Communication

Direct Photoaffinity Labeling of the Kir6.2 Subunit of the ATP-sensitive K⁺ Channel by 8-Azido-ATP*

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ATP-sensitive potassium channels are under complex regulation by intracellular ATP and ADP. The potentiating effect of MgADP is conferred by the sulfonylurea receptor subunit of the channel, SUR, whereas the inhibitory effect of ATP appears to be mediated via the pore-forming subunit, Kir6.2. We determined whether ATP directly interacts with a binding site on the Kir6.2 subunit to mediate channel inhibition by analyzing binding of a photoaffinity analog of ATP (8-azido-[γ-³²P]ATP) to membranes from COS-7 cells transiently expressing Kir6.2. We demonstrate that Kir6.2 can be directly labeled by 8-azido-[γ-³²P]ATP but that the related subunit Kir4.1, which is not inhibited by ATP, is not labeled. Photoaffinity labeling of Kir6.2 is reduced by approximately 50% with 100 μM ATP. In addition, mutations in the NH₂ terminus (R50G) and the COOH terminus (K185Q) of Kir6.2, which have both been shown to reduce the inhibitory effect of ATP upon Kir6.2 channel activity, reduced photoaffinity labeling by >50%. These results demonstrate that ATP binds directly to Kir6.2 and that both the NH₂- and COOH-terminal intracellular domains may influence ATP binding.

ATP-sensitive potassium (K$_{ATP}$)[1] channels play important roles in many tissues by linking the metabolic status of the cell to its membrane potential (1, 2). In pancreatic β-cells, K$_{ATP}$ channels are critical for the regulation of glucose-induced insulin secretion (3, 4) and have recently been shown to be an octameric complex of two subunits, which coassemble with a 4:4 stoichiometry (5–9). The pore-forming subunit, Kir6.2, is a

member of the inwardly rectifying K⁺ channel family (10, 11), whereas the other subunit, the sulfonylurea receptor (SUR1), is a member of the ATP-binding cassette transporter superfamily (12, 13). Unlike most other Kir channels, expression of Kir6.2 alone does not produce functional channel activity; instead, it requires coexpression with SUR1. However, an isoform of Kir6.2 in which the last 26 amino acids have been removed (Kir6.2ΔC26) is capable of expressing functional K⁺ channel activity in the absence of SUR1. Kir6.2ΔC26 retains sensitivity to inhibition by ATP, and mutations in this subunit can significantly reduce the inhibitory effect of ATP (14, 15). This has been taken as evidence that the primary site at which ATP acts to cause K$_{ATP}$ channel closure resides on Kir6.2. However, controversy still remains as to whether ATP binds directly to Kir6.2, whether truncation of Kir6.2 exposes a cryptic blocking site for nucleotides, or whether ATP inhibition is mediated indirectly by binding of the nucleotide to an endogenous subunit that modulates the activity of Kir6.2 (9, 14). In the present study, we show that Kir6.2 directly binds the photoaffinity analog of ATP, 8-azido-ATP, and that this labeling can be reduced by 50% with 100 μM ATP. We also demonstrate that the related inwardly rectifying K⁺ channel subunit Kir4.1, which is not inhibited by ATP, exhibits no significant photoaffinity labeling by 8-azido-[γ-³²P]ATP. Furthermore, we show that mutations in Kir6.2 that reduce the inhibitory effect of ATP on channel activity also reduce photoaffinity labeling. This provides strong evidence that ATP binds directly to Kir6.2.

MATERIALS AND METHODS

Transfection and Preparation of Membranes—COS-7 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum in a humidified atmosphere of 95% air, 5% CO₂ at 37 °C. COS-7 cells were transfected with expression vectors encoding full-length wild-type or mutant mouse Kir6.2, tagged with the Flag epitope at the NH₂ terminus and with a hexahistidine tag at the COOH terminus (Flag-Kir6.2), or tagged with the Flag epitope at the COOH terminus (Kir6.2-Flag), using LipofectAMINEplus (Life Technologies, Inc.) according to the manufacturer's directions. Rat Kir4.1 was tagged with the Flag epitope at the COOH terminus (Kir4.1-Flag). Addition of these epitopes did not significantly affect the functional properties of the channel (data not shown). Two days after transfection, cellular membranes were prepared as described previously (16). Immunoblotting was carried out with an anti-Flag M2 monoclonal antibody (Eastman Kodak).

Photoaffinity Labeling of Flag-Kir6.2 with 8-Azido-[γ-³²P]ATP—8-Azido-[γ-³²P]ATP (500–600 GBq/mmol) was purchased from ICN Biomedicals. Membranes were incubated with 50 μM 8-azido-[γ-³²P]ATP, 2 mM ouabain, 0.1 mM EGTA, 4 mM MgSO₄, and 40 mM Tris-Cl (pH 7.5) in a total volume of 6 μl for 10 min on ice. After UV irradiation (at 254 nm, 4.4–8.2 milliwatts/cm²) for 15 s to 3 min, 500 μl of TE buffer (40 mM Tris-HCl (pH 7.5), 0.1 mM EGTA) was added to the mixture, and free 8-azido-[γ-³²P]ATP was removed by centrifugation (15,000 × g, 10 min, 4 °C). The pellet was solubilized with 100 μl of RIPA buffer (20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 0.15 M NaCl, 10 μg/ml leupeptin, 100 μg/ml (p-amidinophenyl)-methane-sulfonyl fluoride). The lysate was kept on ice for 15 min and centrifuged to remove insoluble material. Flag-Kir6.2 was immunoprecipitated from the supernatant with the anti-Flag M2 antibody. Samples were electrophoresed on a 10% SDS-polyacrylamide gel and autoradiographed. Bound 8-azido-[γ-³²P]ATP to Kir6.2 was measured by scanning with a radioimaging analyzer (BAS2000, Fuji Photo Film Co.). Experiments were carried out in duplicate.

Electrophysiological Studies—Macroscopic currents were recorded from giant inside-out patches excised from *Xenopus* oocytes expressing Kir6.2ΔC26, as described previously (17). Currents were recorded at a holding potential of 0 mV in response to repetitive voltage ramps from

FIG. 1. **Inhibition of Kir6.2ΔC26 by 8-azido-ATP.** *A*, macroscopic currents recorded from a giant inside-out patch on an oocyte injected with mRNA encoding Kir6.2ΔC26. Currents were elicited in response to a series of voltage ramps from −110 mV to +100 mV. ATP or 8-Azido-ATP was added to the internal solution as indicated by the *bar*. *B*, mean dose-response relationship for ATP (*n* = 6) or 8-azido-ATP (*n* = 6). Test solutions were alternated with control solutions, and the slope conductance (*G*) is expressed as a fraction of the mean (*G*$_c$) of that obtained in control solution before and after exposure to ATP. Conductance was measured between −20 and −100 mV and is the mean of five voltage ramps. The *solid lines* are the best fit of the data to the Hill equation (see "Materials and Methods") using the mean values for K_i and *h* given in the text.

−110mV to +100 mV, at 20–24 °C. The pipette solution contained 140 mM KCl, 1.2 mM MgCl$_2$, 2.6 mM CaCl$_2$, and 10 mM HEPES (pH 7.4 with KOH), and the internal (bath) solution contained 110 mM KCl, 2 mM MgCl$_2$, 1 mM CaCl$_2$, 30 mM KOH, 10 mM EGTA, and 10 mM HEPES (pH 7.2 with KOH) and 8-azido-ATP as indicated. ATP dose-response relationships were measured by alternating the control solution with a test ATP solution, and the extent of inhibition by ATP was expressed as a fraction of the mean of the value obtained in the control solution before and after ATP application (17). ATP dose-response curves were fit to the Hill equation $G/G_c = 1/(1 + ([ATP]/K_i)^h)$, where [ATP] is the ATP concentration, K_i is the ATP concentration at which inhibition is half-maximal, and *h* is the slope factor (Hill coefficient).

<center>RESULTS</center>

8-Azido-ATP Inhibition of Kir6.2ΔC26 Currents—We first examined the ability of 8-azido-ATP to inhibit Kir6.2ΔC26 currents. Fig. 1 shows that this nucleotide blocks Kir6.2ΔC26 currents rather less potently than ATP, half-maximal inhibition (*K$_i$*) occurring at 2.8 ± 0.4 mM (*n* = 6) compared with 172 ± 7 μM for ATP (*n* = 6). The Hill coefficients were 0.9 ± 0.2 for 8-azido-ATP and 1.3 ± 0.1 for ATP.

8-Azido-ATP Labeling of Kir6.2—We next investigated the direct interaction of Kir6.2 with ATP using the photoaffinity ATP analog, 8-azido-[γ-^{32}P]ATP. Flag-Kir6.2 and Kir4.1-Flag were transiently expressed in COS-7 cells, and expression levels were monitored by immunoblot analysis of membrane fraction preparations (Fig. 2*A*). Membranes were incubated with 50 μM 8-azido-[γ-^{32}P]ATP for 10 min on ice and irradiated with UV light. Flag-Kir6.2 and Kir4.1-Flag were immunoprecipitated with an anti-Flag M2 antibody and subjected to electrophoresis. The autoradiogram in Fig. 2*B* shows an approximately 43-kDa photoaffinity-labeled protein to be immunoprecipitated from Flag-Kir6.2 transfected cells (Fig. 2*B*, *lanes 3* and *4*) but not from untransfected cells (Fig. 3*A*, *lanes 3* and *4*). The molecular mass of this photoaffinity-labeled membrane protein
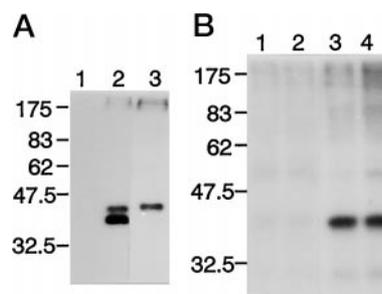
FIG. 2. **Photoaffinity labeling of Flag-Kir6.2 with 8-azido-[γ-^{32}P] ATP.** *A*, membranes (10 μg) from COS-7 cells expressing Kir4.1-Flag and Flag-Kir6.2 were separated by 10% SDS-polyacrylamide gel electrophoresis and detected by immunoblotting with anti-Flag monoclonal antibody M2. *Lane 1*, untransfected cells; *lane 2*, Kir4.1-Flag transfected cells; *lane 3*, Flag-Kir6.2 transfected cells. *B*, membranes (80 μg) were incubated with 50 μM 8-azido-[γ-^{32}P] ATP for 10 min on ice. Proteins were photoaffinity-labeled by UV irradiation (at 254 nm, 8.2 milliwatts/cm^2) for 15 s (*lanes 1* and *3*) and 30 s (*lanes 2* and *4*). Kir4.1-Flag and Flag-Kir6.2 were immunoprecipitated with antibody M2 after solubilization and analyzed as described under "Materials and Methods."
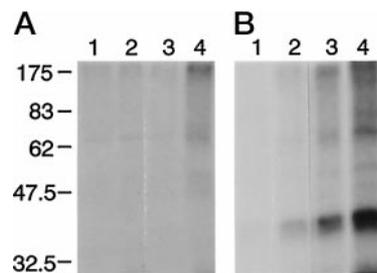
FIG. 3. **Concentration dependence of photoaffinity labeling of Flag-Kir6.2 with 8-azido-[γ-^{32}P] ATP.** Membranes (100 μg) from untransfected COS-7 cells (*A*) or COS-7 cells expressing Flag-Kir6.2 (*B*) were incubated with 8-azido-[γ-^{32}P] ATP at 10 μM (*lane 1*), 50 μM (*lane 2*), 100 μM (*lane 3*), and 200 μM (*lane 4*). Proteins were photoaffinity-labeled with UV irradiation (at 254 nm, 4.4 milliwatts/cm^2) for 3 min. Flag-Kir6.2 was immunoprecipitated with antibody M2 after solubilization as described under "Materials and Methods."

is identical to that of Flag-Kir6.2 identified by Western blotting (Fig. 2*A*). Neither of the two bands observed in the Western blot of Kir4.1-Flag transfected cells (Fig. 2*A*) exhibited any significant photoaffinity labeling (Fig. 2*B*, *lanes 1* and *2*). Attachment of the Flag epitope at the COOH terminus of Kir4.1 is unlikely to hinder immunoprecipitation because photoaffinity-labeled Kir6.2-Flag (COOH-terminal tag) was precipitated as efficiently as the NH$_2$-terminal fusion, Flag-Kir6.2 (data not shown). Flag-Kir6.2 could also be photoaffinity-labeled with 8-azido-[α-^{32}P]ATP (data not shown).

Concentration Dependence—Membranes from Flag-Kir6.2 transfected cells were incubated with different concentrations of 8-azido-[γ-^{32}P]ATP and photoaffinity-labeled (Fig. 3*B*). Photoaffinity labeling increased with increasing concentrations of 8-azido-[γ-^{32}P]ATP, and no saturation was observed at the highest concentration tested (200 μM). No protein smaller than 50-kDa showed specific photoaffinity labeling in membranes from untransfected COS-7 cells, even with 200 μM 8-azido-[γ-^{32}P]ATP (Fig. 3*A*).

Competition of Photoaffinity Labeling by ATP—To determine whether photoaffinity labeling of Flag-Kir6.2 was specific, competition by ATP was examined (Fig. 4). Membranes were preincubated with 100 μM, 1 mM, and 2 mM ATP on ice for 10 min, and then 50 μM 8-azido-[γ-^{32}P]ATP was added. Photoaffinity labeling of Flag-Kir6.2 was reduced as the concentration of ATP was increased. Quantitation by radioimaging analysis revealed that photoaffinity labeling was reduced by approximately 50% in the presence of 100 μM ATP.
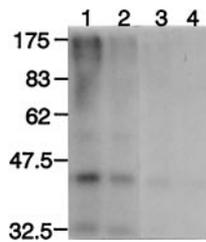
FIG. 4. **Inhibition of 8-azido-[γ-^{32}P] ATP photoaffinity labeling of Flag-Kir6.2 by cold ATP.** Membranes (100 μg) from COS-7 cells expressing Flag-Kir6.2 were incubated without (*lane 1*) or with 100 μM (*lane 2*), 1 mM (*lane 3*), and 2 mM (*lane 4*) cold ATP for 10 min on ice, and 50 μM 8-azido-[γ-^{32}P]ATP was added to the mixture. The mixture was incubated for 10 min on ice, and proteins were photoaffinity-labeled with UV irradiation (at 254 nm, 4.4 milliwatts/cm^2) for 3 min. Flag-Kir6.2 was immunoprecipitated after solubilization as described under "Materials and Methods."

Effects of Kir6.2 Mutations on 8-Azido-ATP Labeling—We have previously identified two mutations that reduce the apparent ATP sensitivity of Kir6.2ΔC26 from a K_i of ~100 μM to ~4 mM. These are R50G in the NH$_2$ terminus and K185Q in the COOH terminus (14, 15). Neither mutation affects the level of channel expression as examined by electrophysiological methods and immunoblotting. The effect of these mutations on 8-azido-[γ-^{32}P]ATP binding was examined. Membranes prepared from cells expressing equivalent amounts of the wild-type and mutant forms of Flag-Kir6.2 (Fig. 5A) were labeled using 100 μM 8-azido-[γ-^{32}P]ATP (Fig. 5B). Quantitation of the labeling by radioimaging analysis revealed that photoaffinity labeling of the mutants R50G and K185Q was reduced by 50 and 65%, respectively, as compared with wild-type Flag-Kir6.2.

DISCUSSION

The results we present here provide the first biochemical evidence that ATP binds directly to Kir6.2. We demonstrate that Kir6.2 can be specifically labeled by the ATP photoaffinity analog 8-azido-[γ-^{32}P]ATP in the absence of SUR and that this labeling can be significantly reduced by competition with ATP. Furthermore, the related subunit Kir4.1, which is not inhibited by ATP, exhibits no photoaffinity labeling by 8-azido-[γ-^{32}P]ATP. Further evidence of the direct and specific interaction of ATP with Kir6.2 is provided by the observation that mutations in Kir6.2 that reduce the inhibitory effect of ATP on channel activity also reduce the photoaffinity labeling.

We have previously reported that SUR1 binds 8-azido-ATP with high affinity (16). SUR1 was efficiently photoaffinity-labeled with 8-azido-[^{32}P]ATP by UV irradiation even after the removal of unbound 8-azido-ATP. In contrast, Kir6.2 was not photoaffinity-labeled with 8-azido-[^{32}P]ATP by UV irradiation after the removal of unbound 8-azido-ATP (data not shown). Photoaffinity labeling with 8-azido-[γ-^{32}P]ATP did not appear to saturate even at a concentration 200 μM (Fig. 3). These results indicate that Kir6.2 has much lower affinity for 8-azido-ATP than SUR1. This is consistent with the result of the lower affinity of Kir6.2 for 8-azido-ATP (K_i = 2.8 mM) as compared with ATP itself (K_i = ~100 μM). Introduction of the reactive azido group at the 8′ position may account for this reduced affinity because Kir6.2 demonstrates high specificity toward the adenine moiety of ATP (15). Labeling with 8-azido-[γ-^{32}P]ATP required 0.1 mM ATP for approximately 50% displacement, a value that is consistent with that found for half-maximal inhibition of Kir6.2ΔC26 currents (~0.1 mM).
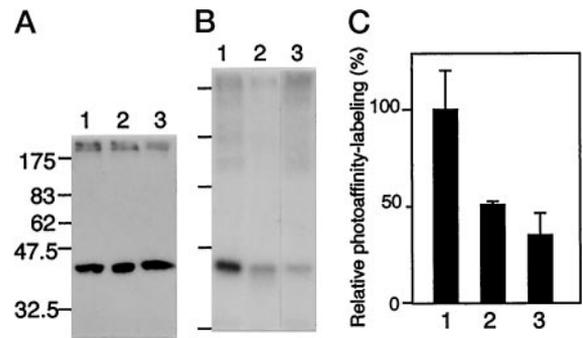
FIG. 5. **Mutations in Flag-Kir6.2 reduce photoaffinity labeling with 8-azido-[γ-^{32}P]ATP.** *Lanes 1*, wild type; *lanes 2*, R50G; *lanes 3*, K185Q. *A*, membranes (10–20 μg) from COS-7 cells expressing equivalent amounts of wild-type or mutant Flag-Kir6.2 were separated by 10% SDS-polyacrylamide gel electrophoresis and detected by immunoblotting with anti-Flag monoclonal antibody M2. *B*, membranes (50–100 μg) from COS-7 cells expressing equivalent amounts of wild-type and mutant Flag-Kir6.2 were photoaffinity-labeled with 100 μM 8-azido-[γ-^{32}P]ATP. *C*, relative photoaffinity labeling of wild-type and mutant Flag-Kir6.2, expressed as percentages of that observed for wild-type Flag-Kir6.2.

Mutations in both the NH$_2$- and COOH-terminal intracellular domains have been identified that significantly reduce ATP inhibition of Kir6.2ΔC26 currents (14, 15). The mutations R50G in the NH$_2$ terminus and K185Q in the COOH terminus both reduce the K_i for inhibition of Kir6.2ΔC26 from ~100 μM to ~4 mM (14, 15). In support of the fact that channel inhibition is mediated by a direct interaction of ATP with Kir6.2, we found that both of these mutations also exhibit significantly reduced photoaffinity labeling with 8-azido-[γ-^{32}P]ATP (Fig. 5). Neither mutation affected the channel gating kinetics (15) and so are predicted to influence ATP sensitivity by effects on ATP binding and/or the link between binding and gating. However, although we show that mutations in both the NH$_2$- and COOH-terminal intracellular domains influence labeling, it remains unclear whether the effect of these mutations reflects a direct interaction of these residues with ATP or whether their effects on ATP binding are mediated indirectly.

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