Original Article

ATP Sensitivity of the ATP-Sensitive K^+ Channel in Intact and Permeabilized Pancreatic β-Cells

Andrei I. Tarasov, Christophe A.J. Girard, and Frances M. Ashcroft

ATP-sensitive K^+ channels (K_{ATP} channels) couple cell metabolism to electrical activity and thereby to physiological processes such as hormone secretion, muscle contraction, and neuronal activity. However, the mechanism by which metabolism regulates K_{ATP} channel activity, and the channel sensitivity to inhibition by ATP in its native environment, remain controversial. Here, we used α-toxin to permeabilize single pancreatic β-cells and measure K_{ATP} channel ATP sensitivity. We show that the channel ATP sensitivity is approximately sevenfold lower in the permeabilized cell than in the inside-out patch and that this is caused by interaction of Mg-nucleotides with the nucleotide-binding domains of the SUR1 subunit of the channel. The ATP sensitivity observed in permeabilized cells accounts quantitatively for K_{ATP} channel activity in intact cells. Thus, our results show that the principal metabolic regulators of K_{ATP} channel activity are MgATP and MgADP.
Diabetes **55:2446–2454, 2006**

ATP-sensitive K^+ channels (K_{ATP} channels), comprising pore-forming Kir6.2 and regulatory sulfonylurea receptor (SUR) subunits, serve as metabolic sensors that link cell metabolism to membrane electrical activity. They contribute to multiple physiological processes but are of particular importance in insulin secretion from pancreatic β-cells (1,2). At substimulatory glucose levels, K_{ATP} channels are open, preventing membrane electrical activity. An increase in plasma glucose stimulates β-cell metabolism, causing K_{ATP} channels to close and thereby elicit membrane depolarization, Ca^{2+}-dependent electrical activity, Ca^{2+} influx, and insulin secretion. The key importance of K_{ATP} channels in insulin release is also illustrated by the fact that their mutation, or impaired metabolic regulation, results in diabetes or hyperinsulinism (3,4).

Although K_{ATP} channel closure is a key step in insulin secretion, the precise mechanism(s) by which glucose metabolism regulates K_{ATP} channel activity remains controversial. It is widely contended that the principal determinant is the intracellular concentration of the metabolic ligands ATP and ADP, which exert opposing actions on the channel: ATP closes it by binding to Kir6.2, whereas MgADP stimulates channel activity by interaction with the nucleotide-binding domains (NBDs) of SUR1 (5). However, the sensitivity of the channel in its native environment to these nucleotides, and their contribution to the regulation of channel activity, remain unclear. A particular puzzle is why channel activity is readily observed in cell-attached patches on intact β-cells, where intracellular concentration of ATP ($[ATP]_i$) is at least 1 mmol/l, when in excised inside-out membrane patches, the channel is almost completely blocked by 1 mmol/l MgATP (1).

Several hypotheses have been advanced to resolve this conundrum. These include *1*) a lower ATP concentration in the vicinity of the channel because of the activity of local ATPases (6), *2*) the presence of membrane lipids like phosphatidylinositol 4,5-bisphosphate (PIP_2) (7) and long-chain acyl-CoAs (LC-CoAs) (8), and *3*) the presence of MgADP (9,10). The idea that [ATP] is lower just beneath the membrane than in the bulk cytosol does not appear to be correct (11,12). However, modulation of ATP sensitivity by MgADP, phosphoinositides, and LC-CoAs remain possibilities. Yet, although there is no doubt that all of these agents reduce K_{ATP} channel sensitivity to ATP (7–9), their relative contributions under physiological conditions are uncertain. Furthermore, it is still a matter of debate whether changes in either [MgATP], [MgADP], or other agents couple glucose metabolism to K_{ATP} channel activity (rev. in 13).

In this article, we used α-toxin to permeabilize single β-cells and measure K_{ATP} channel ATP sensitivity in its native environment. We show that channel ATP sensitivity is sevenfold lower in the permeabilized cell than in the inside-out patch and that this is caused by interaction of Mg-nucleotides with the NBDs of SUR1. Phosphoinositides and LC-CoAs are not involved. The ATP sensitivity observed in permeabilized cells can account quantitatively for K_{ATP} channel activity in intact cells, in both the presence and absence of glucose.

RESEARCH DESIGN AND METHODS

β-Cells were isolated from NMRI mice pancreata (14) and cultured in pancreatic islet medium containing 11 mmol/l glucose and 6% (vol/vol) fetal bovine serum (hCell Technology, Reno, NV) at 37°C in 5% CO_2 in air. Cells were used 1–4 days after isolation.

Kir6.2 was tagged at the COOH-terminus with enhanced yellow fluorescent protein (15). This construct is abbreviated in the text as Kir6.2. The lysine residues in the Walker A motifs of NBD1 (K719) and NBD2 (K1384) of SUR1 were mutated to alanine and methionine, respectively (16). We refer to this construct as SUR1-KAKM (16). HEK293 cells were cultured in Dulbecco's

From the University Laboratory of Physiology, Oxford University, Oxford, U.K.

Address correspondence and reprint requests to Prof. F.M. Ashcroft, University Laboratory of Physiology, Parks Road, Oxford, OX1 3PT, U.K. E-mail: frances.ashcroft@physiol.ox.ac.uk.

Received for publication 19 March 2006 and accepted in revised form 5 June 2006.

Additional information for this article can be found in an online appendix at http://diabetes.diabetesjournals.org.

$[ATP]_i$, intracellular concentration of ATP; $[ATP]_{sm}$, submembrane ATP concentration; K_{ATP} channel, ATP-sensitive K^+ channel; LC-CoA, long-chain acyl-CoA; NBD, nucleotide-binding domain; PIP_2, phosphatidylinositol 4,5-bisphosphate; SUR, sulfonylurea receptor.

DOI: 10.2337/db06-0360

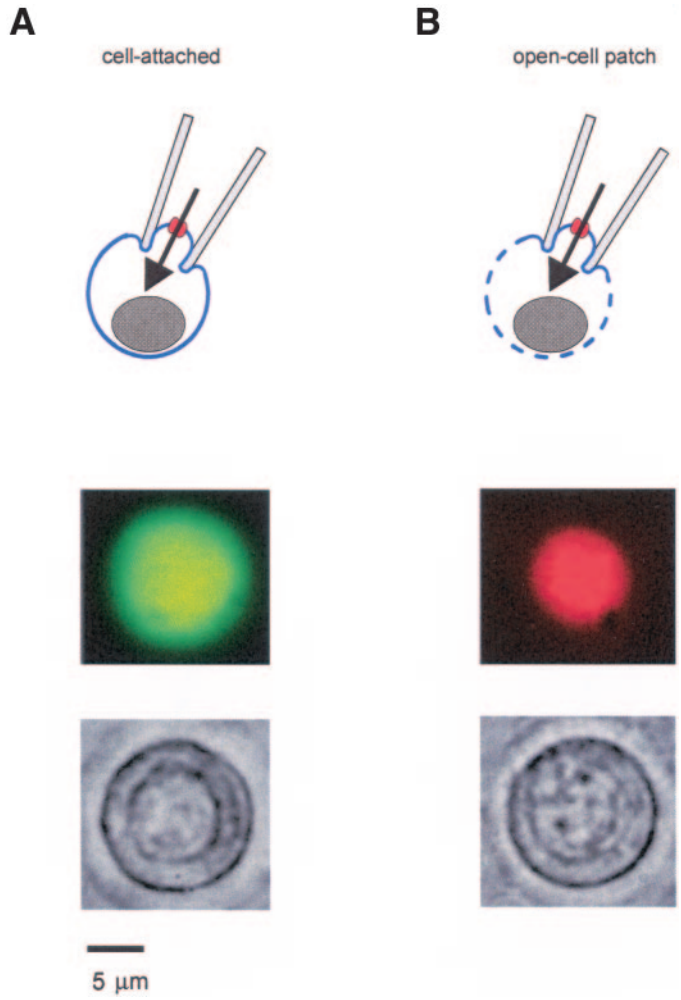

FIG. 1. Schematic of cell-attached (*A*, *top panel*) and open-cell (*B*, *top panel*) patch-clamp configurations. The same single β-cell before (*A*, *middle panel*) and after (*B*, *middle panel*) permeabilization with α-toxin. The external solution contained the membrane-permeant dye fluorescein diacetate (green fluorescence) and the membrane-impermeant nuclear stain propidium iodide (red fluorescence). Intact cells accumulate fluorescein diacetate but exclude propidium iodide (*A*). After permeabilization, fluorescein diacetate diffuses out, and propidium iodide enters the cell (*B*). Photomicrographs (*A* and *B*, *bottom panels*) show the gross structure of the cell remains unchanged after permeabilization.

modified Eagle's medium containing 5 mmol/l glucose, 10% (vol/vol) fetal bovine serum, 2 mmol/l glutamine, 1 mmol/l pyruvate, and 1% nonessential amino acids (Invitrogen) at 37°C in 5% CO_2. They were transfected with 0.2 μg Kir6.2 cDNA and 0.8 μg of either SUR1 or SUR1-KAKM cDNAs using FuGENE6 (Roche Biochemicals), according to the manufacturer's instructions. Transfected cells were identified by enhanced yellow fluorescent protein epifluorescence and used 1–4 days after transfection.

Cell permeabilization. *Staphylococcus aureus* α-toxin (100–200 hemolytic units/ml, equivalent to 10–20 μg protein/ml) was used for cell permeabilization. See supplementary information in the online appendix (available at http://diabetes.diabetesjournals.org) for the advantages of this approach compared with other methods of cell permeabilization. Briefly, a cell-attached patch was obtained in external solution (Fig. 1*A*). The external solution was then replaced with internal solution containing 0.1 mmol/l MgATP. α-Toxin was then added to obtain the open-cell configuration (Fig. 1*B*). Permeabilization was visualized by fluorescein diacetate and propidium iodide fluorescence, using an excitation wavelength of 450–490 nm and an emission wavelength of 520 nm (Fig. 1). Once permeabilization was achieved, cells were perfused with intracellular solution lacking α-toxin and dye. ATP concentration-responses were then measured (i.e., in the open-cell configuration); in some experiments, the patch was subsequently excised and the ATP concentration-response remeasured in the inside-out configuration.

Electrophysiology

Patch recordings. Single-channel currents were recorded from cell-attached, open-cell, and inside-out β-cell patches at −60 mV, filtered at 1 kHz, and digitized at 2 kHz. Macroscopic currents were evoked from HEK cells by 3-s ramps from −110 to +100 mV (holding potential 0 mV), filtered at 1 kHz, and digitized at 133 Hz. Conductance was measured as the slope of the current-voltage relation between −20 and −100 mV for five ramps. Except where indicated, recordings were initiated within 5 min of removal of the culture medium.

The pipette solution contained (in mmol/l): 140 KCl, 10 HEPES (pH 7.2 with KOH), 1.1 $MgCl_2$, and 2.6 $CaCl_2$. The external solution was (in mmol/l): 137 NaCl, 5.6 KCl, 10 HEPES (pH 7.4 with NaOH), 1.1 $MgCl_2$, and 2.6 $CaCl_2$. The internal solution contained (in mmol/l): 30 KCl, 110 K-aspartate, 0.084 $CaCl_2$, 2 $MgSO_4$, 10 HEPES (pH 7.2 with KOH), 0.5 EGTA, and Mg-nucleotides as indicated. The Mg^{2+}-free internal solution was (in mmol/l): 30 KCl, 110 K-aspartate, 2.6 $CaCl_2$, 10 HEPES (pH 7.2 with KOH), 0.5 EGTA, and 5 EDTA. In both internal solutions, free intracellular [Ca^{2+}] was ~30 nmol/l. Solutions were perfused continuously. Experiments were conducted at 21–23°C.

Concentration-inhibition curves were fit with the following equation (Eq. 1): $X/X_c = 1/[1+([ATP]/IC_{50})^h]$, where IC_{50} is the ATP (or ADP) concentration producing half-maximal block, h is the Hill coefficient, and X/X_c is the channel activity, or conductance, in the test solution (X) expressed as a fraction of that in nucleotide-free solution (X_c). Data were fit using the least squares method.

For kinetic analysis, K_{ATP} channel activity (NP_O) was recorded for periods of >1-min duration from patches containing <5 active channels, filtered at 5 kHz and digitized at 10 kHz. Open probability (P_O) was calculated from an all-points amplitude histogram. The number of channels (N) was taken as the maximum number of superimposed openings. Events were identified using a 50% amplitude threshold, and an idealized record was constructed. Burst and interburst times were calculated from rate constants obtained by fitting a three-state model to the idealized record, using a QuB software maximal interval likelihood algorithm (17). P_O values calculated from the model did not differ from those measured experimentally.

Whole-cell recordings. Methods and solutions for standard whole-cell and perforated patch recordings were as previously described (14), except that the standard whole-cell intracellular solution did not contain ATP (see online appendix). Rundown of whole-cell K_{ATP} currents was corrected as described in the online appendix.

All data are means ± SE. The Mann-Whitney U test and Wilcoxon paired test were used to assess statistical significance; $P < 0.05$ was considered significant.

RESULTS

K_{ATP} channel ATP sensitivity: open-cell versus excised patch. Most studies of the ATP sensitivity of the K_{ATP} channel in its native environment have been carried out on cardiomyocytes, using detergents to permeabilize the plasma membrane (6,18). Although this approach has been tried in β-cells, it is less reliable because of their smaller size (19). We therefore used *S. aureus* α-toxin to permeabilize single β-cells in a controlled manner. The pores produced by α-toxin are 1.5 nm in diameter and exclude substances with a molecular mass of >3 kDa, enabling permeation of ATP (500 Da) but not proteins.

Before permeabilization, K_{ATP} channel activity in cell-attached patches was low, but it increased 32 ± 11-fold (n = 8) after exposure to α-toxin (Fig. 2*A*). Subsequent addition of ATP to the internal solution rapidly and reversibly inhibited channel activity. However, the ATP sensitivity was sevenfold lower than in the inside-out patch, the half-maximal inhibitory concentration (IC_{50}) being 156 μmol/l ATP compared with 22 μmol/l after patch excision (Fig. 2*B*, Table 1). Furthermore, significant K_{ATP} current was present at physiologically relevant $[ATP]_i$: at 1 mmol/l ATP, channel activity was 16% of maximal in cell-attached patches on permeabilized cells (open-cell patch) compared with <1% in the inside-out patch.

Removal of Mg^{2+} abolished the difference in ATP sensitivity between open-cell and inside-out patches (Fig. 2*C*,

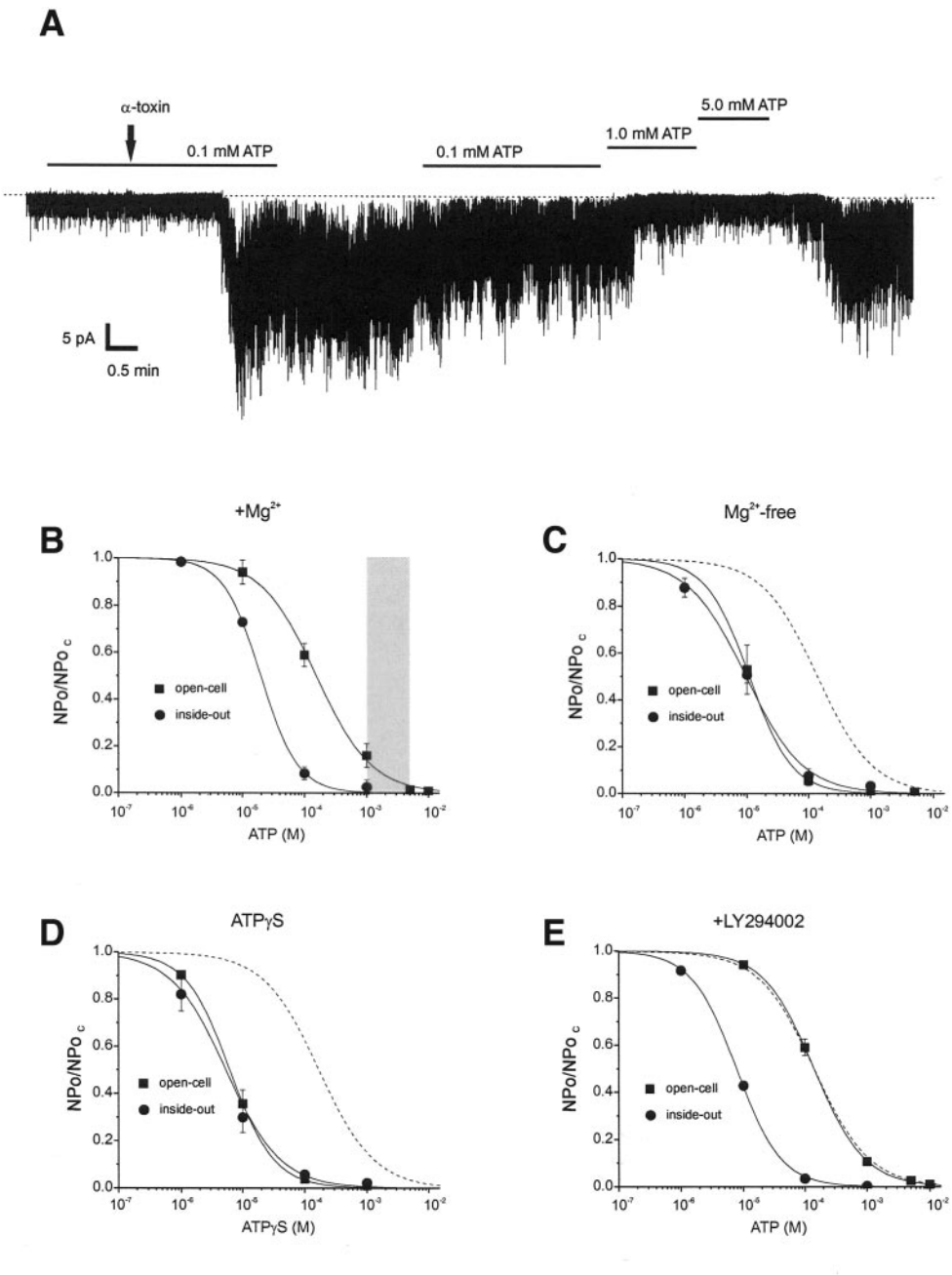

FIG. 2. *A*: Single-channel currents recorded at −60 mV from the same patch on a pancreatic β-cell exposed to internal solution, before and after permeabilization. The bath contained internal solution throughout the experiment, and α-toxin (5 μg/ml) and ATP were added as indicated. *B* and *C*: Mean ATP concentration-inhibition relationships for native β-cell K_{ATP} channels measured first in the open-cell and then in the inside-out patch in the presence (*B*) (open-cell, $n = 7$; inside-out, $n = 4$) or absence (*C*) (open-cell, $n = 10$; inside-out, $n = 5$) of Mg^{2+}. *D* and *E*: Mean concentration-inhibition curve for ATPγS (*D*) (open-cell, $n = 5$; inside-out, $n = 5$) and ATP in the presence of 100 μmol/l LY294002 (*E*) (open-cell, $n = 5$; inside-out, $n = 5$) measured in open-cell and inside-out patches. The gray bar (*B*) indicates the physiologically relevant range of $[ATP]_i$. The solid lines (*B–E*) indicate the best fit of Eq. 1 to the mean data. The dashed lines (*C–E*) indicate the fitted curve for data obtained in open-cell patches given in Fig. 2*B*. K_{ATP} channel activity (NPo) is expressed as a fraction of that in nucleotide-free solution (NPo_c).

Table 1), suggesting that it may result from ATP hydrolysis. Consistent with this idea, the IC_{50} for K_{ATP} channel inhibition by MgATPγS, a poorly hydrolyzable ATP analog, was similar in both configurations (Fig. 2*D*, Table 2).

Role of lipid kinases. One way in which ATP hydrolysis might influence channel ATP sensitivity is via MgATP-dependent synthesis of the phosphoinositides PIP_2 and PIP_3, which enhance the activity of, and decrease the ATP sensitivity of, the K_{ATP} channel (7). However, the lipid kinase inhibitor LY294002 (20) was without significant effect on channel ATP sensitivity in both open-cell and excised patches (Fig. 2*E*, Table 2). This suggests that the activity of lipid kinases is not primarily responsible for the difference in ATP sensitivity in open-cell and inside-out patches.

Role of SUR1. An alternative possibility is that MgATP hydrolysis in the permeabilized cell leads to generation of MgADP, which stimulates K_{ATP} channel activity by interaction with the NBDs of SUR1 (5,10,16). To test this idea, we used recombinant β-cell K_{ATP} channels expressed in HEK cells. We compared the effect of coexpressing Kir6.2 with wild-type SUR1 or with a mutant SUR1 (SUR1-KAKM) that is insensitive to activation by Mg-nucleotides because

TABLE 1
Sensitivity to ATP of native and recombinant channels

Channel, condition	Configuration	IC_{50} (μmol/l)	*h*	*n*
Native, $+Mg^{2+}$	Open-cell	156 ± 40*	1.1 ± 0.2	10
	Inside-out	22 ± 2	1.5 ± 0.1	5
Native, Mg^{2+} free	Open-cell	15 ± 4.0 (NS)	1.6 ± 0.2	10
	Inside-out	9.6 ± 0.8	1.0 ± 0.2	5
Kir6.2/SUR1, $+Mg^{2+}$	Open-cell	371 ± 27†	1.0 ± 0.1	6
	Inside-out	36 ± 8§	0.9 ± 0.1	7
Kir6.2/SUR1, Mg^{2+} free	Open-cell	47 ± 3§	1.1 ± 0.1	4
Kir6.2/SUR1-KAKM, $+Mg^{2+}$	Open-cell	73 ± 14‡	0.9 ± 0.1	4
	Inside-out	31 ± 6	1.2 ± 0.2	7
Kir6.2/SUR1-KAKM, Mg^{2+} free	Open-cell	43 ± 10§	1.1 ± 0.1	4

Data are means ± SE. Results from native K_{ATP} channels (in β-cells) or recombinant K_{ATP} channels expressed in HEK cells (Kir6.2/SUR1; Kir6.2/SUR1-KAKM). Mg^{2+} was present in the internal solution as indicated. *$P < 0.005$; †$P < 0.01$; ‡$P < 0.05$; NS, nonsignificant, when compared with equivalent data in the inside-out patch configuration. §Nonsignificant compared with open-cell data in Mg^{2+}-containing solution for Kir6.2/SUR1-KAKM. *h*, Hill coefficient; *n*, number of patches.

of mutation of lysine residues in the NBDs critical for ATP binding and/or hydrolysis (16) (see online appendix).

Permeabilization of HEK cells transfected with Kir6.2/SUR1 caused a large increase in the current recorded from open-cell patches. This could be blocked reversibly by ATP (Fig. 3*A*), indicating that, as in β-cells, K_{ATP} channels are largely inhibited at rest. In the inside-out patch, the IC_{50} for ATP inhibition of Kir6.2/SUR1 was close to that found for the native channel (Table 1). Importantly, the ATP sensitivity of Kir6.2/SUR1 was 10-fold higher in the inside-out patch than in the open-cell configuration in the presence of, but not absence of, Mg^{2+} (Fig. 3*B*, Table 1). Thus, the recombinant K_{ATP} channel behaves like its native counterpart.

In the absence of Mg^{2+}, ATP blocked Kir6.2/SUR1-KAKM and Kir6.2/SUR1 with similar efficacy in open-cell patches (Fig. 3*C*, Table 1). However, in the presence of Mg^{2+}, Kir6.2/SUR1-KAKM channels were substantially more ATP sensitive than wild-type channels ($P < 0.01$) (Fig. 3*C*, Table 1). These data are in harmony with the idea that the difference in ATP sensitivity between inside-out and open-cell patches is dependent on Mg-nucleotide binding/hydrolysis at the NBDs of SUR1. They also provide strong support for the idea that generation of PIP_2/PIP_3 is not involved.

Effects of MgADP. We hypothesize that MgADP generated by ATP hydrolysis at SUR1, or elsewhere in the cell, might be responsible for the lower ATP sensitivity of the K_{ATP} channel in open-cell patches. If this idea is correct, phosphocreatine should enhance ATP inhibition of K_{ATP} channels in mouse β-cells by enabling endogenous creatine kinases to minimize MgADP levels by rapid regeneration of MgATP (21). In nucleotide-free solution, 1 mmol/l phosphocreatine had no significant effect on channel activity in either the open-cell (Fig. 4*A*) or inside-out (13,21) patch. However, in the presence of MgATP, phosphocreatine caused a dramatic fall in channel activity in open-cell patches (Fig. 4*A*), and the IC_{50} for ATP inhibition decreased to that found in the absence of Mg^{2+} (Table 2). This result supports the idea that the presence of endogenous MgADP accounts for the lower ATP sensitivity of the K_{ATP} channel in the open-cell configuration.

This line of argument further predicts that addition of MgADP to the inside-out patch should reduce channel ATP sensitivity to that found in open-cell patches. Although total cytosolic MgADP is 0.6–1.5 mmol/l in β-cells exposed to glucose-free solutions (9,22), most is bound to cytosolic proteins, and free $[ADP]_i$ is estimated to be <100 μmol/l (23,24). Figure 4*B* shows that 100 μmol/l MgADP reduced the ATP sensitivity of the channel in the inside-out patch. The IC_{50} was 123 μmol/l, closer to that found for open-cell patches (156 μmol/l) than for inside-out patches (22 μmol/l) in ADP-free solution (Table 1). In contrast, in open-cell patches, there was no difference in ATP sensitivity in the absence (156 μmol/l) or presence (149 μmol/l) (Table 2) of MgADP.

In the absence of Mg^{2+}, ADP produced a marked block of channel activity in open-cell patches (Fig. 4*C*), with an IC_{50} of 76 ± 22 μmol/l ($n = 5$), close to that observed in inside-out patches (64 μmol/l) (25). In contrast, ADP had no significant inhibitory effect in the presence of Mg^{2+} (Fig. 4*C*). This reflects the fact that the stimulatory action of MgADP on SUR1 (10,16) now masks the inhibitory effect of ADP on Kir6.2 (5), thereby producing an apparent

TABLE 2
Sensitivity of native K_{ATP} channels to adenine nucleotides

Adenine nucleotide	Configuration	IC_{50} (μmol/l)	*h*	*n*
ATPγS	Open-cell	8.2 ± 0.6 (NS)	1.2 ± 0.1	5
	Inside-out	5.1 ± 1.0	0.9 ± 0.1	5
ATP + 100 μmol/l ADP	Open-cell	149 ± 57 (NS)	1.4 ± 0.1	5
	Inside-out	123 ± 38	1.6 ± 0.2	10
ATP + 1 mmol/l PCr	Open-cell	14 ± 3	1.2 ± 0.1	6
ATP + 100 μmol/l LY294002	Open-cell	137 ± 24*	1.1 ± 0.1	5
	Inside-out	9.0 ± 1.4	1.5 ± 0.3	5

Data are means ± SE. Results from native β-cell K_{ATP} channels exposed to Mg^{2+}-containing internal solution with the additions indicated. *$P < 0.005$; NS, nonsignificant, compared with equivalent data in the inside-out patch configuration. *h*, Hill coefficient; *n*, number of patches; PCr, phosphocreatine.

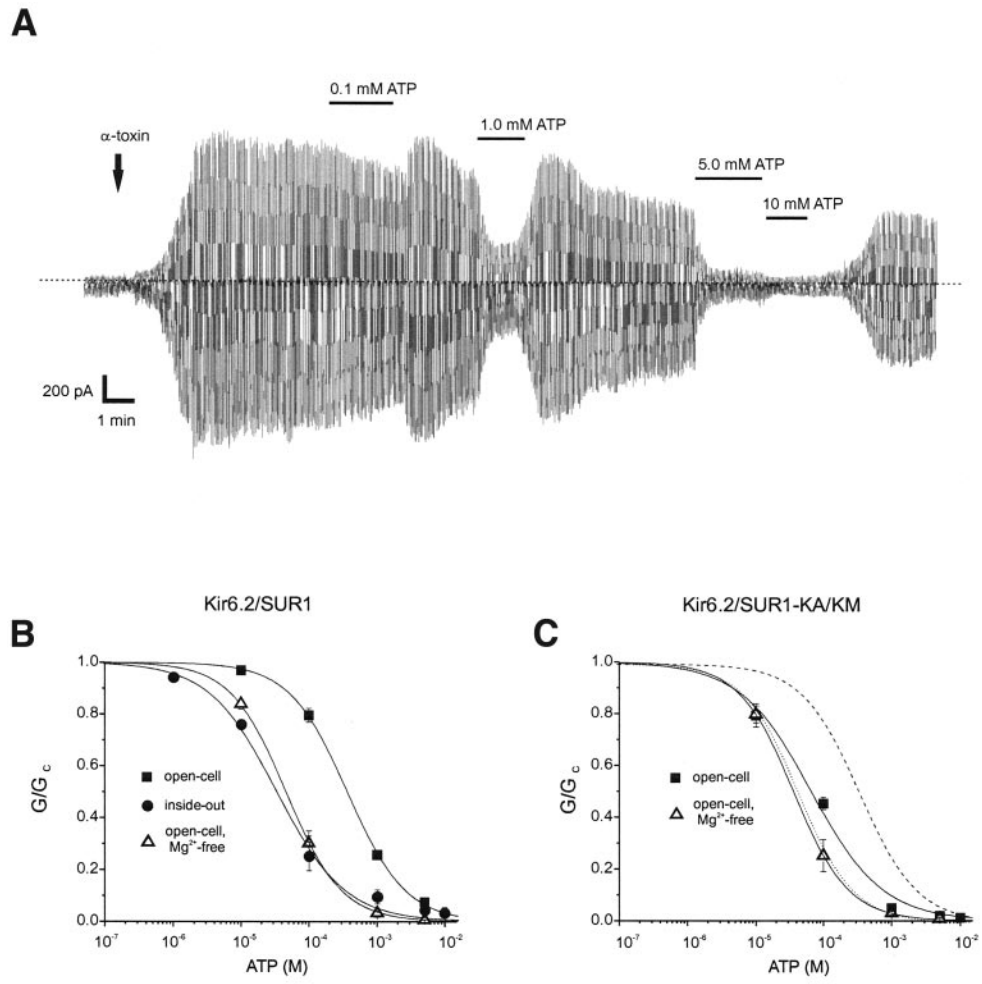

FIG. 3. *A*: Currents recorded from Kir6.2/SUR1 channels expressed in HEK cells in response to 3-s voltage ramps from −110 to +100 mV at a holding potential of 0 mV. *B* and *C*: Mean ATP concentration-inhibition curves measured in open-cell, open-cell Mg^{2+}-free, and inside-out patches for Kir6.2/SUR1 (*B*) (open-cell, $n = 6$; open-cell Mg^{2+}-free, $n = 4$; inside-out, $n = 7$) or Kir6.2/SUR1-KAKM (*C*) (open-cell, $n = 4$; open-cell Mg^{2+}-free, $n = 4$). K_{ATP} conductance (G) is expressed as a fraction of that in nucleotide-free solution (G_c). The solid lines (*B* and *C*) indicate the best fit of Eq. 1 to the mean data. The dashed and dotted lines (*C*) indicate the fitted curves for data obtained for Kir6.2/SUR1 in the open-cell configuration with and without Mg^{2+}, respectively.

reduction in inhibition. In open-cell patches, the stimulatory effect of MgADP is greater than that of MgATP (compare Fig. 2*B* and Fig. 4*C*), in accordance with the idea that MgADP is the stimulatory ligand at SUR1 and that MgATP must first be hydrolyzed to MgADP (13,18).

MgADP might be produced by ATP hydrolysis at the NBDs of SUR1 or be generated by ATPases in the vicinity of the channel. The latter idea seems less likely. Na/K-ATPase, which consumes up to 30% of ATP in β-cells (24), should not be operative under our conditions because the internal solution contained no Na^+. Moreover, the addition of 15 mmol/l Na^+ to the internal solution had no effect on K_{ATP} channel activity in open-cell patches (Fig. 4*D*). We anticipate that activity of Ca^{2+}-ATPases should also be low because Ca^{2+} was buffered to ~30 nmol/l in all internal solutions. Furthermore, thapsigargin (0.5 μmol/l), a specific inhibitor of the endoplasmic reticulum Ca^{2+}-ATPases (26), produced a ~10% block of K_{ATP} activity ($n = 13$, $P < 0.05$) (Fig. 4*D*). Thus, these data favor the idea that MgADP is produced by ATP hydrolysis at SUR1 itself, or a protein tightly associated with it.

Single-channel studies. Previous studies have shown that in inside-out patches, MgADP enhances K_{ATP} channel open probability (P_O) by increasing the duration of bursts of openings and reducing the interburst interval (9). Similar effects were observed in inside-out patches when MgADP was added to MgATP-containing solution (27) (Table 3). In open-cell patches exposed to MgATP, the burst duration and P_O were not significantly different from those found for inside-out patches exposed to MgATP plus MgADP (Fig. 5*A*, Table 3). However, when Mg^{2+} was removed, both parameters were reduced to values similar to those found for inside-out patches in MgATP alone. Thus, these data provide additional support for the idea that MgADP (produced by MgATP hydrolysis) is present in the open-cell configuration and is responsible for the lower ATP sensitivity (and higher P_O) of the K_{ATP} channel seen in the permeabilized cell. In cell-attached patches on intact cells, P_O was intermediate between that found for open-cell and inside-out patches because of a longer interburst interval (Table 3). We attribute this to a higher $[ATP]_i$ (~3 mmol/l in the intact cell, see above) because ATP is known to prolong the interburst interval (28).

Estimation of $[ATP]_i$. We estimated $[ATP]_i$ from the level of K_{ATP} channel activity observed before permeabilization (Fig. 2*A*) and the ATP dose-response curve measured in the same patch after permeabilization (Fig. 2*B*). In β-cells, $[ATP]_i$ measured within 5 min of removal from culture

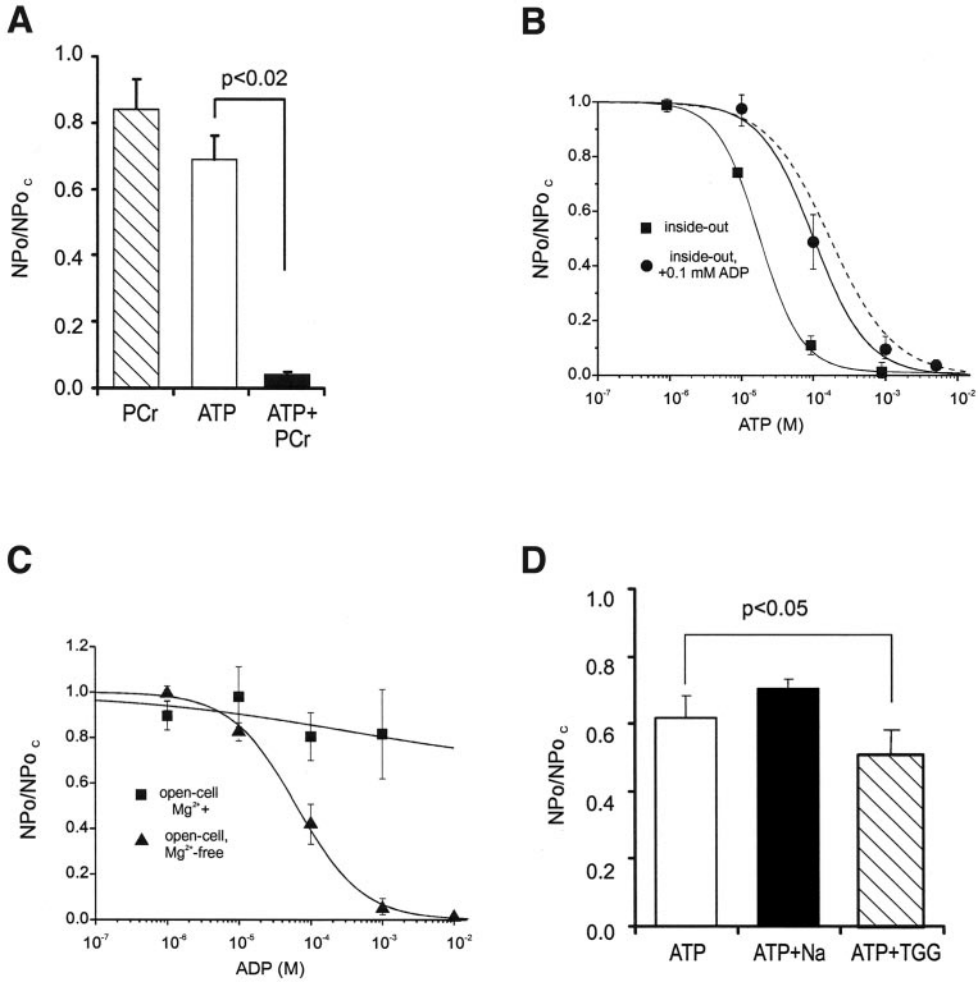

FIG. 4. ***A*****: Mean β-cell K_{ATP} channel activity (NPo) measured in the open-cell configuration in the presence of 1 mmol/l phosphocreatine (PCr), 100 μmol/l ATP, and 100 μmol/l ATP plus 1 mmol/l phosphocreatine. Data are normalized to K_{ATP} channel activity (NPo_c) in nucleotide-free solution.** ***B*****: Mean ATP concentration-inhibition curve measured in inside-out patches from β-cells in the absence ($n = 5$) or presence of 100 μmol/l MgADP ($n = 8$). The solid line is the best fit of Eq. 1 to the mean data. The dashed line is the best fit of Eq. 1 to data obtained in open-cell patches in the absence of MgADP (taken from Fig. 2*B*).** ***C*****: Mean ADP concentration-inhibition curve for native β-cell K_{ATP} channels measured in open-cell patches with ($n = 5$) or without ($n = 10$) Mg^{2+}.** ***D*****: Mean K_{ATP} channel activity measured in open-cell patches in the presence of 100 μmol/l ATP alone, 100 μmol/l ATP plus 15 mmol/l Na^+, and 100 μmol/l ATP plus 0.5 μmol/l thapsigargin (TGG).**

medium containing 11 mmol/l glucose was 2.9 ± 0.7 mmol/l ($n = 6$). After a 30-min preincubation in glucose-free solution, $[ATP]_i$ decreased to 0.9 ± 0.3 mmol/l ($n = 10$).

Glucose dependence of K_{ATP} channel activity. Finally, we assessed whether the ATP sensitivity of the K_{ATP} channel measured using the permeabilized cell approach is consistent with glucose-dependent changes in K_{ATP} currents. Using the perforated patch configuration to ensure metabolism is preserved, whole-cell K_{ATP} currents were 0.51 ± 14 ns/pF ($n = 20$) for β-cells incubated in glucose-free medium for 30 min. The glucose dependence of the K_{ATP} current was then measured. When expressed relative to 0 mmol/l glucose, K_{ATP} currents were half-maximally blocked by 4.4 ± 0.9 mmol/l ($n = 7$) glucose and almost fully blocked by 20 mmol/l, in agreement with previous studies (29).

Subsequently, the standard whole-cell configuration was established, and the maximal K_{ATP} current was measured in the same cells after washout of ATP from the cell interior and correction for rundown. This was 2.6 ± 0.5 ns/pF ($n = 15$). Thus, only 19% of the total K_{ATP} current is

TABLE 3
Analysis of single-channel properties

Condition	Configuration	τ_{burst} (ms)	τ_{closed} (ms)	P_O	n
Intact cell	Cell-attached	31 ± 6	427 ± 93	0.09 ± 0.03	12
100 μmol/l MgATP	Inside-out	13 ± 3	489 ± 155	0.06 ± 0.03	8
100 μmol/l MgADP + 100 μmol/l MgATP	Inside-out	35 ± 12	221 ± 56	0.16 ± 0.05	9
100 μmol/l MgATP	Open cell	39 ± 8	99 ± 38	0.21 ± 0.02	7
100 μmol/l ATP, Mg^{2+} free	Open cell	18 ± 4	500 ± 136	0.05 ± 0.02	5

All experiments were carried out on β-cells, using the configurations and internal solutions indicated. $\tau_{burst,}$ mean burst duration; $\tau_{closed,}$ mean interburst interval.

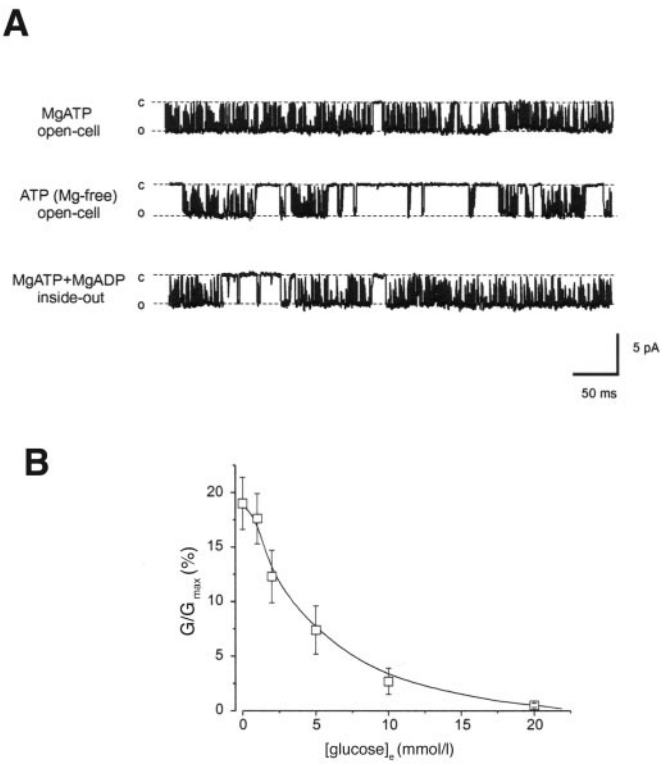

FIG. 5. ***A*****: Typical single-channel K_{ATP} currents recorded from β-cell open-cell or inside-out patches in the presence of Mg^{2+} and nucleotides as indicated.** ***B*****: Mean relationship between the whole-cell K_{ATP} conductance and the external glucose concentration ($[glucose]_e$) measured using the perforated patch configuration in β-cells ($n = 6$). The whole-cell K_{ATP} conductance (G) is expressed as a percentage of the maximal whole-cell K_{ATP} conductance measured in the standard whole-cell configuration (G_{max}).**

present in glucose-free solution. Figure 5*B* shows the relationship between whole-cell K_{ATP} current and glucose concentration measured in the perforated-patch configuration: data are expressed as a fraction of the maximal K_{ATP} current measured in the same cell, using the standard whole-cell configuration. In 10 mmol/l glucose, K_{ATP} currents were 3% of maximal, corresponding to an estimated $[ATP]_i$ of 3.2 mmol/l (from Fig. 2*A*). The estimated $[ATP]_i$ in 0 mmol/l glucose was 0.8 mmol/l. These values are consistent both with measured values (11,24) and those we estimated using α-toxin (0.9 and 2.9 mmol/l $[ATP]_i$ for 0 and 11 mmol/l glucose, respectively).

DISCUSSION

Our results reveal that there is a 7- to 10-fold difference in the ATP sensitivity of the K_{ATP} channel in the inside-out patch and in the permeabilized cell. This difference is Mg dependent, requires ATP hydrolysis, and is largely prevented by ablating nucleotide binding/hydrolysis at the NBDs of SUR1.

Origin of the difference in ATP sensitivity. We tested whether the lower ATP sensitivity found in the permeabilized cell results from a reduced submembrane ATP concentration ($[ATP]_{sm}$) (6) or by the presence of channel modulators such as membrane lipids (7,8) and/or MgADP (9,10). The first possibility can be excluded. Kir6.2/SUR1-KAKM channels are insensitive to Mg-nucleotides and therefore monitor $[ATP]_{sm}$ via Kir6.2. If local ATPases lower $[ATP]_{sm}$, then in the open-cell configuration, Mg^{2+} removal should reduce ATP inhibition of Kir6.2/SUR1-KAKM as it does for wild-type channels. Because this was not the case, local ATP depletion cannot be the primary cause of the lower ATP sensitivity found in open-cell patches. Moreover, previous studies have failed to demonstrate that $[ATP]_{sm}$ is lower than that of the bulk cytosol (11,12).

Two pieces of evidence indicate that Mg-dependent generation of phosphoinositides (PIP_2, etc.) is also not responsible for the disparity in ATP sensitivity between excised and open-cell patches. First, this mechanism cannot account for the similar IC_{50} of Kir6.2/SUR1-KAKM (in open-cell patches) in the presence and absence of Mg^{2+}. Second, inhibition of lipid kinases was without effect. The former argument also excludes the involvement of LC-CoAs and other agents that do not require nucleotide binding/hydrolysis by SUR1 for their action.

Our results show that binding/hydrolysis of Mg-nucleotides by the NBDs of SUR1 is principally responsible for the disparate ATP sensitivities found in excised and open-cell patches. Mutations that abolish interaction of Mg-nucleotides with SUR1 prevent this difference. It is also abolished by phosphocreatine, which lowers MgADP levels by conversion to MgATP: we conclude that this reflects the fall in MgADP because our data exclude a role for regulation by local changes in MgATP (see above). Finally, in excised patches, the addition of MgADP at concentrations similar to those estimated in β-cells reduces channel ATP sensitivity to that found in the open-cell configuration.

How does MgADP elevation act? A key question concerns the origin of the MgADP that mediates the shift in K_{ATP} channel ATP sensitivity in permeabilized cells. There are three possibilities: the activity of local ATPases, ATP hydrolysis by the NBDs of SUR1 itself, or both. We are unable to determine definitively which of these is the most important. One way to do so would be to use a mutation that prevents ATP hydrolysis at the NBDs of SUR1, but that does not affect either MgADP binding or the mechanism by which nucleotide binding is transduced into pore opening. However, such mutations have not been identified: those that abolish ATP hydrolysis also impair MgADP binding (30) and MgADP-dependent channel activation (16).

Our results do not support the idea that classical transport ATPases are primarily responsible for our observations. Na/K- and Ca^{2+}-ATPases are likely to be inactive in our experiments because the intracellular solution contained no Na^+, and Ca^{2+} was buffered to ~30 nmol/l. Furthermore, the endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin had only a small effect. It is possible that under physiological conditions, when intracellular $[Ca^{2+}]$ is not clamped at a low level, Ca^{2+}-ATPases may influence submembrane ATP. However, this does not seem to be the case in our permeabilized cells. Hence, we favor the idea that the lower ATP sensitivity of the K_{ATP} channel in its native environment is caused by the hydrolytic activity of SUR1 itself or by proteins intimately associated with it. For example, creatine kinase physically associates with cardiac K_{ATP} channels (31) and enhances the hydrolytic activity of NBD2 of SUR2A (18). It also regulates the activity of β-cell K_{ATP} channels (21).

What happens on patch excision remains a puzzle. Previous studies have shown that occupation of NBD2 by MgADP stimulates K_{ATP} channel activity (16,18). The MgADP-bound state may be reached by direct binding of MgADP or by binding and hydrolysis of MgATP. The rate

of MgATP hydrolysis appears to influence the occupancy of this state: elevation of ADP reduces MgATP hydrolysis and increases channel activity, whereas phosphocreatine and creatine kinase increase hydrolysis and reduce channel activity (18,21). Thus, if the channel spends more time in the MgADP-bound state in open-cell patches exposed to MgATP, then either the rate of MgADP dissociation must increase or the rate of ATP binding/hydrolysis must slow down on patch excision. We do not know why this might happen, but the (functional) loss of regulatory cytosolic proteins or cytoskeletal elements are possible explanations.

Role of PIP_2 and LC-CoAs. Our data indicate that changes in PIP_2 and/or LC-CoAs are not responsible for the disparity in ATP sensitivity between excised and open-cell patches. This does not mean that they do not affect K_{ATP} channel activity. On the contrary, it is well established that both agents stimulate channel activity when added exogenously to inside-out patches (7,32) and that heterologous overexpression of lipid kinases elevates PIP_2 in INS-1 β-cells, thereby increasing K_{ATP} currents and reducing insulin secretion (33). Conversely, agents that lower the effective concentration of PIP_2 in the plasma membrane by screening membrane surface charges (e.g., polycations) reduce K_{ATP} channel activity (32,34), and overexpression of PIP_2-insensitive Kir6.2 subunits depolarizes INS-1 β-cells (33). Thus there is no doubt that PIP_2 modulates K_{ATP} channel activity. The key question is the extent to which it does so under physiological conditions, and the physiological significance of this regulation compared with that of adenine nucleotides.

Although it remains possible that the ATP sensitivity seen in the absence of Mg^{2+}, or in the excised patch, is influenced by the endogenous level of PIP_2 in the plasma membrane, our results clearly show that PIP_2 does not underlie the difference in ATP sensitivity between excised and open-cell patches. There is also no evidence that changes in PIP_2 contribute to the metabolic regulation of K_{ATP} channel activity under physiological conditions. Elevation of MgATP on glucose metabolism would be expected to enhance PIP_2 generation and open K_{ATP} channels, although glucose actually closes K_{ATP} channels (35). Furthermore, wortmannin, which prevents PIP_2 generation, either has no effect (36) or causes a small increase in glucose-stimulated insulin secretion (37), whereas it should be inhibitory if PIP_2 generation were involved in glucose-stimulated insulin release. Our data provide strong support for the idea that metabolic regulation of K_{ATP} channel activity is mediated entirely by changes in adenine nucleotide concentrations. However, they do not exclude a role for PIP_2 regulation in the response to hormonal stimulation.

Estimated $[ATP]_i$. From the activity of the K_{ATP} channel in cell-attached patches on intact β-cells, we estimate $[ATP]_i$ to range between 0.9 mmol/l in 0 mmol/l glucose and 2.9 mmol/l in 10 mmol/l glucose. This compares favorably with values measured by other methods (9,11,22,24). The $[ATP]_{sm}$ sensed by the K_{ATP} channel has not been measured in β-cells previously, but it was 1.4 mmol/l in COS cells and 4.6 mmol/l in oocytes (12), values similar to those we obtained in HEK cells (2.5–3.3 mmol/l) (see online appendix). The agreement between our estimate of $[ATP]_i$ and that measured by other methods provides support for the idea that the ATP concentration-inhibition curve we measured in the permeabilized cell is a good approximation for that in the intact cell. Consequently, our results further suggest that Mg-nucleotide interactions with the NBDs of SUR1 may underlie the reduced ATP sensitivity of the K_{ATP} channel in the intact (as well as permeabilized) cell.

The ATP concentration-inhibition curve measured in permeabilized cells can account for glucose-dependent changes in K_{ATP} channel activity and supports the proposal that control of β-cell firing frequency by high glucose concentrations is mediated by K_{ATP} channels (38). Our results also demonstrate that under physiological conditions, the K_{ATP} channel operates at very low P_O, as postulated previously (39,40). This is advantageous because it prevents the random opening or closing of a single K_{ATP} channel from altering the membrane potential and producing fluctuations in insulin secretion (39). The magnitude of the K_{ATP} current at physiologically relevant glucose levels is also very small. Nevertheless, a decrease from 7% of maximum (in 5 mmol/l glucose) to 3% (in 10 mmol/l) can cause a marked change in membrane potential because of the high-input resistance of the β-cell membrane (40).

Conclusion. Our results resolve the long-standing controversy over why the ATP sensitivity of the K_{ATP} channel is different in the intact β-cell. We show that this is caused by increased Mg-nucleotide stimulation at the NBDs of SUR1. This shifts the ATP concentration-inhibition curve into a range of intracellular ATP levels over which glucose-induced changes in ATP occur. Thus, changes in K_{ATP} channel activity in response to glucose might be mediated by the increase in MgATP, the associated fall in MgADP, or both.

ACKNOWLEDGMENTS

This study was supported by the E.U. (BioSim, EuroDia), the Wellcome Trust, and the Royal Society. F.M.A. is a Royal Society Research Professor. A.T. is a Junior Research Fellow at Wolfson College Oxford.

We thank Prof. Hagan Bayley for the gift of α-toxin and Dr. Peter Proks for help with kinetic analysis of single channels.

REFERENCES

1. Ashcroft FM, Rorsman P: Electrophysiology of the pancreatic beta-cell. *Prog Biophys Mol Biol* 54:87–143, 1989
2. Seino S, Miki T: Physiological and pathophysiological roles of ATP-sensitive K+ channels. *Prog Biophys Mol Biol* 81:133–176, 2003
3. Dunne MJ, Cosgrove KE, Shepherd RM, Aynsley-Green A, Lindley KJ: Hyperinsulinism in infancy: from basic science to clinical disease. *Physiol Rev* 84:239–275, 2004
4. Ashcroft FM: ATP-sensitive potassium channelopathies: focus on insulin secretion. *J Clin Invest* 115:2047–2058, 2005
5. Tucker SJ, Gribble FM, Zhao C, Trapp S, Ashcroft FM: Truncation of Kir6.2 produces ATP-sensitive K+ channels in the absence of the sulphonylurea receptor. *Nature* 387:179–183, 1997
6. Nichols CG, Lederer WJ: The regulation of ATP-sensitive K+ channel activity in intact and permeabilized rat ventricular myocytes. *J Physiol* 423:91–110, 1990
7. Shyng SL, Nichols CG: Membrane phospholipid control of nucleotide sensitivity of KATP channels. *Science* 282:1138–1141, 1998
8. Gribble FM, Proks P, Corkey BE, Ashcroft FM: Mechanism of cloned ATP-sensitive potassium channel activation by oleoyl-CoA. *J Biol Chem* 273:26383–26387, 1998
9. Kakei M, Kelly RP, Ashcroft SJ, Ashcroft FM: The ATP-sensitivity of K+ channels in rat pancreatic B-cells is modulated by ADP. *FEBS Lett* 208:63–66, 1986
10. Nichols CG, Shyng SL, Nestorowicz A, Glaser B, Clement JP 4th, Gonzalez G, Aguilar-Bryan L, Permutt MA, Bryan J: Adenosine diphosphate as an intracellular regulator of insulin secretion. *Science* 272:1785–1787, 1996
11. Kennedy HJ, Pouli AE, Ainscow EK, Jouaville LS, Rizzuto R, Rutter GA: Glucose generates sub-plasma membrane ATP microdomains in single

islet beta-cells: potential role for strategically located mitochondria. *J Biol Chem* 274:13281–13291, 1999
12. Gribble FM, Loussouarn G, Tucker SJ, Zhao C, Nichols CG, Ashcroft FM: A novel method for measurement of submembrane ATP concentration. *J Biol Chem* 275:30046–30049, 2000
13. Tarasov A, Dusonchet J, Ashcroft F: Metabolic regulation of the pancreatic beta-cell ATP-sensitive K^+ channel: a pas de deux. *Diabetes* 53 (Suppl. 3):S113–S122, 2004
14. Sakura H, Ashcroft SJ, Terauchi Y, Kadowaki T, Ashcroft FM: Glucose modulation of ATP-sensitive K-currents in wild-type, homozygous and heterozygous glucokinase knock-out mice. *Diabetologia* 41:654–659, 1998
15. Tsuboi T, Lippiat JD, Ashcroft FM, Rutter GA: ATP-dependent interaction of the cytosolic domains of the inwardly rectifying K+ channel Kir6.2 revealed by fluorescence resonance energy transfer. *Proc Natl Acad Sci U S A* 101:76–81, 2004
16. Gribble FM, Tucker SJ, Ashcroft FM: The essential role of the Walker A motifs of SUR1 in K-ATP channel activation by Mg-ADP and diazoxide. *Embo J* 16:1145–1152, 1997
17. Qin F, Auerbach A, Sachs F: Estimating single-channel kinetic parameters from idealized patch-clamp data containing missed events. *Biophys J* 70:264–280, 1996
18. Zingman LV, Alekseev AE, Bienengraeber M, Hodgson D, Karger AB, Dzeja PP, Terzic A: Signaling in channel/enzyme multimers: ATPase transitions in SUR module gate ATP-sensitive K+ conductance. *Neuron* 31:233–245, 2001
19. Dunne MJ, Findlay I, Petersen OH, Wollheim CB: ATP-sensitive K+ channels in an insulin-secreting cell line are inhibited by D-glyceraldehyde and activated by membrane permeabilization. *J Membr Biol* 93:271–279, 1986
20. Rosado JA, Sage SO: Phosphoinositides are required for store-mediated calcium entry in human platelets. *J Biol Chem* 275:9110–9113, 2000
21. Krippeit-Drews P, Backer M, Dufer M, Drews G: Phosphocreatine as a determinant of K(ATP) channel activity in pancreatic beta-cells. *Pflugers Arch* 445:556–562, 2003
22. Detimary P, Jonas JC, Henquin JC: Stable and diffusible pools of nucleotides in pancreatic islet cells. *Endocrinology* 137:4671–4676, 1996
23. Ghosh A, Ronner P, Cheong E, Khalid P, Matschinsky FM: The role of ATP and free ADP in metabolic coupling during fuel-stimulated insulin release from islet beta-cells in the isolated perfused rat pancreas. *J Biol Chem* 266:22887–22892, 1991
24. Erecinska M, Bryla J, Michalik M, Meglasson MD, Nelson D: Energy metabolism in islets of Langerhans. *Biochim Biophys Acta* 1101:273–295, 1992
25. Dabrowski M, Tarasov A, Ashcroft FM: Mapping the architecture of the ATP-binding site of the KATP channel subunit Kir6.2. *J Physiol* 557:347–354, 2004
26. Treiman M, Caspersen C, Christensen SB: A tool coming of age: thapsigargin as an inhibitor of sarco-endoplasmic reticulum Ca(2+)-ATPases. *Trends Pharmacol Sci* 19:131–135, 1998
27. Larsson O, Ammala C, Bokvist K, Fredholm B, Rorsman P: Stimulation of the KATP channel by ADP and diazoxide requires nucleotide hydrolysis in mouse pancreatic beta-cells. *J Physiol* 463:349–365, 1993
28. Nichols CG, Lederer WJ, Cannell MB: ATP dependence of KATP channel kinetics in isolated membrane patches from rat ventricle. *Biophys J* 60:1164–1177, 1991
29. Gopel S, Kanno T, Barg S, Galvanovskis J, Rorsman P: Voltage-gated and resting membrane currents recorded from B-cells in intact mouse pancreatic islets. *J Physiol* 521:717–728, 1999
30. Ueda K, Komine J, Matsuo M, Seino S, Amachi T: Cooperative binding of ATP and MgADP in the sulfonylurea receptor is modulated by glibenclamide. *Proc Natl Acad Sci U S A* 96:1268–1272, 1999
31. Crawford RM, Ranki HJ, Botting CH, Budas GR, Jovanovic A: Creatine kinase is physically associated with the cardiac ATP-sensitive K+ channel in vivo. *FASEB J* 16:102–104, 2002
32. Fan Z, Makielski JC: Anionic phospholipids activate ATP-sensitive potassium channels. *J Biol Chem* 272:5388–5395, 1997
33. Lin CW, Yan F, Shimamura S, Barg S, Shyng SL: Membrane phosphoinositides control insulin secretion through their effects on ATP-sensitive K^+ channel activity. *Diabetes* 54:2852–2858, 2005
34. Schulze D, Krauter T, Fritzenschaft H, Soom M, Baukrowitz T: Phosphatidylinositol 4,5-bisphosphate (PIP2) modulation of ATP and pH sensitivity in Kir channels: a tale of an active and a silent PIP2 site in the N terminus. *J Biol Chem* 278:10500–10505, 2003
35. Ashcroft FM, Harrison DE, Ashcroft SJ: Glucose induces closure of single potassium channels in isolated rat pancreatic beta-cells. *Nature* 312:446–448, 1984
36. Gao Z, Konrad RJ, Collins H, Matschinsky FM, Rothenberg PL, Wolf BA: Wortmannin inhibits insulin secretion in pancreatic islets and β-TC3 cells independent of its inhibition of phosphatidylinositol 3-kinase. *Diabetes* 45:854–862, 1996
37. Zawalich WS, Tesz GJ, Zawalich KC: Inhibitors of phosphatidylinositol 3-kinase amplify insulin release from islets of lean but not obese mice. *J Endocrinol* 174:247–258, 2002
38. Henquin JC: ATP-sensitive K+ channels may control glucose-induced electrical activity in pancreatic B-cells. *Biochem Biophys Res Commun* 156:769–775, 1988
39. Cook DL, Satin LS, Ashford ML, Hales CN: ATP-sensitive K+ channels in pancreatic beta-cells: spare-channel hypothesis. *Diabetes* 37:495–498, 1988
40. Ashcroft F, Rorsman P: Type 2 diabetes mellitus: not quite exciting enough? *Hum Mol Genet* 13 (Spec. no. 1):R21–R31, 2004