Activation and inhibition of K-ATP currents by guanine nucleotides is mediated by different channel subunits

STEFAN TRAPP, STEPHEN J. TUCKER, AND FRANCES M. ASHCROFT

University Laboratory of Physiology, Parks Road, Oxford OX1 3PT, United Kingdom

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ABSTRACT The ATP-sensitive potassium channel (K-ATP channel) plays a key role in insulin secretion from pancreatic β-cells. It is closed by glucose metabolism, which stimulates secretion, and opened by the drug diazoxide, which inhibits insulin release. Metabolic regulation is mediated by changes in ATP and MgADP concentration, which inhibit and potentiate channel activity, respectively. The β-cell K-ATP channel consists of a pore-forming subunit, Kir6.2, and a regulatory subunit, SUR1. The site at which ATP mediates channel inhibition lies on Kir6.2, while the potentiatory action of MgADP involves the nucleotide-binding domains of SUR1. K-ATP channels are also activated by MgGTP and MgGDP. Furthermore, both nucleotides support the stimulatory actions of diazoxide. It is not known, however, whether guanine nucleotides mediate their effects by direct interaction with one or more of the K-ATP channel subunits or indirectly via a GTP-binding protein. We used a truncated form of Kir6.2, which expresses independently of SUR1, to show that GTP blocks K-ATP currents by interaction with Kir6.2 and that the potentiatory effects of GTP are endowed by SUR1. We also showed that mutation of the lysine residue in the Walker A motif of either the first (K719A) or second (K1384M) nucleotide-binding domain of SUR1 abolished both the potentiatory effects of GTP and GDP on K-ATP currents and their ability to support stimulation by diazoxide. This argues that the stimulatory effects of guanine nucleotides require the presence of both Walker A lysines.

ATP-sensitive potassium channels (K-ATP channels) are inhibited by intracellular ATP and activated by MgADP, thereby coupling membrane potential and K-fluxes to cell metabolism. They play important physiological and pathophysiological roles in various tissues, including cardiac, smooth and skeletal muscle, neurones, and pancreatic β-cells (1, 2). K-ATP channels are inhibited by the antidiabetic sulfonylureas, diazoxide, and the potentiatory effects of MgADP (3). Like ATP, guanine nucleotides have a dual effect on K-ATP channel activity: high concentrations (>1 mM) block channel activity, while low concentrations (100 μM) stimulate activity (4). We have therefore investigated whether GTP and GDP interact with Kir6.2 to cause channel inhibition and if their potentiatory effects involve interaction with SUR1.

SUR1 is an ATP-binding cassette transporter protein (7, 8) and has two nucleotide-binding domains (NBDs) that contain highly conserved Walker A (WA) and Walker B (WB) consensus sequences (9). In other ATP-binding cassette transporters and ATPases, these motifs are involved in the binding and hydrolysis of ATP (8, 10, 11). Two residues are of particular importance: an aspartate residue within the Walker B motif coordinates the Mg2+ ion of MgATP, while a lysine in the Walker A motif is critical for ATP hydrolysis. In SUR1, these residues are involved in the ability of MgADP to potentiate K-ATP channel activity (12, 13).

In addition to ADP, low concentrations of the guanine nucleotides GTP, GTPγS, GDPβS, and GDP activate K-ATP channels in insulin-secreting cells (6). As is the case for ADP, this effect of guanine nucleotides requires the presence of intracellular Mg2+. The mechanism by which GTP and GDP mediate their stimulatory effects on K-ATP channel activity is unknown but has been suggested to involve either a GTP-binding protein or a direct interaction of the guanine nucleotide with the K-ATP channel complex (13). In support of the former idea is the finding that both AlF4− and vanadate, which are potent activators of G proteins, enhance the activity of the β-cell K-ATP channel (14). However, although most G proteins are activated by GTP, they are inhibited by GDP and GDPβS, which does not accord with the effects of these nucleotides on K-ATP channel activity. Furthermore, the enhancement of K-ATP channel activity by GTPγS is readily reversible (6), whereas GTPγS causes permanent activation of G proteins. This suggests that guanine nucleotides may regulate the K-ATP channel directly. We have therefore also explored whether, like ADP, guanine nucleotides interact with the NBDs of SUR1 to modulate K-ATP channel activity. We examined the effects of mutating the Walker A lysine in either the first (K719A) or second (K1384M) NBD. These mutations have been shown to abolish the activatory effects of ADP on K-ATP channel activity (13).

MATERIALS AND METHODS

Molecular Biology. Site-directed mutagenesis of rat SUR1 (GenBank no. L40624; ref. 7) was carried out by subcloning the appropriate fragments into the pALTER vector (Promega). A 26 amino acid C-terminal deletion of mouse Kir6.2 (GenBank no. D50581) was made by the introduction of a stop codon at the appropriate residue using site-directed mutagenesis.

Abbreviations: NBD, nucleotide-binding domain; K-ATP channel, ATP-sensitive potassium channel.
**Electrophysiology.** *Xenopus* oocytes were defolliculated and injected with \( \approx 2 \) ng each of mRNAs encoding Kir6.2 and either wild-type (wt) or mutant SUR1, or with \( \approx 2 \) ng Kir6.2ΔC26 mRNA (15). Control oocytes were injected with water. The final injection volume was \( \approx 50 \) nl per oocyte. Isolated oocytes were maintained in modified Barth’s solution (15) supplemented with 100 units/ml penicillin, 100 \( \mu \)g/ml streptomycin, and 5 mM pyruvate. Currents were studied 1–4 days after injection. Macromolecular currents were recorded from giant inside-out patches (15) using an EPC7 patch-clamp amplifier (List Electronics, Darmstadt, Germany). The holding potential was 0 mV and currents were evoked by repetitive 3-sec voltage ramps from \(-110 \) mV to 100 mV. Current and voltage signals were filtered at 5 kHz and stored on digital audio tape. Currents were subsequently digitized at 1 kHz (filter, 0.5 kHz) using a Digidata 1200 Interface and PCLAMP 6.0 software (Axon Instruments, Burlingame, CA), for analysis. The mean current amplitude at \(-100 \) mV, measured in nucleotide-free solution immediately after patch excision, varied between \(-0.5 \) and \(-5 \) nA for wild-type Kir6.2 coexpressed with either wild-type or mutant SUR1, and was between \(-0.2 \) and \(-1 \) nA for Kir6.2ΔC26 currents.

**Solutions.** The pipette (external) solution contained 140 mM KCl, 1.2 mM MgCl\(_2\), 2.6 mM CaCl\(_2\), 5 mM Hapes (pH 7.4). The intracellular (bath) solution contained: 10 mM NaCl, 107 mM KCl, 10 mM EGTA, 2 mM MgCl\(_2\), 1 mM CaCl\(_2\), 10 mM Hapes (pH 7.2 with KOH; final K\(^{+}\) \( \approx 140 \) mM). Nucleotides (Li-GTP, Na-GDP) were added to this solution as indicated: since the intracellular solution contained Mg\(^{2+}\), GTP and GDP will also exist as their Mg\(^{2+}\) complexes. Exchange of solutions was achieved by positioning the patch electrode in the mouth of one of a series of adjacent inflow pipes containing the test solutions.

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**Fig. 1.** Effects of GTP and GDP on wild-type K-ATP currents and Kir6.2ΔC26 currents. (A and B) Macroscopic currents recorded from two different inside-out patches in response to a series of voltage ramps from \(-110 \) to \(+100 \) mV. Oocytes were coinjected with mRNAs encoding wild-type Kir6.2 and SUR1 (A) or Kir6.2ΔC26 (B) mRNAs. The holding potential was 0 mV. The dotted line indicates the zero current level. GTP or GDP was added to the intracellular (bath) solution as indicated by the solid line. (C) Mean GTP dose-response relationship for Kir6.2ΔC26 currents (solid squares, \( n = 6 \)). Test solutions were alternated with control solutions and the slope conductance \( (G) \) is expressed as a percentage of the mean \( (G_c) \) of that obtained in control solution before and after exposure to GTP. The line is the best fit of the data to the Hill equation using the mean values obtained for \( K_i \) (6.0 mM) and \( h \) (1.2).
Data Analysis. All data are given as mean ± 1 SEM. The symbols in the figures indicate the mean and the vertical bars 1 SEM. The slope conductance \((G)\) was measured by fitting a straight line to the data between -20 mV and -100 mV; the average of five consecutive ramps was calculated in each solution. Test solutions were alternated with control solutions and \(G\) was expressed as a fraction of the slope conductance in control solution \((G_c)\). GTP dose-response relationships were fit to the Hill equation: \(G/G_c = 1/(1 + ([\text{GTP}] / K_I)^h)\), where \([\text{GTP}]\) is the GTP concentration, \(K_I\) is the ATP concentration at which inhibition is half maximal, and \(h\) is the slope factor (Hill coefficient). Statistical significance was tested using Student’s \(t\) test.

RESULTS

The Inhibitory and Stimulatory Effects of GTP Are Mediated by Kir6.2 and SUR1, Respectively. We first recorded wild-type K-ATP currents from giant inside-out patches excised from Xenopus oocytes coinjected with wild-type Kir6.2 and SUR1. As previously described (15), the patch conductance was low in the cell-attached configuration but increased spontaneously upon excision of the patch into a nucleotide-free solution. These currents were blocked by 1 mM ATP (15), demonstrating that oocytes coinjected with wild-type Kir6.2 and wild-type SUR1 express ATP-sensitive potassium currents. No such currents were observed in control oocytes.

Application of 1 mM GTP to the intracellular solution enhanced the wild-type K-ATP current amplitude to 203 ± 30% (\(n = 7\)) of that in the absence of nucleotide (Fig. 1A Left). By contrast, when GTP was increased to 10 mM, the nucleotide inhibited K-ATP currents by 35 ± 13% (\(n = 4\)) (Fig. 1A Right). One explanation of these findings is that GTP has both stimulatory and inhibitory actions on the K-ATP channel, and that at low GTP concentrations the potentiatory effects of the nucleotide predominate, whereas at higher GTP concentrations the inhibitory action becomes more important (2). Similar results were observed for GDP (see Fig. 3B).

Fig. 1B shows that GTP produced only a concentration-dependent decrease of Kir6.2ΔC26 currents; no potentiation was observed, even at low GTP concentrations. The dose-response curve (Fig. 1C) was half-maximal \((K_i)\) at 6.0 ± 0.3 mM GTP \((n = 7)\). The Hill coefficient \((h)\) was 1.2 ± 0.1, which suggests that interaction of a single GTP molecule with Kir6.2 is sufficient to inhibit the channel. These data argue that the site at which GTP mediates K-ATP channel inhibition lies on Kir6.2. Since GTP has an additional stimulatory action when Kir6.2 is coexpressed with SUR1, our results further suggest that the site(s) involved in GTP activation is located on SUR1.

Effects of GTP. To explore the possibility that the potentiatory action of GTP involves the NBDs of SUR1, we examined the effects of the nucleotide on K-ATP channels in which the \(W_A\) motif of SUR1 was mutated. We refer to these as K719A (wild-type Kir6.2 coexpressed with K719A–SUR1) and K1384M (wild-type Kir6.2 coexpressed with K1384M–SUR1). In contrast to wild-type K-ATP currents, neither K719A nor K1384M currents were activated by GTP (Fig. 2A). Instead, increasing GTP concentrations simply produced a dose-dependent inhibition of both K719A and K1384M currents (Fig. 2B). For K719A currents, the \(K_i\) for current inhibition was 2.7 ± 0.6 mM and the Hill coefficient was 1.0 ± 0.2 \((n = 5)\), and for K1384M currents, the \(K_i\) was 3.3 ± 0.5 mM and the Hill coefficient was 1.4 ± 0.2 \((n = 5)\). These results argue that the stimulatory effect of GTP involves the \(W_A\) lysines of SUR1. Both K719 and K1384 appear to be required.

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**Fig. 2** Effects of GTP on wild-type and mutant K-ATP channels. (A) Macroscopic currents recorded from three different inside-out patches in response to a series of voltage ramps from −110 to +100 mV. Oocytes were coinjected with mRNAs encoding Kir6.2 and either wild-type SUR1, K719A-SUR1, or K1384M-SUR1 mRNAs. The holding potential was 0 mV. The broken line indicates the zero current level. GTP (0.1 mM) was added to the intracellular (bath) solution as indicated by the solid line. (B) Mean slope conductance of wild-type or mutant currents recorded in the presence of the GTP concentration indicated \((G)\), expressed as a percentage of its value in the absence of the nucleotide \((G_c)\). The broken line indicates the control (GTP-free) conductance level. The number of patches is indicated above the bars.
for this effect, since mutation of a single WA lysine abolished the potentiatory effect of GTP.

**Effects of GDP.** We next analyzed the effects of GDP. Wild-type K-ATP currents were strongly activated by 100 μM GDP, increasing currents to 275 ± 19% (n = 21) of their value in nucleotide-free solution (Fig. 3). This potentiation appeared to be greater than that observed for MgADP, which stimulated wild-type K-ATP currents by ~160% (13). Neither K719A nor K1384M currents were activated by 100 μM GDP. Since mutation of a single WA lysine, at either NBD, was sufficient to abolish the stimulatory action of GDP, it appears that both NBDs of SUR1 may be needed to promote channel activation by GDP.

The dose-response curve for MgGDP modulation of wild-type K-ATP currents was bell-shaped, with maximal activation at 1 mM GDP (Fig. 3B). The reduced activation observed with 10 mM GDP may arise because of an additional inhibitory effect of the nucleotide at high concentration, as suggested by the block of WA mutant currents by 10 mM GDP (Fig. 3C). The lack of an inhibitory effect of 1 mM GDP on K719A or K1384M currents suggests that at this concentration GDP exerts only a stimulatory effect on wild-type K-ATP currents. MgGDP (10 mM) blocked K719A and K1384M currents by 30 ± 5% and by 55 ± 6%, respectively (Fig. 3C). The extent of this inhibition suggests that GDP does not interact as strongly as ADP with the inhibitory binding site, since as little as 100 μM ADP blocked K719A and K1384M currents by >60% (13).

**Effects of Diazoxide.** A number of studies have shown that the ability of the potassium channel opener diazoxide to enhance native K-ATP currents depends on the presence of hydrolyzable intracellular nucleotides such as Mg-ATP, Mg-GTP, or Mg-GDP (16, 17). This effect of adenine nucleotides requires the WA lysines of the NBDs of SUR1 and is reduced or abolished when these residues are mutated (13). We therefore examined the ability of guanine nucleotides to support the action of diazoxide on wild-type, K719A, and K1384M currents.

Fig. 4 shows that 200 μM of diazoxide stimulated wild-type K-ATP currents in the presence of 10 μM of GTP or GDP, with GDP being the more effective. Likewise, diazoxide was more effective in the presence of 100 μM GDP than 100 μM GTP. Although increasing GTP from 10 μM to 100 μM caused greater diazoxide-induced stimulation, this was not the case for GDP. Indeed, the relative increase in current was actually less with 100 μM GDP. It seems probable that this is because the K-ATP current is already almost fully activated by 100 μM GDP, so that subsequent addition of diazoxide has little effect. The fact that the K-ATP currents were enhanced to a similar final magnitude by diazoxide in the presence of 10 μM and 100 μM GDP supports this idea. A similar explanation may account for the lack of a marked stimulatory effect of diazoxide in the presence of 100 μM ADP (13).
Diazoxide produced a slight (but significant, $P < 0.05$) block of K719A currents when either GTP or GDP was present. A similar degree of inhibition was observed for wild-type K-ATP currents in the absence of any nucleotide (13) and argues that an inhibitory effect of diazoxide is unmasked in the absence of nucleotide stimulation. Diazoxide was without effect on K1384M currents in the presence of guanine nucleotides. However, since diazoxide has an intrinsic weak inhibitory action, this result suggests that mutation of K1384 to methionine does not completely abolish the ability of GDP to support the stimulatory action of diazoxide.

**DISCUSSION**

Our results demonstrate that MgGTP and MgGDP have a dual regulatory action on cloned K-ATP currents. As previously reported for native K-ATP currents (2, 6), both nucleotides potentiate K-ATP currents at low concentrations but inhibit K-ATP currents at high concentrations. Our data show that these effects are mediated by separate mechanisms and involve different K-ATP channel subunits.

The inhibitory action of GTP is mediated by interaction with the Kir6.2 subunit, since it is also observed when Kir6.2ΔC26 is expressed in the absence of SUR1. The efficacy of block was comparable to that observed for wild-type Kir6.2 when coexpressed with mutant SUR1: half-maximal inhibition was 6.0 mM for Kir6.2ΔC26 currents, 2.7 mM for K719A currents, and 3.3 mM for K1384M currents. This argues that mutation of K719 to alanine or of K1384 to methionine abolishes the potentiatory effect of MgGTP and unmasks the inhibitory effect of the nucleotide intrinsic to Kir6.2. The inhibitory site discriminates strongly between GTP and ATP, since half-maximal inhibition of Kir6.2ΔC26 currents is produced by $\sim 100 \mu$M ATP (5), compared with 6 mM GTP. When Kir6.2ΔC26 currents are coexpressed with SUR1, the ATP sensitivity is enhanced ($K_i = 10 \mu$M). This may explain why the K719A and K1384M currents appear slightly more sensitive to GTP than Kir6.2ΔC26 currents. Because Kir6.2ΔC26 currents are blocked by ADP (5), it seems probable that the inhibitory effect of GDP is also mediated by Kir6.2.

Unlike Kir6.2ΔC26 currents, wild-type K-ATP (Kir6.2/SUR1) currents are activated by both MgGTP and MgGDP. This indicates that the potentiatory effects of guanine nucleotides are endowed by SUR1. In support of this idea, mutation of either of the $W_A$ lysines of SUR1 abolished K-ATP current activation by GTP or GDP. These data also argue that...
activation of the K-ATP channel may be mediated by interaction of GTP and GDP with the NBDs of SUR1, rather than indirectly via activation of a G protein. Because the effects of these nucleotides on native K-ATP channels require the presence of Mg\(^2+\) (2, 6), it is likely that it is the Mg-bound form of the nucleotide, rather than the free acid, which interacts with SUR1. The ability of GDP, as well as GTP, to potentiate wild-type K-ATP currents is also inconsistent with the potentiatory mechanism involving G-protein activation. It remains possible, however, that in native β-cells, in addition to the direct effects of guanine nucleotides, G-protein regulation of the K-ATP channel may also occur.

The simplest explanation of our results is that the potentiatory effects of GTP and GDP are mediated by interaction of the guanine nucleotides with the NBDs of SUR1. It is apparent that both NBDs must be involved in K-ATP current activation since single mutation of a single W\(\_\)lysine was sufficient to abolish the effect. One explanation for the inability of guanine nucleotides to enhance K719A or K1384M currents is that mutation of the W\(\_\)lysines abolishes binding of either GTP or GDP. Alternatively, these mutations may not alter nucleotide binding but instead prevent the conformational change (induced by guanine nucleotides), which leads to K-ATP channel activation. Although we cannot exclude the former possibility, we favor the latter idea because mutation of the W\(\_\)lysines in other ATP-binding cassette transporters, and in ATPases, does not markedly affect nucleotide binding, but instead primarily decreases ATP hydrolysis (8, 10, 11). It is therefore possible that both GTP and GDP hydrolysis are impaireed by the W\(\_\)mutations. This raises the question of whether the conformational change that facilitates K-ATP channel opening requires hydrolysis of MgGTP (or MgGDP) at the NBDs of SUR1. The fact that neither GTP nor GDP activate native K-ATP currents in the absence of Mg\(^{2+}\) (13), or K719A or K1384M currents in the presence of Mg\(^2+\), is consistent with this hypothesis.

Our results confirm previous findings that in addition to ATP, both GTP and GDP are capable of supporting diazoxide action. We further show that mutation of the W\(\_\)lysine at NBD1 (K719A) completely abolishes, while mutation of that at NBD2 (K1384M) very substantially reduces, the ability of GTP or GDP to support diazoxide activation. These results are similar to those found for adenine nucleotides (13). It has previously been postulated that diazoxide may act by reversing the inhibitory effect of ATP—for example, by displacing ATP from its inhibitory site. Our results argue that this is unlikely to be the case because both GTP and GDP supported diazoxide activation at concentrations that had no inhibitory effect.

We observed that 100 μM GDP is a more potent stimulator of channel activity than 100 μM MgGTP, stimulating the current \(\approx 8\) times more strongly. Likewise, GDP was more effective at supporting the potentiatory effect of diazoxide. Thus, the nucleotide diphosphate seems to be the more potent activator of K-ATP currents. It is not possible to determine whether this is the case for adenine nucleotides because of the inhibitory effects of low concentrations of ATP and ADP (13).

Finally, our data support the idea that guanine nucleotides activate and inhibit K-ATP currents by separate mechanisms. The potentiatory site resides on SUR1 and does not discriminate between guanine and adenine nucleotides, whereas the inhibitory site lies on Kir6.2 and has a marked preference for adenine nucleotides. At least for the cloned K-ATP channel, these effects are sufficient to account for guanine nucleotide regulation and suggest that G-proteins are not involved.

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