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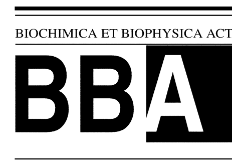

# The nucleotide regulatory sites on the mitochondrial $K_{ATP}$ channel face the cytosol [1]

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**Abstract**

The mitochondrial $K_{ATP}$ channel (mito$K_{ATP}$) is richly endowed with regulatory sites for metabolites and drugs, but the topological location of these sites is unknown. Thus, it is not known whether ATP, GTP and acyl CoA esters regulate mito$K_{ATP}$ from the matrix or cytosolic side of the inner membrane, nor whether they all act from the same side. The experiments reported in this paper provide an unambiguous answer to these questions. Electrophysiological experiments in bilayer membranes containing purified mito$K_{ATP}$ showed that current is blocked asymmetrically by ATP. $K^+$ flux experiments using proteoliposomes containing purified mito$K_{ATP}$ showed that mito$K_{ATP}$ is unipolar with respect to regulation by $Mg^{2+}$, ATP, GTP, and palmitoyl CoA and that all of these ligands react on the same pole of the protein. This demonstration was made possible by the new finding that mito$K_{ATP}$ is 85–90% oriented inward or outward in liposomes, depending on the presence or absence of $Mg^{2+}$ in the reconstitution buffer. $K^+$ flux experiments in respiring rat liver mitochondria showed that mito$K_{ATP}$ was inhibited by palmitoyl CoA and activated by GTP when these agents were added to the external medium. Given that the inner membrane is impermeant to these ligands and that mito$K_{ATP}$ is unipolar with respect to nucleotide regulation, it follows that the regulatory sites on mito$K_{ATP}$ face the cytosol. © 1997 Elsevier Science B.V.

*Keywords:* ATP-sensitive potassium channel; Mitochondrion; Potassium transport; Potassium channel opener; Channel reconstitution; Potassium channel regulation

Abbreviations: BLM, bilayer lipid membrane; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; mito$K_{ATP}$, mitochondrial $K_{ATP}$ channel; PBFI, potassium-binding benzofuran isophthalate; $TEA^+$, tetraethylammonium cation; TES, *N*-tris(hydroxymethyl)methylaminoethenesulfonic acid; TMPD, $N,N,N',N'$-tetramethyl-*p*-phenylenediamine; 4-Br-A23187, 4-bromo-A23187 (nonfluorescent)

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## 1. Introduction

In previous work [1–5], we showed that the mitochondrial $K_{ATP}$ channel (mito$K_{ATP}$) is inhibited with high affinity by adenine nucleotides, long-chain acyl-CoA esters, and glyburide. Inhibition by ATP and palmitoyl CoA is reversed with high affinity by guanine nucleotides and $K^+$ channel openers such as cromakalim and diazoxide. The rich variety of its regulation suggests that mito$K_{ATP}$ has an important physiological function, but the nature of this function remains to be established.

It is clear that opening of mito$K_{ATP}$ will shift the balance between $K^+$ uptake and efflux and thereby increase the steady-state volume of mitochondria [5,6]. Substrate oxidation, in turn, is tightly controlled by matrix volume, a phenomenon that was first reported in 1948 by Lehninger and Kennedy [7]. The volume effect can be summarized as follows: contracted mitochondria oxidize substrates slowly and fatty acids hardly at all; whereas mildly expanded mitochondria oxidize all substrates at rapid rates. Volume-activation of electron transport has been demonstrated in liver, heart and brown adipose tissue mitochondria [8–11]. Volume changes secondary to hormonal stimulation of liver [10] and brown adipose tissue [12] have also been observed in the intact cell. It was demonstrated conclusively by Nicholls et al. [8] that regulation of oxidation is mediated strictly by changes in matrix volume, independently of the means used to change volume.

In view of these findings, the working hypothesis that volume-regulation by mito$K_{ATP}$ plays a central role in cell signalling pathways calling for activation of electron transport and stimulation of fatty acid oxidation [3–5,11,13] is entirely sound. Nevertheless, the hypothesis remains to be proven. A crucial issue in its evaluation is knowing where the nucleotide regulatory sites reside. Do they face the matrix, as suggested by Inoue [14] and Halestrap [11], or do they face the cytosol?

We addressed these questions using three techniques: (i) measurement of $K^+$ flux in liposomes reconstituted with purified mito$K_{ATP}$; (ii) measurement of electrical activity in BLM containing purified mito$K_{ATP}$; and (iii) measurement of $K^+$ flux in intact mitochondria using light scattering. The reconstitutions provided us with a very useful handle on the problem. We found that mito$K_{ATP}$ is 90% oriented inward or outward with respect to ATP access, depending on the presence or absence of $Mg^{2+}$ in the reconstitution buffer. This enabled us to demonstrate that mito$K_{ATP}$ is unipolar with respect to regulation by $Mg^{2+}$, ATP, GTP, and palmitoyl CoA and that all of these ligands react on the side of the protein facing the cytosol.

## 2. Materials and methods

### *2.1. Assays of $K^+$ flux in proteoliposomes containing reconstituted mito$K_{ATP}$ isolated from rat liver mitochondria*

Purification and reconstitution followed the protocols described previously [4,15]. Briefly, inner membrane vesicles were prepared according to McEnery et al. to remove $F_1$-ATPase and other peripheral proteins [16], and proteins were extracted with 3% Triton X-100. After high-speed centrifugation, the supernatant was loaded onto a 5 ml DEAE-cellulose column, and the active fraction was eluted with buffer containing 300 mM KCl. The purified mito$K_{ATP}$ fraction was added to a 10:1 mixture of L-α-phosphatidylcholine (Avanti) and cardiolipin in a buffer containing 10% octylpentaoxyethylene, 300 μM PBFI, and a buffer of defined composition. In standard protocols, this internal medium contained 100 mM TEA-$SO_4$, 0.14 mM KCl, 1 mM TEA-EDTA, and 25 mM TEA-HEPES, pH 6.8. Variations in internal medium are described in Section 3.

Detergent was removed on sequential Bio-Beads SM-2 columns (Bio-Rad), and extraliposomal PBFI was removed by sequential passage over two Sephadex G-25-300 columns (1:10 v/v ratio). The resulting stock of proteoliposomes (about 50 mg lipid/ml) was stored on ice during the experiment.

15 μl of stock of proteoliposomes were added to 2 ml of external medium containing 150 mM KCl, 1 mM TEA-EDTA, and 25 mM TEA-HEPES, pH 7.4. Electrophoretic $K^+$ flux was initiated by addition of 1 μM FCCP to provide charge-compensating proton flux. As previously reported [1,15], identically prepared pure liposomes exhibited no $K^+$ flux upon addition of 1 μM FCCP, confirming the low permeability of these liposomes to $K^+$. $K^+$ flux into proteo-

liposomes was calculated from changes of intraliposomal PBFI fluorescence, which is enhanced in the presence of $K^+$. Fluorescence was followed with an SLM 8000 Fluorimeter (SLM, Urbana, IL) with excitation set at 344 nm (band pass 8 nm) and emission set at 485 nm (band pass 8 nm). Each preparation was calibrated to permit conversion of fluorescence to internal [$K^+$], as previously described [15,17].

### 2.2. Assays of $K^+$ flux in intact rat liver mitochondria

Electrophoretic uptake of $K^+$ or $TEA^+$ into respiring mitochondria is driven by the high membrane potential and is accompanied by electroneutral uptake of acetate and succinate. Uptake of salts and water results in osmotic swelling of mitochondria and a consequent decrease in light scattered by the mitochondrial suspension. Under proper conditions, the light scattering variable, $\beta$, is linearly related to volume, and $d\beta/dt$ is proportional to the rate of cation uptake [18,19]. $\beta$ normalizes reciprocal absorbance ($A^{-1}$) for mitochondrial concentration, P (mg/ml):

$$\beta \equiv (P/P_s) \cdot (A^{-1} - a)$$

where $P_s$ (= 1 mg/ml) is introduced to make $\beta$ a scaled, dimensionless quantity, and $a$ is a machine constant equal to 0.25 with our apparatus. Absorbance is measured at 520 nm and sampled at 0.6-s intervals with a Brinkmann PC 700 probe colorimeter connected via an analog/digital converter to a computer for conversion to $A^{-1}$, real-time plotting, and data storage. A linear regression routine is used to obtain rates, $d\beta/dt$ ($min^{-1}$), from the traces.

The standard assay medium for light scattering studies is described in Ref. [2]. It contains either $K^+$ or $TEA^+$ salts of chloride (45 mM), succinate (3 mM), acetate (25.4 mM), TES (5 mM), and EGTA (0.1 mM), pH 7.4. 1 mM $MgCl_2$ was added where indicated. Media were supplemented with rotenone (2 μg/mg) and cytochrome *c* (10 μM) and maintained at 25°C. Endogenous cytochrome *c* is mobilized in salt medium and may diffuse away when the outer membrane is ruptured by matrix swelling [20]. Under the conditions tested, cytochrome *c* in fact had little or no effect on mito$K_{ATP}$-dependent $K^+$ flux; however any reduction of respiration would cause a hidden artefactual reduction of $K^+$ flux, so cytochrome *c* was added to all media. The instrument was zeroed before each run; consequently there was no interference from cytochrome *c* absorbance.

Stock mitochondria were added to assay medium in a final concentration of 0.1 mg protein/ml. The mitochondria used in these studies exhibited respiratory control ratios of 4–6.

### 2.3. Electrophysiology of mito$K_{ATP}$ in the lipid bilayer (BLM)

Stock vesicles containing mito$K_{ATP}$ were incorporated into BLM using protocols described by Cuppoletti et al. [21]. A lipid solution was painted across the aperture ($0.9 \cdot 10^{-3}$ $cm^2$) in a clean, dry chamber [22]. The lipid was a 3:1 (w/w) mixture of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] (POPS) and 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphoethanolamine (POPE), 40 mg/ml in n-decane (Sigma). The bilayer was formed under gradient conditions with 150 mM KCl on the cis-side and 50 mM KCl on the *trans*-side. Both solutions contained 20 mM Tris-HCl buffer (pH 7.2). Proteoliposomes loaded with 150 mM KCl were painted over the cis-side of the aperture using a fire-polished glass micropipette. Fusion was induced by addition of 5 mM $CaCl_2$ to the cis-side of the chamber and by application of ±100 mV across the membrane. Experimental data were collected using an amplifier (Dagan model 8900, Minneapolis, MN) connected on-line with an IBM PC and storage oscilloscope (model 549, Tektronix). Membrane conductivity was determined using standard voltage-clamp methodology.

The sources of chemicals and drugs used in this paper were the same as previously described [4].

## 3. Results

### 3.1. Activation of the inhibited $K_{ATP}$ channel by $Mg^{2+}$ chelation

Inhibition of $K^+$ flux through mito$K_{ATP}$ by ATP, ADP, and palmitoyl CoA requires the presence of $Mg^{2+}$ [1,4]. To fully exploit this behavior, it was

necessary to show that removal of $Mg^{2+}$ is sufficient to reverse ATP inhibition. The traces contained in Fig. 1 demonstrate that the fully inhibited channel can be activated by $Mg^{2+}$ removal. Normal $K^+$ flux was observed in the presence of ATP without $Mg^{2+}$ (Fig. 1, trace a) and was strongly inhibited by addition of $Mg^{2+}$ to the assay medium (Fig. 1, trace b). $K^+$ flux was restored by the addition of EDTA during the assay (Fig. 1, trace c). Palmitoyl CoA inhibition of $K^+$ flux was also reversed by chelation after inhibition was in place (data not shown).

### 3.2. Inhibition of reconstituted mito$K_{ATP}$ by intraliposomal and extraliposomal ATP

The three sets of traces in Fig. 2 illustrate the basic finding that the composition of reconstitution buffer strongly affects ATP inhibition of $K^+$ flux through mito$K_{ATP}$. Different reconstitution buffers (internal media) were used for each set of traces. For the data in Fig. 2A, standard reconstitution buffer containing EDTA was used. For the data in Fig. 2B, reconstitution buffer contained ATP (0.5 mM), $Mg^{2+}$ (3 mM), and no EDTA. For the data in Fig. 2C, reconstitution buffer contained $Mg^{2+}$ (3 mM) and no ATP or EDTA. We emphasize that ATP and $Mg^{2+}$ were added prior to detergent removal and liposome formation.

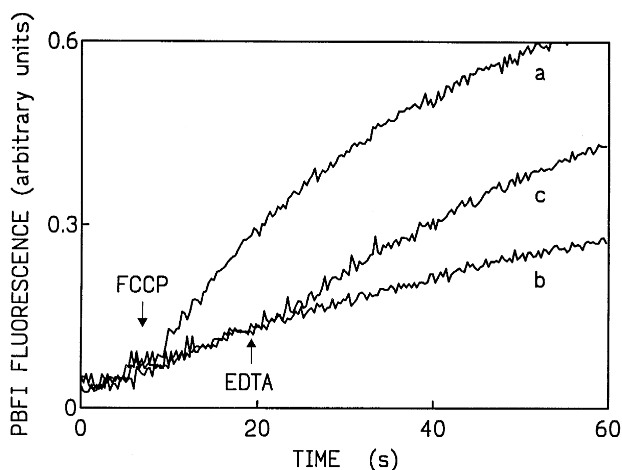



Fig. 1. Activation of the ATP-inhibited mito$K_{ATP}$ by $Mg^{2+}$ removal. Shown are PBFI fluorescence traces from liposomes reconstituted with purified mito$K_{ATP}$. Increasing PBFI fluorescence reflects increasing intraliposomal $[K^+]$ due to $K^+$ transport. Electrophoretic $K^+$ influx was initiated by adding 1 μM FCCP to KCl assay medium at 10 s. Trace a, assay medium contained 0.5 mM ATP and no $Mg^{2+}$. Trace b, assay medium contained 3 mM $Mg^{2+}$ and 0.5 mM ATP. Trace c, assay medium contained $Mg^{2+}$ and ATP, as in trace b, and TEA-EDTA (10 mM, with pH adjusted to leave final assay pH unaffected) was added at 20 s. Proteoliposomes were reconstituted in internal medium containing EDTA.

The results in Fig. 2A (EDTA vesicles) demonstrate 90% inhibition of $K^+$ flux by external MgATP (Fig. 2A, trace d) relative to control (Fig. 2A, trace a). ATP did not inhibit $K^+$ flux in the absence of $Mg^{2+}$ (Fig. 2A, trace c). These results, including the requirement for $Mg^{2+}$, confirm previous findings [1].

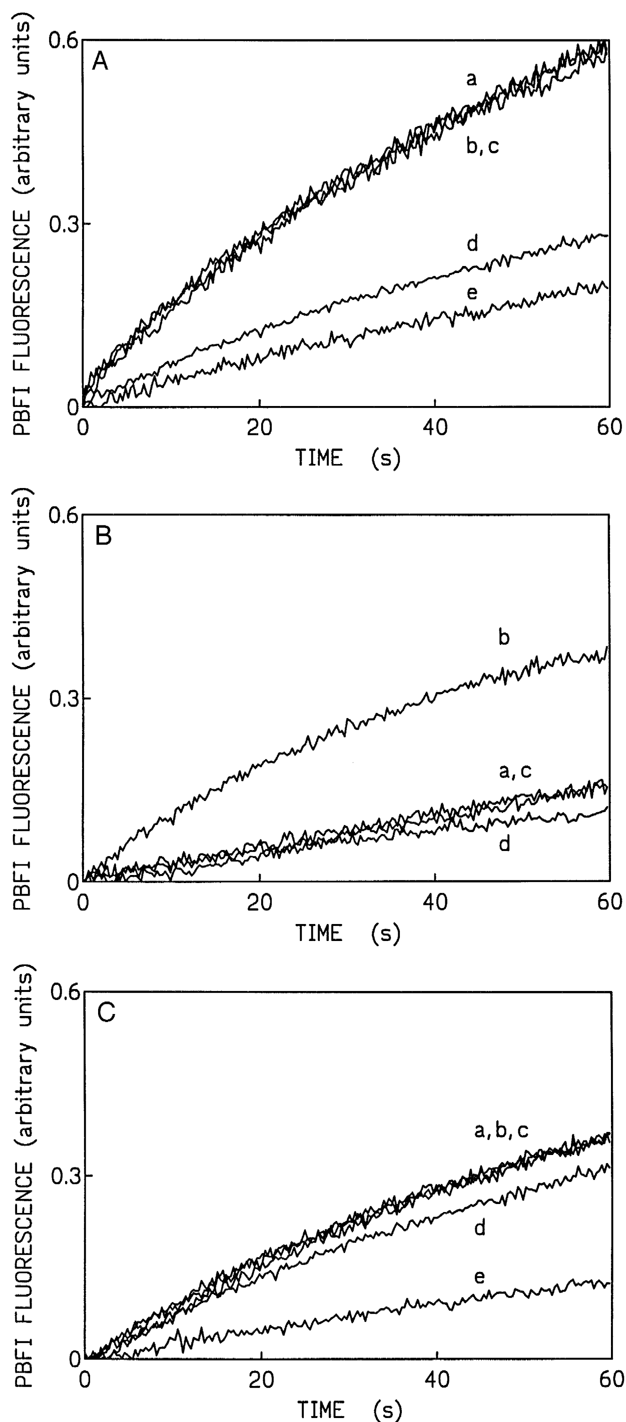
The results in Fig. 2B (MgATP vesicles) demonstrate that the presence of ATP and $Mg^{2+}$ in the reconstitution buffer had a profound effect on the response of mito$K_{ATP}$. Almost no $K^+$ flux was observed when assayed in medium lacking ATP and $Mg^{2+}$ (Fig. 2B, trace a). $K^+$ flux was restored when MgATP vesicles were exposed to 0.5 μM 4-Br-A23187 in an assay medium containing 5 mM EDTA to remove intraliposomal $Mg^{2+}$ (Fig. 2B, trace b). $K^+$ flux was again inhibited when 4-Br-A23187 was added to assay medium containing 3 mM $Mg^{2+}$ and no EDTA (Fig. 2B, trace c). These results show that $K^+$ flux in MgATP vesicles is 90% inhibited by internal MgATP.

The results in Fig. 2C (Mg vesicles) demonstrate that the presence of $Mg^{2+}$ alone in the reconstitution buffer also had a profound, but different, effect on the response of mito$K_{ATP}$. Normal $K^+$ flux was observed in EDTA-containing medium (Fig. 2C, trace a) and was not affected by external ATP in the absence of $Mg^{2+}$ (Fig. 2C, trace c). Furthermore, external ATP plus $Mg^{2+}$, which inhibited $K^+$ flux by 90% in EDTA vesicles, had almost no effect on $K^+$ flux in Mg vesicles (Fig. 2C, trace d).

### 3.3. The polarity of reconstituted mito$K_{ATP}$

The findings contained in Fig. 2 are consistent with two interpretations which cannot be distinguished unequivocally by the preceding experiments. Either the channel has bipolar nucleotide regulatory sites, or its orientation in the liposomal membrane is reversed by the presence of $Mg^{2+}$ or ATP during liposome formation. Special protocols were devised to distinguish between these alternatives.

We prepared MgATP vesicles in which $K^+$ flux was 90% inhibited in the absence of external ATP, as was demonstrated in Fig. 2B. We preincubated these liposomes in 1 mM EDTA with a low dose of 4-Br-A23187 that was (i) sufficient to remove $Mg^{2+}$ in the concentrated suspension, but (ii) insufficient to catalyze $Mg^{2+}$ flux after 130-fold dilution into the assay medium. The test of the first requirement is to show that the A23187 pretreatment was sufficient to activate $K^+$ flux. The test of the second requirement is to show that adding $Mg^{2+}$ to the assay medium does not re-inhibit $K^+$ flux, because final [A23187] is too low to catalyze $Mg^{2+}$ uptake.




Both criteria were satisfied by preincubation with 0.5 μM 4-Br-A23187. Pretreatment fully activated $K^+$ flux (Fig. 3, trace a) by comparison to control with high 4-Br-A23187 added to the assay medium (see Fig. 2B, trace b). Furthermore, $K^+$ flux was not inhibited when assayed in 3 mM $Mg^{2+}$ (Fig. 3, trace b), showing that dilution of A23187 rendered it ineffective over the time period studied. Results of the test experiment are shown in trace *c* of Fig. 3: these $Mg^{2+}$-depleted MgATP vesicles were inhibited only 10% by external ATP and $Mg^{2+}$. When the assay medium contained $Mg^{2+}$ and 0.5 μM 4-Br-A23187 $K^+$ flux was inhibited 85% by internal MgATP (Fig. 3, trace d). This result was obtained with three separate reconstitutions.

Fig. 2. The effects of reconstitution buffer composition on behavior of reconstituted mito$K_{ATP}$. Each panel contains PBFI fluorescence traces from liposomes reconstituted with purified mito$K_{ATP}$ in different internal media. $K^+$ influx is reflected in increasing PBFI fluorescence. A: EDTA Vesicles. Vesicles were reconstituted in standard internal medium containing 1 mM EDTA and assayed in external medium, as described in Section 2. Trace a, assay medium contained no $Mg^{2+}$ and no ATP. Trace b, assay medium contained 3 mM $Mg^{2+}$ and no ATP. Trace c, assay medium contained no $Mg^{2+}$ and 0.5 mM ATP. Trace d, assay medium contained 3 mM $Mg^{2+}$ and 0.5 mM ATP. Trace e, assay medium as used for trace a, but FCCP was omitted (baseline). B: MgATP Vesicles. Vesicles were reconstituted in standard internal medium containing 0.5 mM ATP, 3 mM $Mg^{2+}$, and no EDTA. Trace a, assay medium contained no $Mg^{2+}$ and no ATP. Trace b, assay medium as used for trace a, plus 0.5 μM 4-Br-A23187 and 5 mM EDTA. Trace c, assay medium contained 3 mM $Mg^{2+}$, 0.5 μM 4-Br-A23187, and no EDTA or ATP. Trace d, assay medium as used for trace b, but FCCP was omitted (baseline). C: Mg vesicles. Vesicles were reconstituted in standard internal medium containing 3 mM $Mg^{2+}$ and no EDTA or ATP. Trace a, assay medium contained no $Mg^{2+}$ and no ATP. Trace b, assay medium contained 3 mM $Mg^{2+}$ and no ATP. Trace c, assay medium contained no $Mg^{2+}$ and 0.5 mM ATP. Trace d, assay medium contained 3 mM $Mg^{2+}$ and 0.5 mM ATP. Trace e, assay medium as used for trace a, but FCCP was omitted (baseline).

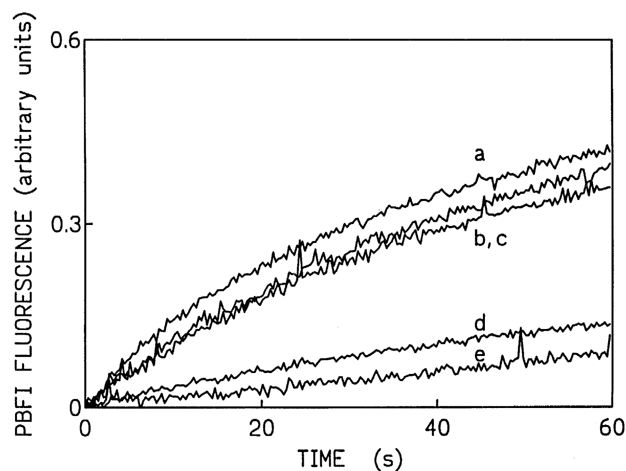



Fig. 3. Demonstration that ATP inhibition of mito$K_{ATP}$ is unipolar. This figure contains PBFI fluorescence traces from liposomes reconstituted with purified mito$K_{ATP}$ in internal medium containing 3 mM $Mg^{2+}$ and 0.5 mM ATP, as described in legend to Fig. 2B. These liposomes were preincubated at 4°C for 5 min with 0.5 μM 4-Br-A23187 and 1 mM EDTA, then diluted 130-fold into the assay media. Trace a, $K^+$ flux was activated by pretreatment to remove intraliposomal $Mg^{2+}$. Assay medium contained no $Mg^{2+}$ and no ATP. Trace b, residual 4-Br-A23187 is inadequate to restore inhibition by internal ATP. Assay medium contained 3 mM $Mg^{2+}$ and no ATP. Trace c, $K^+$ flux in MgATP vesicles cannot be inhibited by external MgATP; therefore, the nucleotide binding sites are intraliposomal. Assay medium contained 3 mM $Mg^{2+}$ and 0.5 mM ATP. Trace d, assay medium contained 0.5 μM 4-Br-A23187 and 3 mM $Mg^{2+}$. Trace e, control. Assay medium as used for trace a, but FCCP was omitted.

The experiments contained in Figs. 2 and 3 establish that composition of the reconstitution buffer determines mito$K_{ATP}$ orientation, that ATP acts only on one side of mito$K_{ATP}$, and that ATP and $Mg^{2+}$ act on the same side. These statements also apply to GTP and palmitoyl CoA. External GTP activates the ATP-inhibited mito$K_{ATP}$ when added to EDTA vesicles on the same side as ATP [4], whereas external GTP had no effect on the inhibited $K^+$ flux observed in MgATP vesicles (see Fig. 8). Similarly, palmitoyl CoA was unable to inhibit $K^+$ flux in Mg vesicles (data not shown), indicating that palmitoyl CoA could not gain access to the internalized binding sites.

### 3.4. Polarity of mito$K_{ATP}$ following incorporation in BLM

Fig. 4 contains a typical single channel record from BLM after fusion with liposomes containing purified mito$K_{ATP}$. (The same liposomes exhibited ATP-sensitive $K^+$ flux.) In the experiment shown in Fig. 4, both chambers contained 150 mM KCl and 5 mM $Ca^{2+}$, and ATP was added to the chamber *trans* to the direction of $K^+$ flux driven by 100 mV. ATP blocked the current almost completely. On the other hand, when ATP was added to the opposite chamber, there was no inhibition (not shown). We observed this asymmetry in over ten experiments. These results provide independent evidence for the contention that mito$K_{ATP}$ is asymmetric with respect to ATP inhibition.

### 3.5. Orientation of mito$K_{ATP}$ in intact mitochondria

The preceding experiments set the stage for determining the side on which nucleotides and $Mg^{2+}$

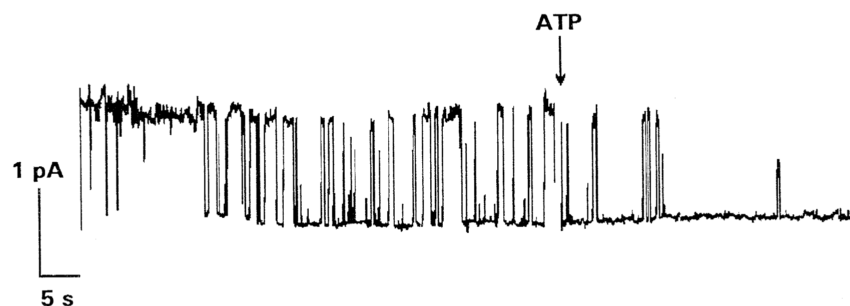



Fig. 4. ATP-sensitive single channel currents from purified mito$K_{ATP}$ incorporated in lipid bilayer membranes. This figure contains a single channel record (1.6 pA in 150/150 mM KCl, +100 mV) from BLM after fusion with liposomes containing purified mito$K_{ATP}$. 1 mM ATP was added to the *trans*-side of the membrane. ATP also inhibited $K^+$ current when the potential was reversed, but no effect was observed when ATP was added to the *cis*-side of the membrane. Similar results were obtained in 10 bilayer experiments. Media contained 150 mM KCl, 5 mM $Ca^{2+}$ and 20 mM Tris-HCl (pH 7.2) on both sides of the chamber.

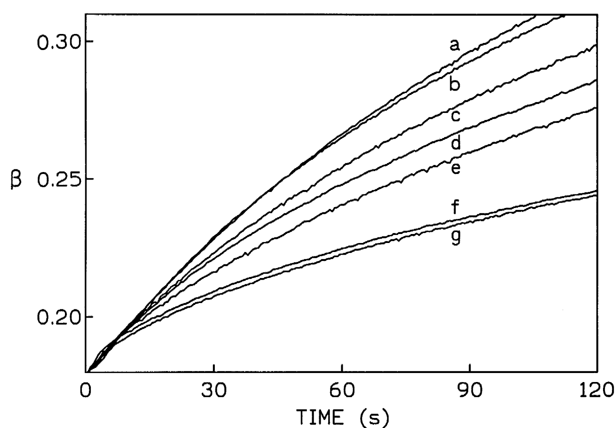



Fig. 5. Palmitoyl CoA inhibits $K^+$ flux in intact mitochondria. Light-scattering kinetics for mitochondria suspended in $K^+$ media are shown (see Section 2). Trace a, control trace in assay medium containing 1 mM $Mg^{2+}$ and no ATP. Traces b–f, assay medium containing 1 mM $Mg^{2+}$ and 133, 200, 250, 300, and 2000 nM of palmitoyl CoA, respectively. Trace g, maximally inhibited $K^+$ flux in assay medium containing 1 mM $Mg^{2+}$ and 0.1 mM ATP. Palmitoyl CoA inhibited $K^+$ flux to the level observed in the presence of saturating ATP. Palmitoyl CoA had no effect if $Mg^{2+}$ was absent from assay medium. These results are representative of three experiments. Ascorbate/TMPD was used as substrate.

interact with mito$K_{ATP}$ in situ. The traces in Fig. 5 demonstrate progressive inhibition of respiration-induced $K^+$ uptake by increasing doses of palmitoyl CoA. Like ATP, palmitoyl CoA was without effect in the absence of $Mg^{2+}$ (data not shown). Fig. 6 contains dose–response curves for inhibition by palmitoyl CoA and ATP in the presence of 1 mM $Mg^{2+}$. The $K_{1/2}$ for palmitoyl CoA in intact mitochondria is about 262 nM ($n_H \approx 3.8$), very similar to the value for inhibition of $K^+$ flux through reconstituted mito$K_{ATP}$ [4]. The $K_{1/2}$ for ATP is about 2.5 μM ($n_H \approx 1$). The finding that externally added palmitoyl CoA and ATP inhibit ATP-sensitive $K^+$ flux is consistent with interaction with external sites on mito$K_{ATP}$.

Fig. 7 contains dose–response curves for activation of $K^+$ flux in mitochondria by GTP. Assay media also contained 100 μM ATP and 1 mM $Mg^{2+}$, which completely inhibit $K^+$ flux through mito$K_{ATP}$ in intact mitochondria. GTP restored $K^+$ flux to fully active rates with $K_{1/2} = 4.6$ μM ($n_H \approx 1$), similar to values observed with reconstituted mito$K_{ATP}$ [4]. Since mitochondria contain no transport systems for GTP, this result shows that GTP is acting on the cytosolic face of mito$K_{ATP}$.

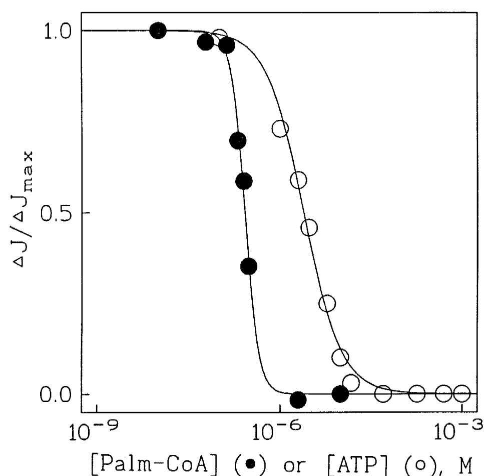



Fig. 6. Dose–response curves for inhibition of $K^+$ flux in mitochondria by palmitoyl CoA and ATP. Relative ATP-sensitive $K^+$ uptake into respiring rat liver mitochondria, $\Delta J/\Delta J_{max}$, is plotted versus concentrations of palmitoyl CoA (●) or ATP (○), in the presence of 1 mM $Mg^{2+}$. $\Delta J_{max}$ is the maximum ATP-sensitive $K^+$ flux (i.e., the difference between control fluxes in the absence and presence of saturating ATP). $\Delta J$ is the difference between fluxes in the presence and absence of palmitoyl CoA or ATP. $K_{1/2}$ values and Hill coefficients are given in text.

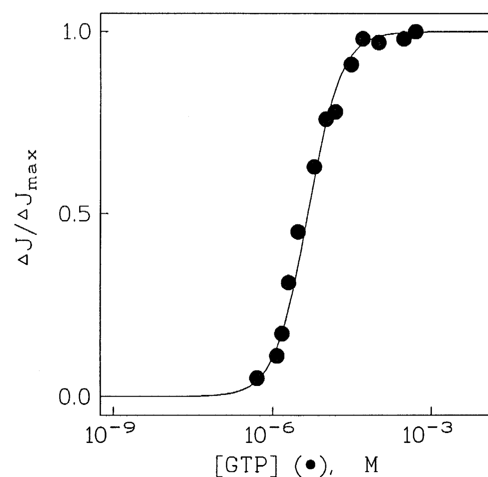



Fig. 7. Dose–response curves for activation of ATP-inhibited $K^+$ flux in mitochondria by GTP. Relative ATP-sensitive $K^+$ uptake into respiring rat liver mitochondria, $\Delta J/\Delta J_{max}$, is plotted versus concentrations of GTP (●) in the presence of 1 mM $Mg^{2+}$. $\Delta J_{max}$ is defined in Fig. 6. $\Delta J$ is the difference between fluxes in the presence and absence of GTP, with both fluxes being measured in 0.1 mM ATP. $K_{1/2}$ value and Hill coefficient are given in text.

The $K^+$ channel opener, cromakalim, was previously shown to restore $K^+$ flux from the fully inhibited state in MgATP to control rates observed in the absence of MgATP [3]. In additional experiments (not shown), we observed that neither cromakalim nor GTP activated $K^+$ flux beyond control rates, whether or not mito$K_{ATP}$ was inhibited by palmitoyl CoA or ATP. Cromakalim is a membrane permeant drug (see Fig. 8); consequently, this result shows that mito$K_{ATP}$ is fully asymmetric in intact mitochondria and does not have nucleotide regulatory sites facing in both directions.

### *3.6. Accessibility of internalized mito$K_{ATP}$ receptor to $K^+$ channel openers*

The experiments in Fig. 8 were carried out in MgATP vesicles, in which the nucleotide-binding sites are oriented inward. These preparations were assayed for the ability of GTP, cromakalim, and diazoxide to activate the inhibited channels. As can be seen from the figure, the $K^+$ channel openers fully activated the channel, whereas GTP was without its normal activating effect. The $K_{1/2}$ values for cromakalim (1.7 μM) and diazoxide (0.6 μM) are very close to the values obtained in preparations with outward orientation [3]. Thus, these hydrophobic drugs can activate from either side of the membrane, whereas the impermeant GTP cannot.

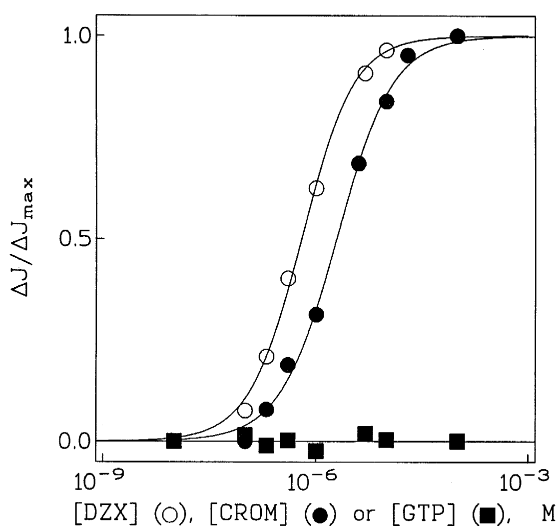



Fig. 8. Effects of $K^+$ channel openers and GTP on inward-oriented mito$K_{ATP}$ (MgATP vesicles). The relative ATP-sensitive $K^+$ uptake into liposomes reconstituted with mito$K_{ATP}$, $\Delta J/\Delta J_{max}$, is plotted versus concentration of GTP (■), cromakalim (●) and diazoxide (○), which were added to the KCl assay medium. Vesicles were reconstituted in internal medium containing 3 mM $Mg^{2+}$ and 0.5 mM ATP without EDTA, which induces an inward orientation of the nucleotide-receptors. Two experiments yielded essentially the same results. $\Delta J_{max}$ is the difference between control fluxes in the activated (0.5 μM 4-Br-A23187 and 5 mM EDTA added to assay) and inhibited (no additions) states of MgATP vesicles. $\Delta J$ is the difference between fluxes in the presence or absence of drug or GTP.

## 4. Discussion

Mito$K_{ATP}$ is exquisitely sensitive to metabolites, including adenine nucleotides (inhibitors), long-chain acyl CoA esters (inhibitors) and guanine nucleotides (activators) [1,4]. Metabolite inhibition exhibits an absolute requirement for $Mg^{2+}$, which appear to react independently with the receptor [4]. In vivo, these ligands exist on both sides of the inner membrane, and no experiments have heretofore been performed to determine which pool regulates mito$K_{ATP}$. The cell physiology of mito$K_{ATP}$ regulation is a new and important area of investigation. To establish whether the mito$K_{ATP}$ receptor sites face the cytosol or the mitochondrial matrix is a prerequisite for such studies.

Our experiments yielded an unambiguous answer to this question with results that may be summarized as follows. (i) In proteoliposomes, orientation of mito$K_{ATP}$ is determined by the composition of the reconstitution buffer. The ATP regulatory sites were oriented outward when reconstituted in EDTA medium and inward when reconstituted in the presence of $Mg^{2+}$. It is interesting that we previously observed a similar orienting effect of $Mg^{2+}$ on the reconstituted $K^+/H^+$ antiporter [23]. We also showed in liposomes that nucleotides, palmitoyl CoA, and $Mg^{2+}$ regulate from one and the same side of the channel. (ii) In BLM, mito$K_{ATP}$ is also unipolar, with ATP inhibition being seen from only one side. (iii) In intact mitochondria, nucleotide regulatory sites on mito$K_{ATP}$ are accessible from the external medium. Thus, external GTP maximally activates, and external palmitoyl CoA maximally inhibits, mito$K_{ATP}$ under

conditions in which these ligands are not transported across the inner membrane.

Taken together, our results establish the topology of mito$K_{ATP}$ as it exists in the inner membrane of intact mitochondria: the regulatory domains face the cytosol or, more specifically, the intermembrane space between inner and outer mitochondrial membranes. This conclusion does not exclude the possibility that other ligands may regulate from the matrix side, but none has yet been identified.

This result conflicts with the conclusion of Inoue et al. [14] that mito$K_{ATP}$ is inhibited by ATP added to the matrix side of the membrane. In those experiments, patch clamp was applied to fused giant mitoplasts that had undergone severe osmotic swelling and exposure to 20 mM $Ca^{2+}$. No experiments were undertaken to establish the sidedness of the fused mitoplasts. A possible explanation for the discrepancy is that the membranes were inverted during this fusion process as occurs, for example, with submitochondrial particles. This discrepancy must be resolved by future experiments.

Cromakalim and diazoxide are able to activate the ATP-inhibited mito$K_{ATP}$ in either orientation, with the regulatory sites facing inward (Fig. 8) or outward [3]. Thus, the intracellular location of the mito$K_{ATP}$ receptor does not prevent access by hydrophobic drugs, such as $K^+$ channel openers and glyburide in vivo. This important pharmacological point supports our hypothesis [3] that mito$K_{ATP}$ may be a receptor for the cardioprotective actions of $K^+$ channel openers.

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