Compromised ATP binding as a mechanism of phosphoinositide modulation of ATP-sensitive K^+ channels

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Abstract Inhibition of ATP-sensitive K^+ (K_{ATP}) channels by ATP, a process presumably initiated by binding of ATP to the pore-forming subunit, Kir6.2, is reduced in the presence of phosphoinositides (PPIs). Previous studies led to the hypothesis that PPIs compromise ATP binding. Here, this hypothesis was tested using purified Kir6.2. We show that PPIs bind purified Kir6.2 in an isomer-specific manner, that biotinylated ATP analogs photoaffinity label purified Kir6.2, and that this labeling is weakened in the presence of PPIs. Patch-clamp measurements confirmed that these ATP analogs inhibited Kir6.2 channels, and that PPIs decreased the level of inhibition. These results indicate that interaction of PPIs with Kir6.2 impedes ATP-binding activity. The PPI regulation of ATP binding revealed in this study provides a putative molecular mechanism that is potentially pivotal to the nucleotide sensitivity of K_{ATP} channels.

Key words: Inwardly rectifying potassium channel; Purification; Photoaffinity labeling; ATP analog; Phosphoinositide

1. Introduction

ATP-sensitive K^+ channels (K_{ATP} channels) are heteromeric complexes of regulatory sulfonylurea receptor (SUR) subunits and channel-forming Kir6 subunits [1,2]. K_{ATP} channels become permeable to K^+ when intracellular ATP levels are reduced, thus linking the metabolic status of the cell to membrane excitability and permeability. Membrane-bound phosphoinositides (PPIs), which are versatile signaling molecules, modulate K_{ATP} channels, including their ATP sensitivity [3–7]. Electrophysiological studies suggest that PPIs are an essential determinant of the ATP sensitivity of K_{ATP} channels in intact cells [8]. However, the physiological role of this modulation remains unclear, partly because its molecular nature is poorly understood [9,10].

Theoretically, PPI modulation of K_{ATP} channels could result from direct interaction between the lipids and the channel protein, or could be mediated indirectly through signal transduction pathways. It is also possible that changes in lipid composition could affect K_{ATP} channels through a change in the physical chemistry properties of the membrane. So far, research on this subject has been largely restricted to electrophysiological characterization of the effects of PPIs on K_{ATP} channels and mutants. However, such an approach has limitations. For example, studies of single-channel function have not yet determined whether PPIs alter ATP binding. In this study, we used biochemical approaches to address this issue. Our data show that PPIs specifically bind to the channel-forming subunit, Kir6.2, and reduce ATP-binding activity. These observations indicate that compromised ATP binding is a mechanism whereby PPIs reduce channel inhibition by ATP. Competition between the binding of phosphatidylinositol bisphosphate (PIP_2) and ATP to a peptide derived from the C-terminal sequence of Kir6.2 was also reported recently [11]. Our present results are consistent with these data and further extend the finding to include the whole Kir6.2 subunit. A portion of this work was presented previously as an abstract [12].

2. Materials and methods

2.1. Construction of plasmids encoding Kir6.2

A truncated form of the Kir6.2 subunit, Kir6.2ΔC35, was constructed using a PCR-based site-directed mutagenesis kit, ExSite (Stratagene, CA, USA), in a mammalian expression vector, pcDNA3.1 (Invitrogen, CA, USA). The Kir6.2ΔC35 sequence was also subcloned into a yeast expression vector, pYES2/NT (Invitrogen), containing a *GAL1* promoter for high-level inducible protein expression by galactose and repression by glucose, for expression with a $(His)_6$-Xpress tag at its N-terminus for affinity purification and antibody recognition. Kir6.2ΔC35 without SUR subunits expresses functional ATP-sensitive channels in mammalian [13] and yeast cells [14]. For simplicity, in this study Kir6.2ΔC35 is referred as Kir6.2Δ and the tagged Kir6.2ΔC35 as Kir6.2Δ+.

2.2. Heterologous expression in cultured mammalian cells

The pcDNA3.1-Kir6.2Δ vector was introduced into COS-1 cells using a NovaFECTOR kit (VennNova, FL, USA). Transient expression of Kir6.2 typically peaked at 64–72 h after transfection [4].

2.3. Expression of Kir6.2 in yeast cells

Competent *Saccharomyces cerevisiae* NVSc1 cells (Invitrogen) were transformed with pYES2/NT-Kir6.2Δ+ vector. This yeast strain is auxotrophic for uracil so that the transformants with the vector containing a *URA3* auxotrophic gene could be selected on plates of uracil-deficient SC minimal medium. Selected colonies were cultured in a shaking incubator, and protein expression was induced by supplying galactose to the medium. The cells were allowed to grow until reaching OD_{600} 2–3.

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Abbreviations: K_{ATP} channels, ATP-sensitive K^+ channels; PPI, phosphoinositide; 2-N_3-ATP-[γ]bio, 2-azidoadenosine 5′-triphosphate [γ]-biotin; ATP-[γ]azidoanilide-bio, adenosine 5′-triphosphate [γ]azidoanilide-biotin

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2.4. Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting

Protein samples either in liposomes or in solution were pretreated for 10 min at 100°C in 60 mM Tris–HCl, 25% glycerol, 2% SDS, 14 mM 2-mercaptoethanol, and 0.1% bromophenol blue at pH 6.8. Protein content was analyzed by SDS–PAGE in a discontinuous Tris/glycine buffer system according to the Laemmli protocol in 0.75- or 1.5-mm-thick slab gels (9.5% acrylamide, 3% stacking gel). Gels were stained with Coomassie blue or silver-staining methods. Molecular weights of the samples were determined by comparison to markers (Sigma, MO, USA). Immunoblotting was performed on nitrocellulose membranes (S and S). Briefly, proteins were transferred from gels to the membrane in a semidry-blot device. Membranes were washed and blocked in blocking buffer [3% bovine serum albumin (BSA) in Tris-buffered saline (TBS), Tris–HCl 20 mM, NaCl 150 mM, pH 7.5], and then incubated with an adequately diluted primary antibody. The primary antibody was recognized by a secondary antibody conjugated with alkaline phosphatase (AP) or horseradish peroxidase. Streptavidin-AP (Ambion, TX, USA) was used to specifically recognize biotinylated samples. The antibodies or streptavidin were visualized with a color-developing kit (BioRad Labs, CA, USA), or chemiluminescent-staining kit, SuperSignal West Pico (Pierce, IL, USA) or CDP-Star (Ambion). Stained protein bands were scanned and analyzed by densitometry using Scion Image software.

2.5. Protein purification

Affinity chromatography was used to purify Kir6.2Δ+ from fractionated yeast membrane samples. From 2 l of 24-h yeast culture, 6–15 g wet weight of yeast was usually harvested. The cells were broken with glass beads in a power-driven beater (BioSpec, OK, USA). The suspension was briefly centrifuged, and laid on a sucrose gradient (18%, 25%, 30%, 35%, and 45% sucrose) in TED buffer (Tris–HCl 20 mM, EDTA 1 mM, DTT 1 mM, pH 7.4). After centrifugation at 35 000 r.p.m. for 3 h at 4°C in a SW41 Ti rotor (Beckman), the membrane fractions were collected at all gradient interfaces. Membrane fractions with high content of Kir6.2Δ+ were pooled, diluted with 4 volumes of cold water, and centrifuged again. The resulting pellet was dissolved in 25 mM sucrose and 10 mM HEPES–KOH (pH 7.4). The collected membranes were solubilized with 8–20 mM CHAPS, and protein concentration was determined by the Bradford method. Kir6.2Δ+ was isolated by Ni^{2+}-chelate chromatography. The solubilized membrane proteins were applied to a ProBond resin column (Invitrogen). Unbound proteins were washed from the column with loading buffer and low concentrations of imidazole, and proteins bound to the column were further eluted with high concentrations of imidazole. Fractions with high Kir6.2Δ+ content were identified by immunoblotting. Untagged Kir6.2Δ was obtained by incubating Kir6.2Δ+ with an enterokinase, Enterokinase Max (Invitrogen), to cleave the tags. The resulting Kir6.2Δ was then separated from the enzyme and cleaved tags using an EK-Away resin kit (Invitrogen). Whenever necessary, buffer and protein concentration were adjusted by dialyzing in Slide-A-Lyzer Dialysis Cassettes (Pierce), and concentrating in Centricon centrifugal filters (Millipore, MA, USA). Proteinase inhibitor, phenylmethylsulfonyl fluoride (0.1 mM), was included during purification, and leupeptin (300 ng/ml) and pepstatin (1.4 μg/ml) were also included during the initial steps. The pH of all buffers was adjusted to 6.5–7.8 to avoid denaturation.

2.6. Incorporation of Kir6.2 into liposomes

Defined mixtures of phospholipids, after drying under a stream of nitrogen, were dissolved at 50 mg/ml in buffer containing 8 mM CHAPS. Solubilized Kir6.2Δ+ was added to make a final protein concentration of ~200 μg/ml. CHAPS was extracted from the mixture by overnight dialysis at 4°C against an appropriate intravesicle buffer, and this was repeated to remove residual detergent.

2.7. Photoreactive labeling

Freshly purified or incorporated Kir6.2 samples were diluted to 50 or 100 μg/ml in TBS supplemented with 50 μM oxidized glutathione at pH 7.4, into which photoaffinity analogs of ATP were added. The reaction mixtures were incubated for 30 min on ice in covered glass tubes. The mixtures were then irradiated at 254 nm for 45–90 s at room temperature in a UV crosslinker (Stratalinker UV Crosslinker, Model 2400, Stratagene). The lamp delivers UV light to the preparation tube at an intensity of ~4 mW/cm^2.

2.8. PPI-binding assay

Lipid-spotted membranes (Echelon Inc., UT, USA) were blocked

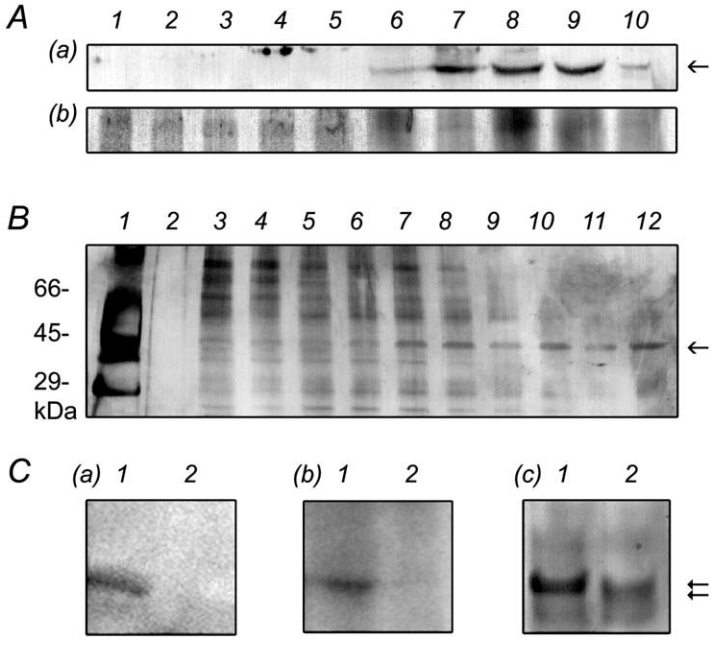


Fig. 1. Purification of Kir6.2 heterogeneously expressed in *S. cerevisiae* NVSc1 cells. A: (a) Western blot of isolated membrane fractions labeled with anti-poly(His) antibody. Lanes 1–5 – samples from mock transformants; lanes 6–10 – samples from cells transformed with pYES2/NT-Kir6.2Δ+. The samples (left to right) were collected from the interfaces of 18, 25, 30, 35, and 45% sucrose and from the bottom of the sucrose gradient. (b) Coomassie blue-stained SDS–PAGE gel. B: SDS–PAGE gel showing the purification stages. Lane 1 – molecular weight marker; lane 2 – blank; lane 3 – total membrane proteins; and lanes 4–12 – washes and eluates from the affinity resin column. C: (a) Western blot of purified Kir6.2Δ+ (lane 1) and a control protein (lane 2) labeled with anti-Kir6.2 antibody. (b) Western blotting with anti-Xpress antibody against an Xpress recognition site on the tag before (lane 1) and after (lane 2) cleavage of the tag with enterokinase. (c) Coomassie blue-stained SDS–PAGE gel. The arrows point to the Kir6.2 bands.

for 1 h in TBST (TBS with 0.01% Tween 20) containing 3% BSA. Solubilized Kir6.2 (1 μg/ml) was incubated with the membrane for 30 min at 4°C in the same buffer. The membranes were washed, incubated for 2 h with an antibody specific for Kir6.2 fusion protein, and then washed again. The membranes were subsequently incubated with a secondary antibody conjugated with AP for 2 h and then visualized.

2.9. Patch-clamp recordings and analysis

The patch-clamp and data-acquisition system is a well-established technique in our lab [7]. In inside-out patch-clamp experiments, the intracellular solution contained (in mM): KCl 140, HEPES 5.5, EGTA 2, pH 7.3 adjusted using KOH, and the extracellular solution contained (in mM): NaCl 130, KCl 10, $CaCl_2$ 1.8, $MgCl_2$ 0.48, HEPES 5.5, and pH 7.4.

3. Results

3.1. Purification of Kir6.2

Expression of Kir6.2Δ+ in yeast cells was detected using a monoclonal anti-polyhistidine antibody (Sigma). Analysis of the subcellular distribution by sucrose gradient ultracentrifugation showed that Kir6.2Δ+ was most abundant in the plasma membrane fraction (Fig. 1A, collected from interfaces of 35–45% sucrose) [15]. During the preliminary experiments, three detergents, Triton X-100, Zwittergent 3-14, and CHAPS, were tested to optimize recovery of Kir6.2Δ+. It appears that 8 mM CHAPS (a concentration close to its upper CMC limit) provided sufficient solubility and repeatable results. Purification was monitored by SDS–PAGE of samples taken from the various eluted fractions (Fig. 1B). Identity of the purified Kir6.2Δ+ was confirmed by labeling with two unrelated antibodies: a monoclonal anti-Xpress antibody (Invitrogen), and an anti-Kir6.2 antibody (Fig. 1C; Kir6.2 antibody was a gift from Dr. D.E. Clapham).

3.2. Isomer-specific binding of PPIs to Kir6.2

The dot blot binding assay was used to examine whether Kir6.2 interacts selectively with PPIs. Fig. 2 illustrates results of the binding assay. Solubilized Kir6.2Δ+ in the detergent-containing buffer bound most strongly to PIP_2 isomers; it also bound PIP_3, PIP, phosphatidic acid, and phosphatidylserine to various extents. In comparison, under this experimental

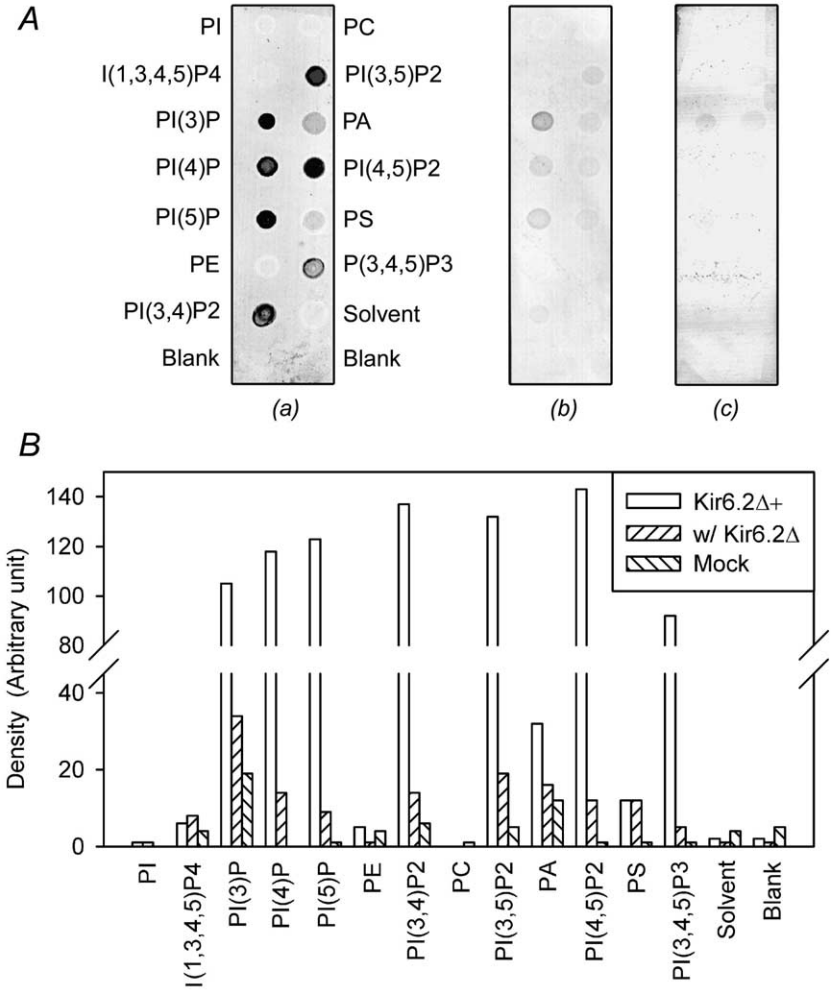


Fig. 2. Specific binding between Kir6.2 and lipids. A: (a) Dot-blot assay with anti-Xpress antibody to detect binding of the tagged Kir6.2 with lipids spotted on the membrane. (b) Dot blotting in the presence of both tagged and untagged Kir6.2 (1:10); the latter was obtained from enterokinase treatment. (c) Dot blotting of a protein (mock) unrelated to Kir6.2 but containing the same tag. B: Plot of density of the dots. Abbreviations used in the figure: $PI(3,4,5)P_3$, phosphatidylinositol 3,4,5-trisphosphate; $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; $PI(3,5)P_2$, phosphatidylinositol 3,5-bisphosphate; $PI(3,4)P_2$, phosphatidylinositol 3,4-bisphosphate; PI(3)P, phosphatidylinositol 3-monophosphate; PI(4)P, phosphatidylinositol 4-monophosphate; PI(5)P, phosphatidylinositol 5-monophosphate; PI, phosphatidylinositol phosphate; PE, phosphatidylethanolamine; PS, phosphatidylserine; PA, phosphatidic acid; PC, phosphatidylcholine; $I(1,3,4,5)P_4$, inositol (1,3,4,5)-tetrakisphosphate.

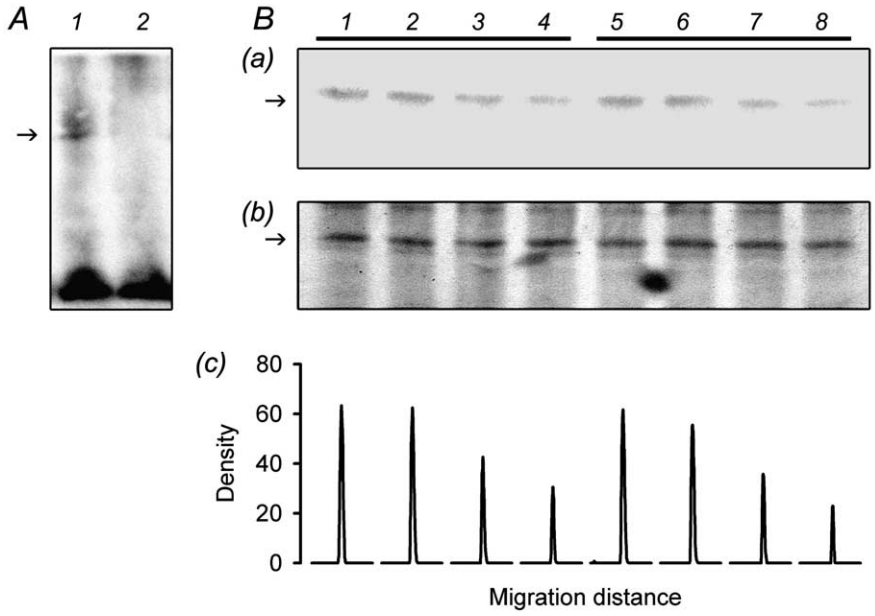
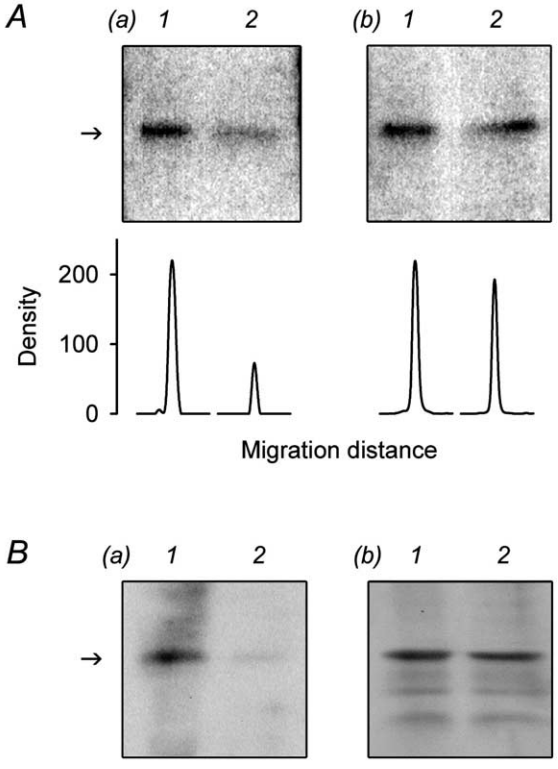


Fig. 3. Specific photoaffinity labeling of Kir6.2 by biotinylated ATP photoaffinity analogs. A: Inhibition of photolabeling of Kir6.2 by ATP. Western blot of Kir6.2Δ+ photolabeled by 100 μM 2-N_3-ATP-[γ]bio detected with streptavidin. Samples in lanes 1 and 2 were photoreacted in the absence and presence of 10 mM ATP, respectively. B: Dose-dependent inhibition of photolabeling by unbiotinylated ATP analog. (a) Western blot with streptavidin of Kir6.2Δ+ photolabeled by 100 μM of either 2-N_3-ATP-[γ]bio or ATP-[γ]azidoanilide-bio in the presence of 0 (lanes 1, 5), 0.01 (lanes 2, 6), 0.2 (lanes 3, 7), and 1 mM (lanes 4, 8) of ATP-[γ]azidoanilide. (b) Coomassie blue-stained SDS–PAGE gel. (c) Plot of density measured from B.

condition, binding of Kir6.2Δ+ to inositol tetrakisphosphate, phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol phosphate, or solvent was insignificant. Kir6.2Δ without the tag (removed by enterokinase), retarded the binding of Kir6.2Δ+ (Fig. 2), confirming that the binding site(s) is located specifically on the Kir6.2 structure.



3.3. *Photolabeling of Kir6.2 by ATP photoaffinity analogs and effect of PPIs*

Three biotinylated ATP photoaffinity analogs (ALT Inc., KY, USA) were tested for specific labeling of Kir6.2. Kir6.2Δ+ transferred onto nitrocellulose membranes after reacting with either 2-azidoadenosine 5′-triphosphate [γ]-biotin (2-N_3-ATP[γ]bio) or adenosine 5′-triphosphate [γ]azidoanilide-biotin (ATP-[γ]azidoanilide-bio) could be detected specifically with streptavidin (Fig. 3), whereas labeling with 8-azidoadenosine 5′-triphosphate [γ]-biotin resulted in poor specificity (i.e. less contrast relative to the background; data not shown). This result is consistent with a report that ATP-[γ]azidoanilide was a better probe than 8-azidoadenosine 5′-triphosphate in cell membrane preparations [16]. The amount of labeled Kir6.2Δ+ was competitively reduced when either ATP or a non-biotinylated ATP analog, ATP-[γ]azidoanilide, was included in the reaction mix (Fig. 3). Inclusion of PIP_2 but not phosphatidylcholine in the reaction considerably reduced the labeling of the solubilized Kir6.2Δ+. The ATP analogs could also label Kir6.2Δ+ incorporated into phosphatidylcho-

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Fig. 4. Effect of phospholipids on photoaffinity labeling of Kir6.2 by ATP photoaffinity analogs. A: Western blots with streptavidin illustrating the effect of phosphatidylinositol 4,5-bisphosphate (a) and phosphatidylcholine (b) on photoaffinity labeling. In both blots, lane 1 is Kir6.2Δ+ photolabeled with 2-N_3-ATP-[γ]bio in the absence of lipids, and lane 2 is in the presence of 100 μM lipids. Corresponding density plots are also given. B: Reduced photoaffinity labeling of Kir6.2 in proteoliposomes containing PPIs. (a) Western blotting with streptavidin of photolabeled Kir6.2Δ+ by 2-N_3-ATP-[γ]bio, in proteoliposomes composed of phosphatidylcholine/phosphatidylethnolamine (lane 1), and phosphatidylcholine/phosphatidylethnolamine/PPIs (lane 2, PPIs extracted from bovine brain, Sigma). (b) Corresponding SDS–PAGE gel stained with Coomassie blue.

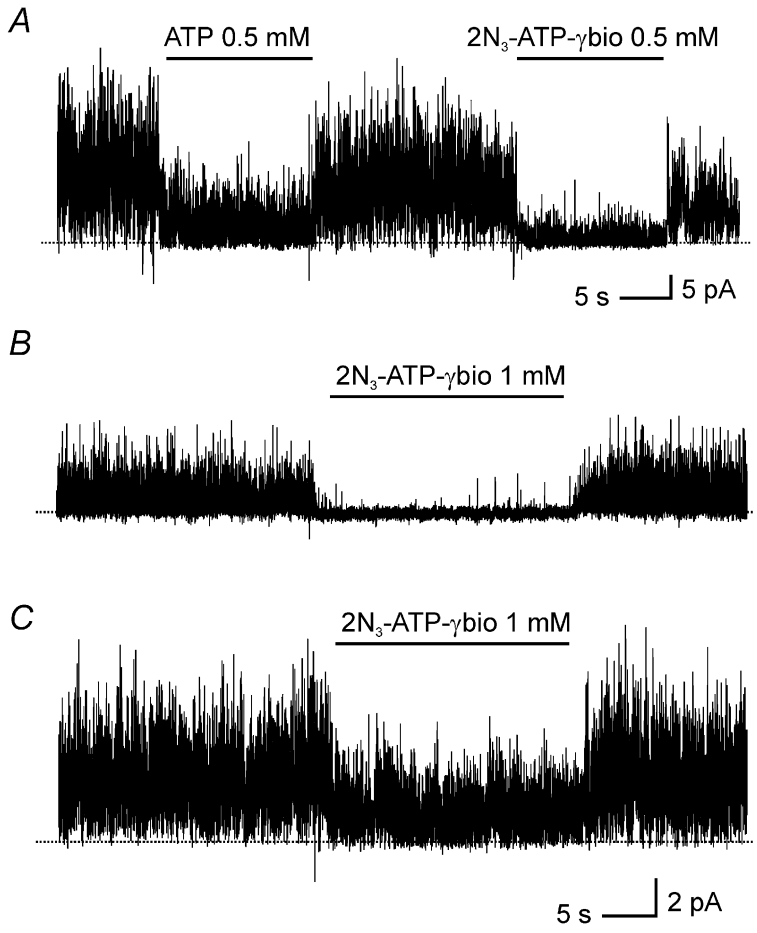

Fig. 5. Effect of 2-N_3-ATP-[γ]bio on Kir6.2 channel current. A: Reversible inhibition of Kir6.2Δ channel current by ATP and 2-N_3-ATP-[γ]bio in an inside-out patch. B,C: Channel inhibition by 2-N_3-ATP-[γ]bio before (B) and after (C) 3-minute treatment with PPIs (0.5 mg/ml).

line/phosphatidylethanolamine (1:5) liposomes. In paired experiments, labeling was weaker when PPIs were included in the proteoliposomes (Fig. 4).

3.4. *Inhibition of Kir6.2 channels by ATP photoaffinity analogs and effect of PPIs*

Functionally, we confirmed that the biotinylated ATP photoaffinity analogs inhibited Kir6.2Δ channels as effectively as ATP (Fig. 5A). Application of PPIs (0.5 mg/ml) reduced the channel inhibition by these ATP analogs (Fig. 5B) in a manner similar to that described for ATP [7].

4. Discussion

Using purified Kir6.2, our study demonstrates that (1) both PPIs and ATP analogs selectively interact with the Kir6.2 subunit, and (2) interaction with ATP is weakened in the presence of PPIs. Previously, indirect electrophysiological evidence led to the hypothesis that PPIs reduce ATP inhibition by interfering with the ATP-binding process [7]. The present results provide more direct evidence in support of this hypothesis.

Electrophysiological as well as biochemical studies have indicated that the primary binding site for ATP inhibition of K_{ATP} channels resides on the Kir6.2 subunit. It has been reported that ATP photoaffinity analogs can label Kir6.2 in extracted cell membranes [16,17], and that ATP analogs can bind to peptides derived from the C-terminal sequence of Kir6.2 [18]. This is the first report that purified Kir6.2 can be photolabeled by ATP analogs, and our results are comparable to those obtained from cell membranes [17]. Also, this is the first report to use biotinylated ATP photoaffinity analogs. The labeling and channel inhibition data not only indicate that these non-radioactive analogs are suitable for studying the ATP-binding site of Kir6.2, but also further confirmed the idea that the structural specificity of this binding is low.

Previous electrophysiological analyses have shown that PPIs affect Kir6.2Δ channels, suggesting that Kir6.2 contains the primary site of channel–PPI interaction [7]. It has been postulated that interaction between lipid headgroups and the C-terminal region of Kir6.2 is essential for the effect of PPIs [4]. Mutagenesis analysis has implicated the involvement of several residues of the C-terminal region in the effect of PPIs [4,19]. The hypothesis of direct lipid–channel interaction has also received support from experiments that show binding of PPIs with peptides derived from the C-terminal sequence [11]. In this study we further demonstrate that PPIs selectively bind purified Kir6.2. Yet must this information be interpreted with caution because the interactions manifested by these experiments may include unphysiological ones. The specificity revealed by the dot-blot assay suggests that the binding of PPIs to Kir6.2 varies in general with the electrostatic characteristic of the lipid headgroup, which is consistent with the effects on channel activity [4]. These data indicate that the

interaction appears primarily between the hydrophilic surfaces of Kir6.2 and lipids, and is therefore likely to also occur in situ.

At present, two possible mechanisms have been proposed for the effect of PPIs on ATP inhibition. The first hypothesis attributes the effect to an indirect consequence of channel-gating changes caused by PPIs [20]. According to this hypothesis, as the result of longer openings in the presence of PPIs, the channel becomes apparently less sensitive to ATP inhibition. We and others [7,8] offered an alternative hypothesis: that PPIs directly compromise ATP binding. The present results are clearly consistent with the hypothesis that direct interaction of PPIs with Kir6.2 interferes with the ATP-binding process, although they are not sufficient to let us postulate how this interference takes place, and they do not disprove the first hypothesis. Indeed, these two hypotheses are not necessarily exclusive of each other. It is very possible that both mechanisms account partly for the effect of PPIs on ATP inhibition.

In studies of the effect of PPIs on K_{ATP} channels, use of purified Kir6.2 allows for control of the lipid environment, and thus for more robust analysis of PPI interaction with the channel-forming protein. However, the use of purified Kir6.2 in a defined lipid environment has the potential to distance the results from physiological significance. In this study, the functional status of Kir6.2 as a channel has not been systematically evaluated. The selective interactions with ATP and PPIs, nonetheless, have indicated that purified Kir6.2 retains the structural conformation necessary for ATP and PPI binding. Purified Kir6.2 therefore appears to be a useful model for structure-function study of these interactions.

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