested that the SUR1 subunit confers the ATP sensitivity of pancreatic β-cell K_{ATP} channels and that the Kir6.2 subunit forms the K^+ ion-permeable domain of the channel (2, 4), direct evidence of the effects of ATP and ADP on SUR1 has not yet been reported. Nichols et al. (12) have shown that mutations in NBF2 abolish MgATP antagonism of ATP inhibition without affecting ATP inhibition itself.

It has been found recently that the apparent affinity for nucleotides of Pgp is greatly increased in the presence of vanadate (14) probably because a stable and reversible inhibitory complex is generated by trapping a nucleotide at the catalytic site. The vanadate-inhibited nucleotide trapping method has been used successfully for the analysis of the mechanism of ATP hydrolysis by MRP (18). Utilizing this technique, we show in the present study that unlike other ABC proteins such as Pgp and MRP, SUR1 strongly and stably binds ATP at NBF1 even in the absence of Mg^{2+}, and that MgADP, through binding at NBF2, antagonizes the Mg^{2+}-independent high affinity ATP binding at NBF1. Accordingly, the mechanism of the interaction of SUR1 with nucleotides is different from that of Pgp and MRP.

**EXPERIMENTAL PROCEDURES**

**Materials**—The 8-azido-[α-32P]ATP and 8-azido-[γ-32P]ATP were purchased from ICN Biomedicals.

**Transfection and Preparation for Crude Plasma Membrane Proteins**—African green monkey cells COS-7 were propagated in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum under 5% CO_2 at 37 °C. COS-7 cells were transfected with hamster SUR1 expression vectors with LipofectAMINETM (Life Technologies, Inc.) according to the manufacturer’s directions. Three days after transfection, membrane proteins were prepared by nitrogen cavitation as described previously (19) (without sucrose density gradient centrifugation). Immunoblotting was done with an antibody against the C-terminal 21 amino acids of hamster SUR1.

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1 The abbreviations used are: SUR, sulfonylurea receptor; ABC, ATP-binding cassette; CFTR, cystic fibrosis transmembrane conductance regulator; Pgp, P-glycoprotein; NBF, nucleotide-binding fold; MRP, multidrug resistance-associated protein; PHHI, persistent hyperinsulinemic hypoglycemia of infancy.

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2 T. Gono, K. Kotake, J. Sanchez, N. Inagaki, and S. Seino, unpublished data.
Fig. 1. Photoaffinity labeling of P-glycoprotein and SUR with 8-azido-[γ-32P]ATP—Membrane proteins (10 μg) from COS-7 cells expressing SUR1 (lanes 1–4) or P-glycoprotein (lanes 5–9) or from untransfected COS-7 cells (lanes 10 and 11) were incubated with 5 μM 8-azido-[γ-32P]ATP or 8-azido-[γ-32P]ATP, 2 mM ouabain, 0.1 mM EGTA, and 40 mM Tris-Cl (pH 7.5) in the presence of 3 mM MgCl2 and 200 μM orthovanadate (lanes 1, 5, 6, 10, and 11), 3 mM MgCl2 (lanes 2 and 7), 1 mM EDTA and 200 μM orthovanadate (lanes 3 and 8), and 1 mM EDTA (lanes 4 and 9) for 10 min at 37 °C. The reactions were in the presence of 20 μM verapamil (lanes 6–9 and 11). Proteins were photoaffinity-labeled with UV irradiation after removal of unbound ligands and analyzed as described under “Experimental Procedures.”

Fig. 2. ATP concentration dependence of photoaffinity labeling of SUR1. A, membrane proteins (8 μg) from COS-7 cells expressing SUR1 were incubated with 8-azido-[γ-32P]ATP at 0.69 μM (lane 1), 1.04 μM (lane 2), 1.56 μM (lane 3), 2.34 μM (lane 4), 3.51 μM (lane 5), 5.25 μM (lane 6), 7.99 μM (lane 7), 11.9 μM (lane 8), 17.8 μM (lane 9), 26.7 μM (lane 10), 40 μM (lane 11), and 60 μM (lane 12) in the presence of 3 mM MgCl2, 200 μM orthovanadate, 2 mM ouabain, and 0.1 mM EGTA for 10 min at 37 °C. B, membrane proteins (8 μg) from COS-7 cells expressing SUR1 were incubated with 8-azido-[γ-32P]ATP of 0.31 μM (lane 1), 0.63 μM (lane 2), 1.25 μM (lane 3), 2.5 μM (lane 4), 5 μM (lane 5), 10 μM (lane 6), 20 μM (lane 7), 40 μM (lane 8), and 80 μM (lane 9) in the presence of 2 mM ouabain, 0.1 mM EGTA, and 1 mM EDTA for 10 min at 37 °C. Proteins were photoaffinity-labeled with UV irradiation after removal of unbound ligands and analyzed as described under “Experimental Procedures.”

RESULTS

Effect of Mg2+ and Vanadate on Nucleotide Binding in SUR1—We investigated whether SUR1 trapped a nucleotide at the catalytic site in the presence of vanadate, as has been reported of Pgp. Membrane proteins from COS-7 cells transiently expressing SUR1 were first preincubated with vanadate, Mg2+, and 5 μM 8-azido-[α-32P]ATP or 8-azido-[γ-32P]ATP, 2 mM ouabain, 0.1 mM EDTA, and 40 mM Tris-Cl (pH 7.5) in a total volume of 5 μl for 10 min at 37 °C. (Variations of these conditions are described in the figure legends.) The reactions were stopped by the addition of 400 μl of ice-cold Tris-EGTA buffer (40 mM Tris-Cl (pH 7.5), 0.1 mM EDTA), and free ATP was removed after centrifugation (15,000 × g, 10 min, 4 °C). Pellets were washed in the same buffer, resuspended in 8 μl of Tris-EGTA buffer, and irradiated for 5 min (at 254 nm, 5 milliwatts/cm²) on ice. Samples were electrophoresed on a 7% SDS-polyacrylamide gel, and autoradiographed. The trapped 8-azido-[γ-32P]ATP in SUR1 was measured by counting the radioactivity of the band of SUR1 excised from an SDS gel or by scanning with a radioimaging analyzer (BAS2000, Fuji Photo Film Co.). Experiments were done in duplicate or triplicate.

Effects of Mutations in NBFs on MgADP Antagonism—ADP, in the absence of Mg2+, weakly antagonized Mg2+-independent high affinity ATP binding with a half-maximal inhibitory concentration (IC50) of approximately 200 μM (Fig. 4A, lanes 1–5). ADP, in the presence of Mg2+, strongly antagonized the ATP binding (IC50 < 10 μM) (Fig. 4A, lanes 6–10). Because Mg2+ did not affect the apparent affinity for ATP (Fig. 2), we assume that Mg2+ increases the affinity for ADP of SUR1. The K1385M mutation reduced MgADP antagonism (IC50, approximately 80 μM) (B, lanes 6–10), although it did not affect ADP antagonism in the absence of Mg2+ (B, lanes 1–5).

DISCUSSION

The apparent affinity for MgATP of Pgp and MRP is low, and it increases in the presence of vanadate (Fig. 1) (14, 18). The nucleotide trapped in Pgp and MRP is ADP, and the metastable state, E-MgADP-Pi, where E is Pgp or MRP, is formed after hydrolysis (14, 18). As are other nucleotide-binding and -hydrolyzing proteins, nucleotide and Mg2+ coordinate bind to Pgp. We show here that SUR1 strongly binds ATP even in the absence of Mg2+, suggesting that the interaction of SUR1 with nucleotides is different from that of Pgp and MRP.
ATP Binding and MgADP Antagonism in SUR1

Mutations of SUR1 in humans cause persistent hyperinsulinemic hypoglycemia of infancy (PHHI) (20), and K$_{ATP}$ channel function in pancreatic β-cells isolated from patients with PHHI is lost or severely impaired (21). Nichols et al. (12) have reported that mutations in NBF2 of SUR1 found in patients with PHHI did not affect the ATP sensitivity of the β-cell K$_{ATP}$ channels in the absence or presence of Mg$^{2+}$, but impaired MgADP antagonism of ATP inhibition. Their conclusions regarding the essential role of NBF2 of SUR1 in MgADP antagonism are supported by the ATP binding and ADP antagonism in vitro shown in this report.

Walker A and B motifs form a portion of a nucleotide-binding pocket (13). The lysine residue in Walker A may interact with the phosphoryl moiety of the bound nucleotide (22) and the aspartic acid in Walker B to coordinate with Mg$^{2+}$ in the MgATP complex (23). Both NBFs are necessary for ATPase and transport activities of Pgp, because mutations of the lysine in Walker A or the aspartic acid in Walker B of either one of the two NBFs impaired those activities (24, 25). The ADP binding affinity of Pgp was decreased when lysine residues in both NBFs were replaced by methionine at the same times (26).

Since substitution of the lysine in Walker A of SUR1 impaired Mg$^{2+}$-independent high affinity ATP binding, the lysine may interact with ATP, as do other ATP-hydrolyzing proteins. The aspartate in Walker B in NBF1 of SUR1, different from other ATP-hydrolyzing proteins which probably act via Mg$^{2+}$, may interact directly with ATP, because replacement of this residue with asparagine-impaired Mg$^{2+}$-independent high affinity ATP binding of SUR1.

It is postulated that drug transport of Pgp is coupled to relaxation of a high energy catalytic site conformation generated by ATP hydrolysis (15) and that ATP hydrolysis provides the free energy necessary to effect a conformational change that leads to the conducting state of CPTR (27). The Mg$^{2+}$-independent high affinity ATP binding in SUR1 could support the ATP-dependent inhibition of K$_{ATP}$ channel activity which is antagonized by MgADP (8, 11). Mg$^{2+}$-independent high affinity ATP binding was less when the reaction was done at 0°C than when it was done at 37°C (data not shown). MgADP antagonism was not observed when MgADP (a final concentration of 100 μM) was added after SUR1 was incubated with 5 μM 8-azido-[α-32P]ATP for 10 min at 37°C (data not shown), but MgADP completely blocked ATP binding when added at the same times (Fig. 4). This suggests that Mg$^{2+}$-independent ATP binding at NBF1 causes a conformational change of SUR1 that makes the binding to ATP tighter and more stable.

Gribble et al. (28) reported recently that both NBFs in SUR1 are not essential for K$_{ATP}$ channel inhibition by ATP, but that they are essential for channel activation by MgADP, using K719A and K1385M SUR1 mutants. However, we have observed that while K719M and K719R mutants severely impair functional expression of K$_{ATP}$ channels, K1385M and K1385R mutants do not. Although whether or not SUR1 has ATP hydrolysis activity is unknown, ATP binding to NBF1 of SUR1 might be important in maintaining K$_{ATP}$ channels in the operative state. It has recently been shown that a truncation of Kir6.2 itself also functions as a K$_{ATP}$ channel, with lower ATP sensitivity (29). Since SUR1 endows the truncated Kir6.2 with higher ATP sensitivity than that of the truncated Kir6.2 alone, both SUR1 and Kir6.2 subunits are probably involved in the regulation of channel activity by ATP and MgADP.

NBFl of SUR1 may be a Mg$^{2+}$-independent high affinity ATP binding site because mutations in NBF1 impaired ATP binding, and mutations at equivalent sites in NBF2 did not (Fig. 3) (12). The K1385M mutation reduced MgADP antagonism, although it did not affect ADP antagonism in the absence of Mg$^{2+}$ (Fig. 4). Because the K1385M mutation did not affect Mg$^{2+}$-independent high affinity ATP binding (Fig. 3), MgADP may antagonize the ATP binding at NBF1 through binding at NBF2, and ADP probably antagonizes the ATP binding as a weaker analog of ATP. SUR1 may have two ATP binding sites, one high affinity and the other low affinity, which may work cooperatively because SUR1 with mutations in NBF1 binds ATP only slightly even when incubated with 40 μM ATP (Fig. 2).

Fig. 3. Immunoblot analysis (A) and photoaffinity labeling with 8-azido-[α-32P]ATP (B and C) of wild-type SUR1 and the SUR1 mutants. Lane 1, wild-type SUR1; lane 2, K719R; lane 3, K719M; lane 4, D584N; lane 5, K1385R; lane 6, K1385M; lane 7, D1506N. A, membrane proteins (8–20 μg) from COS-7 cells expressing equivalent amounts of SUR1 were separated by 7% SDS-polyacrylamide gel electrophoresis, and SUR1 and mutant SUR1s were detected by immunoblotting with antibody against the C-terminal 21 amino acids of hamster SUR1. B and C, membrane proteins (8–20 μg) from COS-7 cells expressing SUR1 were incubated with 5 μM (B) or 40 μM (C) 8-azido-[α-32P]ATP, 2 mM ouabain, 0.1 mM EGTA, and 1 mM EDTA for 10 min at 37°C. Proteins were photoaffinity-labeled with UV irradiation after removal of unbound ligands and analyzed as described under “Experimental Procedures.”

Fig. 4. ADP and MgADP antagonism of ATP binding of wild-type (A) and K1385M mutant (B) SUR1. Membrane proteins (8 μg) from COS-7 cells expressing wild-type (A) or K1385M mutant (B) SUR1 were incubated with 5 μM 8-azido-[α-32P]ATP, 2 mM ouabain, 0.1 mM EGTA, and either 1 mM EDTA (lanes 1–5) or 3 mM MgCl$_2$ (lanes 6–10) in the presence of ADP at 10 μM (lanes 2 and 7), 50 μM (lanes 3 and 8), 100 μM (lanes 4 and 9), and 500 μM (lanes 5 and 10) for 10 min at 37°C. Proteins were photoaffinity-labeled with UV irradiation after removal of unbound ligands and analyzed as described under “Experimental Procedures.”

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