

# The Mitochondrial $K_{ATP}$ Channel as a Receptor for Potassium Channel Openers\*

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The biochemical properties of the mitochondrial  $K_{ATP}$  channel are very similar to those of plasma membrane  $K_{ATP}$  channels, including inhibition by low concentrations of ATP and glyburide (Paucek, P., Mironova, G., Mahdi, F., Beavis, A. D., Woldegiorgis, G., and Garlid, K. D. (1992) *J. Biol. Chem.* 267, 26062–26069). Plasma membrane  $K_{ATP}$  channels are highly sensitive to the family of drugs known as  $K^+$  channel openers, raising the question whether mitochondrial  $K_{ATP}$  channels are similarly sensitive to these agents. We addressed this question by measuring  $K^+$  flux in intact rat liver mitochondria and in liposomes containing  $K_{ATP}$  channels purified from rat liver and beef heart mitochondria.  $K^+$  channel openers completely reversed ATP inhibition of  $K^+$  flux in both systems. In liposomes, ATP-inhibited  $K^+$  flux was restored by diazoxide ( $K_{1/2} = 0.4 \mu M$ ), cromakalim ( $K_{1/2} = 1 \mu M$ ), and two developmental cromakalim analogues, EMD60480 and EMD57970 ( $K_{1/2} = 6 nM$ ). Similar  $K_{1/2}$  values were observed in intact mitochondria. These potencies are well within the range observed with plasma membrane  $K_{ATP}$  channels. We also compared the potencies of these  $K^+$  channel openers on the plasma membrane  $K_{ATP}$  channel purified from beef heart myocytes. The  $K_{ATP}$  channel from cardiac mitochondria is 2000-fold more sensitive to diazoxide than the channel from cardiac sarcolemma, indicating that two distinct receptor subtypes coexist within the myocyte. We suggest that the mitochondrial  $K_{ATP}$  channel is an important intracellular receptor that should be taken into account in considering the pharmacology of  $K^+$  channel openers.

$K^+$  channel openers (KCOs)<sup>1</sup> activate ATP-inhibited  $K_{ATP}$  channels. As described in several excellent reviews (1–3), mem-

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<sup>1</sup> The abbreviations used are: KCO, potassium channel opener; cell $K_{ATP}$ , plasma membrane  $K_{ATP}$  channel; mito $K_{ATP}$ , mitochondrial  $K_{ATP}$  channel; PBFI, potassium-binding benzofuran isophthalate; TEA<sup>+</sup>, tetraethylammonium cation; TES, *N*-tris(hydroxymethyl)methylaminoethanesulfonic acid; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylene-diamine.

bers of this drug family exhibit a rich and clinically important pharmacology. Thus, cell membrane  $K_{ATP}$  channels (cell $K_{ATP}$ ) in different tissues are considered to mediate the hypotensive and diabetogenic effects of diazoxide (4) and the cardioprotective effects of cromakalim and its derivatives (5). It is important to determine whether these drugs also act on mitochondrial  $K_{ATP}$  channels (mito $K_{ATP}$ ) in their therapeutic range.

In the first reports of KCO actions in mitochondria, Belyaeva *et al.* (6) and Szewczyk *et al.* (7) observed stimulation of  $K^+$  uptake by KCOs in respiring mitochondria. RP66471 was the most potent KCO studied ( $K_{1/2} = 50 \mu M$ ), whereas P1060 and diazoxide were only weakly active at 700  $\mu M$ . Because these concentrations are much higher than  $K_{1/2}$  values observed with cell $K_{ATP}$  (1), these results appear to imply that mitochondrial actions of KCOs are not pharmacologically important.

We now report that diazoxide, cromakalim, and two experimental benzopyran derivatives are very potent activators of  $K^+$  flux through ATP-inhibited mito $K_{ATP}$ , with  $K_{1/2}$  values similar to those observed with cell $K_{ATP}$ . KCO activation of  $K^+$  flux was observed in both intact mitochondria and proteoliposomes containing reconstituted mito $K_{ATP}$ . No effect was observed on uninhibited  $K^+$  flux, which likely explains the low potencies observed by previous workers (6, 7) in assays that did not include  $Mg^{2+}$  and ATP. We also found that mito $K_{ATP}$  and cell $K_{ATP}$  from beef heart differed strongly in their sensitivity to diazoxide, indicating distinct receptor subtypes among  $K_{ATP}$  channels from the same cell. Our results indicate that mito $K_{ATP}$  may be an important intracellular receptor for  $K^+$  channel openers, and they raise the possibility that mito $K_{ATP}$  is the site of action of cardioprotective KCOs.<sup>2</sup>

## EXPERIMENTAL PROCEDURES

*Assays of  $K^+$  Flux in Proteoliposomes Containing Reconstituted Mito $K_{ATP}$  Isolated from Rat Liver Mitochondria*—Mito $K_{ATP}$  was purified and reconstituted into proteoliposomes exactly as described previously (8, 9). Internal medium contained 300  $\mu M$  PBFI, 0.14 mM KCl, 1 mM TEA-EDTA, 25 mM TEA-HEPES, and 100 mM TEA-SO<sub>4</sub> (pH 6.8). Vesicles were added in a final concentration of 0.38 mg lipid/ml to external medium containing 150 mM KCl and 25 mM TEA-HEPES (pH 7.4) at 25 °C. As indicated in the text, external medium also contained 3 mM MgCl<sub>2</sub> or 1 mM TEA-EDTA, and 0.5 mM ATP or no ATP. Electrophoretic  $K^+$  flux was initiated by 1  $\mu M$  carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone, which catalyzes charge compensation.  $K^+$  flux was determined by linear regression of the initial changes in the  $K^+$ -dependent fluorescence of intraliposomal PBFI, which was calibrated for each preparation (8, 9).

*Assays of  $K^+$  Flux in Proteoliposomes Containing Reconstituted Cell $K_{ATP}$  Isolated from Beef Heart Sarcolemmal Vesicles*—Sarcolemmal vesicles were prepared from the left ventricular muscle of fresh beef heart according to a modification (10) of the method of Jones and Besch (11). The sarcolemmal  $K_{ATP}$  channel was solubilized, purified, and

<sup>2</sup> A preliminary report of these findings has been published in abstract form (32).

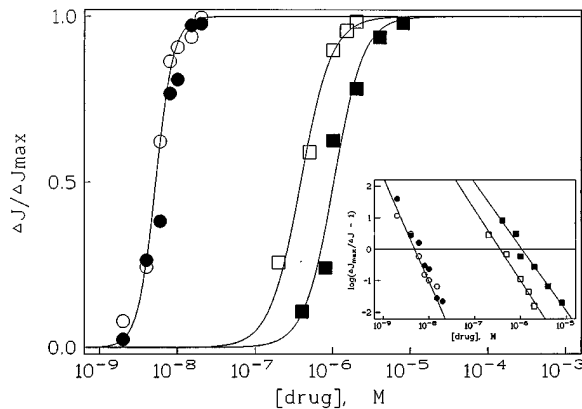


FIG. 1. Activation of  $K^+$  flux by  $K^+$  channel openers in liposomes reconstituted with  $\text{mitoK}_{ATP}$ . Dose-response curves and Hill plots (*inset*) for activation of  $K^+$  flux through  $\text{mitoK}_{ATP}$  by four KCOs: EMD60480 ( $\circ$ ), EMD57970 ( $\bullet$ ), diazoxide ( $\square$ ), and cromakalim ( $\blacksquare$ ). " $\Delta J_{\max}$ " is the maximum ATP-sensitive  $K^+$  flux, *i.e.* the difference between control fluxes in the absence and presence of saturating ATP (0.5 mM). " $\Delta J$ " is the difference between fluxes in the presence or absence of the drug, both measured in the presence of 0.5 mM ATP. In nine separate preparations,  $\Delta J_{\max}$  ranged between 400 and 600  $\mu\text{M/s}$ , similar to values previously reported using these protocols (8). Observed  $K_{1/2}$  values and Hill slopes are given in the text.

reconstituted into proteoliposomes exactly as described above for  $\text{mitoK}_{ATP}$ , except that  $\text{cellK}_{ATP}$  activity was found in the 100 mM KCl fraction (12). Internal and external media were as described for  $\text{mitoK}_{ATP}$ , except that  $\text{Na}^+$  was substituted for  $\text{TEA}^+$  ion, because  $\text{TEA}^+$  inhibits  $K^+$  flux through  $\text{cellK}_{ATP}$ . Assays of cardiac  $\text{cellK}_{ATP}$  were carried out as described for  $\text{mitoK}_{ATP}$ .

**Assays of  $K^+$  Flux in Intact Rat Liver Mitochondria**—6  $\mu\text{l}$  of a stock mitochondrial suspension in 0.25 M sucrose (50 mg protein/ml) were added to 3 ml of " $K^+$  medium" containing  $K^+$  salts of chloride (45 mM), acetate (25.4 mM), TES (5 mM), EGTA (0.1 mM), pH 7.4, plus  $\text{MgCl}_2$  (1 mM) and cytochrome *c* (10  $\mu\text{M}$ ). Substrates for respiration were either succinate (3 mM) and rotenone (2  $\mu\text{g}/\text{mg}$  of protein) or ascorbate (2.5 mM) and TMPD (0.25 mM), as indicated in the figure legends. For assays in " $\text{TEA}^+$  medium,"  $\text{TEA}^+$  was substituted for  $K^+$ . The assay cuvette was maintained at 25  $^{\circ}\text{C}$ , and absorbance data at 520 nm were collected and stored digitally as described previously (13–15). Respiration-driven, electrophoretic uptake of  $K^+$  is accompanied by electroneutral uptake of acetate and osmotically obligated water. The light-scattering variable,  $\beta$ , is linearly dependent on matrix volume; hence,  $K^+$  influx ( $J$ ) is proportional to  $d\beta/dt$ .

**Chemicals and Drugs**—PBFI was purchased from Molecular Probes Inc. (Eugene, OR). Two benzopyranyl derivatives, EMD57970 and EMD60480, were provided by E. Merck (Darmstadt, Germany). All other chemicals and reagents were obtained from Sigma.

## RESULTS

**Activation of  $K^+$  Flux through Reconstituted  $\text{MitoK}_{ATP}$  by  $K^+$  Channel Openers**—Fig. 1 contains dose-response curves for KCO stimulation of  $K^+$  flux in vesicles reconstituted with  $\text{mitoK}_{ATP}$ . Drug assays were carried out in medium containing 3 mM  $\text{Mg}^{2+}$  and 0.5 mM ATP, which fully inhibits  $\text{mitoK}_{ATP}$  ( $K_{1/2}$  (ATP)  $\approx$  22  $\mu\text{M}$ ). The drugs tested were potent activators of  $K^+$  flux, restoring ATP-inhibited flux to, but not beyond, control rates measured in the absence of ATP.

Observed  $K_{1/2}$  values (mean and S.D.) were  $1.05 \pm 0.06$   $\mu\text{M}$  for cromakalim ( $n = 5$ ),  $0.37 \pm 0.03$   $\mu\text{M}$  for diazoxide ( $n = 4$ ),  $6.1 \pm 1.3$  nM for EMD60480 ( $n = 2$ ), and  $6.20 \pm 0.02$  nM for EMD57970 ( $n = 2$ ). As shown in the *inset* to Fig. 1, cromakalim and diazoxide exhibited indistinguishable Hill slopes of  $2.0 \pm 0.5$ , and the benzopyranyl derivatives yielded Hill slopes of  $3.5 \pm 0.3$ . Hill slopes greater than 1.0 may reflect a tetrameric structure of the channel, as observed with other  $K^+$  channels (16), or the existence of multiple binding sites on a regulatory ATP binding cassette, as has been proposed for the sulfonilylurea receptor of the pancreatic  $\beta$  cell (17).

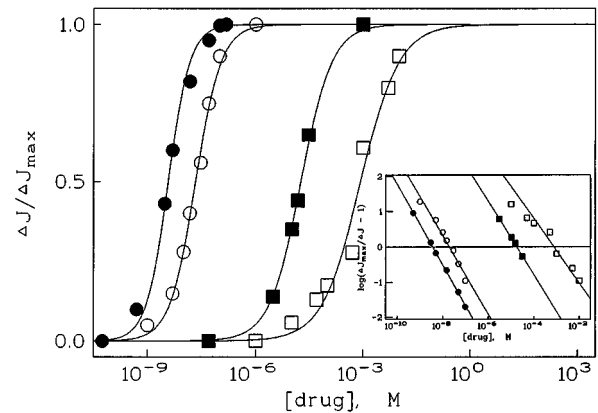


FIG. 2. Activation of  $K^+$  flux by  $K^+$  channel openers in liposomes reconstituted with cardiac sarcolemmal  $K_{ATP}$  channels ( $\text{cellK}_{ATP}$ ). Dose-response curves and Hill plots (*inset*) for activation of  $K^+$  flux through  $\text{cellK}_{ATP}$  by four KCOs: EMD57970 ( $\bullet$ ), EMD60480 ( $\circ$ ), cromakalim ( $\blacksquare$ ), and diazoxide ( $\square$ ). " $\Delta J_{\max}$ " and " $\Delta J$ " are defined as in Fig. 1, but with reference to rates in 2 mM ATP. Observed  $K_{1/2}$  values and Hill slopes are given in the text. Duplicate experiments on an independent preparation yielded similar results.

We also measured KCO activation of  $K^+$  flux in proteoliposomes reconstituted with  $\text{mitoK}_{ATP}$  purified from beef heart mitochondria. Observed  $K_{1/2}$  values from two experiments were 1  $\mu\text{M}$  for cromakalim and 0.4  $\mu\text{M}$  for diazoxide. These results extend a previous observation that cardiac and hepatic  $\text{mitoK}_{ATP}$  behave very similarly.

We stress that these drugs stimulated  $K^+$  flux only when  $K^+$  flux was inhibited by  $\text{Mg}^{2+}$  and ATP. Control (uninhibited)  $K^+$  flux is observed in media containing  $\text{Mg}^{2+}$  alone, ATP alone, and lacking both  $\text{Mg}^{2+}$  and ATP (8). In each of these conditions, KCOs had no effect on control  $K^+$  flux at doses up to 30-fold higher than their respective  $K_{1/2}$  values.

**Activation of  $K^+$  Flux through Reconstituted Cardiac Plasma Membrane  $K_{ATP}$  by  $K^+$  Channel Openers**—The high potency of diazoxide for cardiac  $\text{mitoK}_{ATP}$  was somewhat surprising and suggested a pharmacological distinction from cardiac  $\text{cellK}_{ATP}$ , which is relatively insensitive to diazoxide (18). Accordingly, we evaluated the effects of the same set of KCOs on  $\text{cellK}_{ATP}$  reconstituted from cardiac sarcolemmal vesicles (12, 19). Fig. 2 contains dose-response curves for KCO stimulation of  $K^+$  flux in proteoliposomes reconstituted with cardiac  $\text{cellK}_{ATP}$ . These assays were carried out in media containing 2 mM ATP ( $K_{1/2} = 0.5$  mM). Each of the drugs restored ATP-inhibited  $K^+$  flux to control rates measured in the absence of ATP. As was the case with  $\text{mitoK}_{ATP}$ , these KCOs had no effect on the control rate in the absence of ATP. Observed  $K_{1/2}$  values and Hill slopes (in parentheses) for opening the plasma membrane  $K_{ATP}$  channel were 3.7 nM (1.1) for EMD57970, 22 nM (1.2) for EMD60480, 17  $\mu\text{M}$  (1.1) for cromakalim, and 855  $\mu\text{M}$  (0.9) for diazoxide. Similar values were obtained in a separate preparation. The  $K_{1/2}$  values for the benzopyranyl derivatives are reasonably similar to those observed for  $\text{mitoK}_{ATP}$ ; however, the  $K_{1/2}$  value for diazoxide is about 2000-fold higher. In contradistinction to the finding with  $\text{mitoK}_{ATP}$ , the Hill slopes for activation of  $\text{cellK}_{ATP}$  were indistinguishable from 1 for all of the KCOs tested.

**Activation of ATP-Sensitive  $K^+$  Flux in Intact Mitochondria by  $K^+$  Channel Openers**—The preceding results appear to conflict with previous work (6, 7) showing very low potencies for KCO activation of  $K^+$  flux in mitochondria. Accordingly, it was important to determine whether the high potencies observed in Fig. 1 are also observed *in situ*.

Fig. 3 contains representative light-scattering traces from rat liver mitochondria respiring on ascorbate-TMPD. Swelling in  $K^+$  salts (*trace a*) was sharply inhibited by addition of 100  $\mu\text{M}$

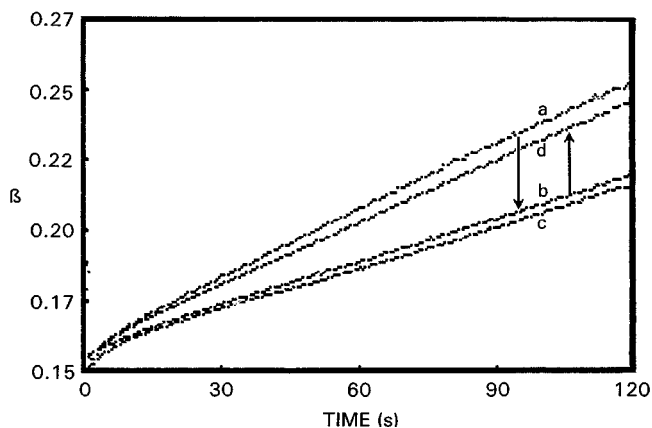


FIG. 3. Activation of  $K^+$  flux by cromakalim in intact mitochondria. Light-scattering kinetics for mitochondria suspended in  $K^+$  media (see "Experimental Procedures"). Trace a,  $K^+$  medium without ATP or cromakalim. Trace b,  $K^+$  medium containing 0.1 mM ATP. Trace c, TEA $^+$  medium without ATP. Trace d,  $K^+$  medium with 0.1 mM ATP and 20  $\mu$ M cromakalim. The down arrow from trace a to trace b shows the inhibitory effect of ATP on swelling kinetics, and the up arrow from trace b to trace d shows the opening effect of cromakalim. Ascorbate-TMPD was used as the respiratory substrate; entirely similar results were obtained with succinate.

ATP (down arrow to trace b) to levels close to those observed in TEA $^+$  salts (trace c). In agreement with previous results (10), higher [ATP] had no further effect in  $K^+$  medium, and ATP had no effect on TEA $^+$  flux (not shown). When 20  $\mu$ M cromakalim was included in the assay medium containing 100  $\mu$ M ATP, ATP inhibition was prevented (up arrow to trace d, Fig. 3). In the absence of ATP, cromakalim had no effect on the control rate up to 100  $\mu$ M, the highest dose tested. When cromakalim was added during the inhibited state, ATP inhibition was reversed (not shown).

Fig. 4 contains dose-response curves for activation of  $K^+$  flux by diazoxide, cromakalim, and EMD60480 in mitochondria. Activation was measured relative to rates in the presence of 100  $\mu$ M ATP and 1 mM  $Mg^{2+}$ , conditions in which the  $K_{1/2}$  for ATP inhibition is 2–3  $\mu$ M (13). The estimated  $K_{1/2}$  values were 2.3  $\mu$ M for diazoxide, 6.3  $\mu$ M for cromakalim, and 5.4 nM for EMD60480.

As in proteoliposomes, these drugs stimulated  $K^+$  flux only when  $K^+$  flux was inhibited by  $Mg^{2+}$  and ATP. In doses 20-fold higher than their respective  $K_{1/2}$  values, these KCOs had no effect on flux through the uninhibited channel. This effect was verified in media containing  $Mg^{2+}$  alone, ATP alone, and lacking both  $Mg^{2+}$  and ATP.

#### DISCUSSION

This is the first report showing that KCOs activate mito $K_{ATP}$  over the same dose range as they activate cell $K_{ATP}$ . This finding was observed in mitochondria and in proteoliposomes reconstituted with mito $K_{ATP}$  and raises the possibility that mito $K_{ATP}$  may be activated by KCOs *in vivo*. Kinetic parameters differed between intact mitochondria and the reconstituted preparations. As previously reported (13), the  $K_{1/2}$  for ATP inhibition is lower in mitochondria (2–3  $\mu$ M) than in proteoliposomes (20–25  $\mu$ M). We now show that the  $K_{1/2}$  values for diazoxide and cromakalim are about 6-fold higher in mitochondria than in liposomes. On the other hand, the  $K_{1/2}$  for EMD60480 is about the same in the two preparations. These differences may reflect regulatory complexity in intact mitochondria, which is lost upon extraction and reconstitution.

In the dose ranges studied, KCOs had no effect on  $K^+$  flux when  $Mg^{2+}$  and/or ATP were omitted from the assay medium. The lack of effect of KCOs on the open channel is also charac-

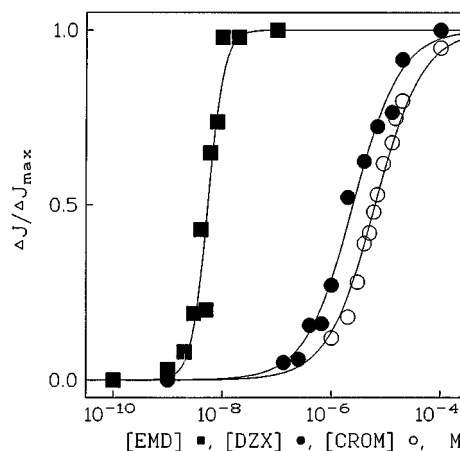


FIG. 4. Dose-response curves for activation of  $K^+$  flux in intact mitochondria. Relative ATP-sensitive  $K^+$  uptake into respiring rat liver mitochondria;  $\Delta J/\Delta J_{max}$  is plotted versus drug concentration. " $\Delta J_{max}$ " and " $\Delta J$ " are defined as in Fig. 1. The dose-response curves reflect activation by EMD60480 (■), diazoxide (●), and cromakalim (○). Assay medium for  $\Delta J$  contained 1 mM  $Mg^{2+}$  and 0.1 mM ATP, which maximally inhibited  $K^+$  uniport in mitochondria (10), and ascorbate-TMPD as respiratory substrates. The  $K_{1/2}$  values reported in the text are means of two independent experiments. For each drug, duplicate  $K_{1/2}$  values were within 5% of each other.

teristic of cell $K_{ATP}$  (20). The finding that KCOs in low doses have no effect on the uninhibited channel is also consistent with the results of Belyaeva *et al.* (6) and Szweczyk *et al.* (7), who did not include  $Mg^{2+}$  and ATP in the assay medium used for their studies.

*Physiological Consequences of Opening and Closing Mito $K_{ATP}$* —Opening of mito $K_{ATP}$  will shift the balance between  $K^+$  uniport and  $K^+/H^+$  antiport, causing transient net  $K^+$  uptake and matrix swelling to a higher steady-state volume (21). Halestrap (22) has established that increasing matrix volume over a fairly narrow range greatly activates electron transport at the point where electrons feed into ubiquinone, and he has suggested (23) that this sequence may be triggered by opening of mito $K_{ATP}$ . Thus, opening of mito $K_{ATP}$  may be a necessary component of the cellular signals calling, for example, for higher ATP production to support increased work in heart or for faster  $\beta$  oxidation of fatty acids to support thermogenesis in brown adipose tissue. Conversely, blocking mito $K_{ATP}$  may interfere with the cell's response to these signals.

*Mito $K_{ATP}$  as a Pharmacological Receptor*—Recognition of mito $K_{ATP}$  as an intracellular receptor for KCOs adds a new dimension to the KCO pharmacology, which has heretofore focused exclusively on plasma membrane  $K_{ATP}$  channels. Pharmacological regulation of  $K_{ATP}$  channels has many important, tissue-dependent consequences (1–3); however, the receptors for these effects have not yet been identified, and a mitochondrial contribution cannot be excluded. The role of  $K_{ATP}$  channels in pancreatic  $\beta$  cells is a case in point. Flatt *et al.* (24) have recently shown that  $Ca^{2+}$ -dependent insulin release from electroporated  $\beta$  cells is stimulated by glyburide and inhibited by diazoxide. Because plasma membrane  $K_{ATP}$  channels are inoperative in the permeabilized cell, these effects point to an intracellular receptor for these agents (24).

A particularly exciting development in heart is the finding by Grover and colleagues (5, 25) and others (26, 27) that KCOs are cardioprotective during experimental ischemia. KCO-treated hearts maintained higher ATP levels and exhibited reduced infarct size and enhanced post-ischemic recovery upon reperfusion. All of these effects were blocked by glyburide, which is contraindicated in patients susceptible to cardiac ischemia. Preconditioning, in which a period of brief ischemia reperfusion

protects the heart against subsequent ischemic damage (28), was also blocked by glyburide (29). These pharmacological effects point to a role of  $K_{ATP}$  channels in myocardial protection; but, again, the receptor for these effects has not been identified, and a mitochondrial site of action cannot be excluded (25).

Exploration of this possibility is aided by the existence of receptor subtypes among  $K_{ATP}$  channels (1). For example, cromakalim is a potent activator of cell $K_{ATP}$  from heart and vascular smooth muscle (29) but has a minimal effect on insulin secretion (4, 30). Diazoxide is a potent vasodilator (4) and also reduces insulin secretion (31) but has little effect on cardiac cell $K_{ATP}$  (18). This raises the question whether mito $K_{ATP}$  and cell $K_{ATP}$  from the same cell differ pharmacologically. Accordingly, we have compared drug sensitivities of cardiac mito $K_{ATP}$  and cell $K_{ATP}$  reconstituted from beef heart. These experiments yielded the following preliminary results: (i) mito $K_{ATP}$  from heart and liver do not differ significantly in their drug sensitivities ( $K_{1/2}$  values); (ii) cardiac mito $K_{ATP}$  and cardiac cell $K_{ATP}$  exhibit similar sensitivities to benzopyran derivatives; however, (iii) cardiac mito $K_{ATP}$  is about 2000 times more sensitive to diazoxide than cardiac cell $K_{ATP}$ . The low sensitivity of reconstituted cardiac cell $K_{ATP}$  to diazoxide is entirely consistent with previous reports (18). The inferred existence of receptor subtypes within the cardiac myocyte may provide a means to determine the site of cardioprotective action of  $K^+$  channel openers.

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