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# Intramitochondrial signaling: interactions among mitoK$_{ATP}$, PKCε, ROS, and MPT

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**Costa AD, Garlid KD.** Intramitochondrial signaling: interactions among mitoK$_{ATP}$, PKCε, ROS, and MPT. *Am J Physiol Heart Circ Physiol* 295: H874–H882, 2008. First published June 27, 2008; doi:10.1152/ajpheart.01189.2007.—Activation of protein kinase Cε (PKCε), opening of mitochondrial ATP-sensitive $K^+$ channels (mitoK$_{ATP}$), and increased mitochondrial reactive oxygen species (ROS) are key events in the signaling that underlies cardioprotection. We showed previously that mitoK$_{ATP}$ is opened by activation of a mitochondrial PKCε, designated PKCε1, that is closely associated with mitoK$_{ATP}$. mitoK$_{ATP}$ opening then causes an increase in ROS production by complex I of the respiratory chain. This ROS activates a second pool of PKCε, designated PKCε2, which inhibits the mitochondrial permeability transition (MPT). In the present study, we measured mitoK$_{ATP}$-dependent changes in mitochondrial matrix volume to further investigate the relationships among PKCε, mitoK$_{ATP}$, ROS, and MPT. We present evidence that *1*) mitoK$_{ATP}$ can be opened by $H_2O_2$ and nitric oxide (NO) and that these effects are mediated by PKCε1 and not by direct actions on mitoK$_{ATP}$, *2*) superoxide has no effect on mitoK$_{ATP}$ opening, *3*) exogenous $H_2O_2$ or NO also inhibits MPT opening, and both compounds do so independently of mitoK$_{ATP}$ activity via activation of PKCε2, *4*) mitoK$_{ATP}$ opening induced by PKG, phorbol ester, or diazoxide is not mediated by ROS, and *5*) mitoK$_{ATP}$-generated ROS activates PKCε1 and induces phosphorylation-dependent mitoK$_{ATP}$ opening in vitro and in vivo. Thus mitoK$_{ATP}$-dependent mitoK$_{ATP}$ opening constitutes a positive feedback loop capable of maintaining the channel open after the stimulus is no longer present. This feedback pathway may be responsible for the lasting protective effect of preconditioning, colloquially known as the memory effect.

mitochondrial ATP-sensitive $K^+$ channel; reactive oxygen species; protein kinase; preconditioning; signaling pathways

IT IS NOW GENERALLY ACCEPTED that mitochondria are both essential effectors of cardioprotection and primary targets of cardioprotective signaling. It is widely agreed both that opening of the mitochondrial permeability transition (MPT) is responsible for the pathogenesis of necrotic cell death following ischemia-reperfusion (9, 13) and that opening of mitochondrial ATP-sensitive $K^+$ channels (mitoK$_{ATP}$) is essential for cardioprotection (21). The conditions that obtain during reperfusion after prolonged ischemia—high levels of calcium, phosphate, and reactive oxygen species (ROS)—cause opening of the MPT (32). Thus mechanisms of cardioprotection must target mitochondria in such a way as to ensure that MPT opening is inhibited. This notion is supported by the recent findings that mitochondria isolated from preconditioned (2) or postconditioned (3) hearts are more resistant to induction of MPT.

Progress has been made in understanding the intramitochondrial signaling pathway that leads to MPT inhibition and the central roles played in this process by mitoK$_{ATP}$, PKCε, and ROS. Most signals reach mitochondria from the cytosol, and bradykinin triggers the protected phenotype by activating guanylyl cyclase to produce cGMP, which then activates a cGMP-dependent protein kinase (PKG) (44). PKG was shown to be the last cytosolic step in the signaling pathway by the demonstration that PKG opened mitoK$_{ATP}$ in isolated mitochondria to the same extent as cromakalim and diazoxide given at concentrations to yield a $V_{max}$ response (6). PKG interaction with mitochondria causes the signal to be transmitted to a PKCε (PKCε1) bound to the mitochondrial inner membrane (MIM), which in turn phosphorylates mitoK$_{ATP}$ and causes it to open (6, 24). The resulting increase in $K^+$ influx with attendant matrix alkalinization causes increased ROS production by complex I of the respiratory chain (1). This increase in ROS then activates a second inner membrane PKCε (PKCε2), which inhibits MPT (7).

A number of questions remain regarding the interactions among PKCε, mitoK$_{ATP}$, ROS, and MPT. In the present study, we show that $H_2O_2$, the PKCε-activating peptide ψεRACK (receptor for activated C kinase), and PMA cause mitoK$_{ATP}$ opening through a PKCε that is bound to the inner membrane and that this mitoK$_{ATP}$ opening depends on phosphorylation. We show for the first time that nitric oxide (NO) and $H_2O_2$ open mitoK$_{ATP}$ indirectly, through their activation of PKCε, and do not act on mitoK$_{ATP}$ directly. We find that PKG reacts with and phosphorylates an unknown mitochondrial outer membrane (MOM) protein and that an intact MOM is necessary for transmission of the signal from the cytosolic surface of the MOM to PKCε on the inner membrane. Exogenous NO and $H_2O_2$ are also able to inhibit MPT through their activation of a second PKCε, and this occurs independently of mitoK$_{ATP}$. Superoxide anion was found not to open mitoK$_{ATP}$, and superoxide-dependent mitoK$_{ATP}$ opening is shown to be due to superoxide dismutation to $H_2O_2$. Finally, we demonstrate mitoK$_{ATP}$-dependent mitoK$_{ATP}$ opening, which occurs via an increase in mitoK$_{ATP}$-dependent ROS, ROS activation of PKCε, and persistent, phosphorylation-dependent mitoK$_{ATP}$ opening. The latter finding provides evidence for the first time of a positive feedback loop within mitochondria that may be responsible for the lasting (memory) effect of preconditioning (15, 47).

## METHODS

*Langendorff-perfused hearts.* Male Sprague-Dawley rats (200–220 g) were briefly anesthetized with carbon dioxide and immediately

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decapitated. Hearts were rapidly excised, submerged in iced Krebs buffer, and perfused by an aortic cannula delivering normothermic (37°C) modified Krebs-Henseleit solution containing (in mM) 118 NaCl, 5.9 KCl, 1.75 $CaCl_2$, 1.2 mM $MgSO_4$, 0.5 EDTA, 25 $NaHCO_3$, and 16.7 glucose at pH 7.4. The perfusate was gassed with 95% $O_2$-5% $CO_2$, which results in a $Po_2$ >600 mmHg at the level of the aortic cannula. Hearts were allowed to stabilize for 25 min, after which diazoxide (30 μM) was perfused for 15 min, followed by a 10-min wash. Hearts were then collected for isolation of mitochondria. Control perfusions were for 50 min without interruption (sham-perfused hearts). The experimental protocols used in these studies were performed in compliance with the American Physiological Society's "Guiding Principles in the Care and Use of Animals" and were approved by the Institutional Animal Care and Use Committee at Portland State University.

*Preparation of mitochondria and mitoplasts.* Heart mitochondria were isolated by differential centrifugation and further purified in a 26% self-generating Percoll gradient exactly as previously described (6, 8). Mitoplasts were prepared from isolated mitochondria by digitonin treatment (58). Protein phosphatase inhibitors were added to all mitochondrial preparations from Langendorff-perfused hearts.

*Matrix volume measurements.* Respiration-driven influx of $K^+$, with accompanying anions and water, causes swelling of the mitochondrial matrix (20). Within well-defined limits, changes in matrix volume are linearly related to the reciprocal of the absorbance of the suspension (1/A), corrected for the extrapolated value at infinite protein concentration (1/A∞) (4): $V = a + b(1/A - 1/A\infty)$. The conversion parameters $a$ and $b$ are estimated to be −0.1026 and 0.5855, respectively. The absolute values of these parameters are unimportant when normalized differences are considered. Thus data in Figs. 2–8 are summarized as "volume change (%)", given by $100 \times [V(x) - V(ATP)]/[V(0) - V(ATP)]$, where $V(x)$ is the observed steady-state volume at 120 s under the given experimental condition and V(ATP) and V(0) are observed values in the presence and absence of ATP, respectively. The assay medium composition was (in mM) 120 KCl, 10 HEPES pH 7.2, 10 succinate, 5 inorganic phosphate, 0.5 $MgCl_2$, and 0.1 EGTA, supplemented with 1 μM rotenone and 0.67 μg/ml oligomycin. Light-scattering changes were followed at 520 nm and 30°C.

It should be noted that mitoK$_{ATP}$-dependent $K^+$ flux has been validated by five independent measurements: light scattering, direct measurements of $K^+$ flux, $H^+$ flux, respiration, and $H_2O_2$ production. Each of these was found to yield quantitatively identical measures of $K^+$ flux when calibrated with the $K^+$ ionophore valinomycin. In each of these assays, the effect of 30 μM diazoxide, to yield a $V_{max}$ response, matched the effect of 1 pmol valinomycin/mg protein (1, 8). Moreover, other pharmacological openers at concentrations to yield a $V_{max}$ response, including cromakalim (8, 21, 22, 25, 52), bimakalim (52), nicorandil (52, 57), pinacidil (38), BMS195095 (23), bepridil (56), sevoflurane (53), as well as activators of PKCε (Refs. 7, 24 and present study) yielded results comparable with those of diazoxide.

MPT was assayed exactly as described by Costa et al. (7). MPT opening was synchronized by sequential additions at 20-s intervals of $CaCl_2$ (100 μM free $Ca^{2+}$), ruthenium red (0.5 μM, to block further $Ca^{2+}$ uptake), and CCCP (250 nM, to synchronize MPT opening; Ref. 50). Mitochondria (0.1 mg/ml) were added to assay medium in the presence of ATP (50 μM). Rates of matrix volume change were obtained by taking the linear term of a second-order polynomial fit of the light scattering trace, calculated over the initial 2 min after MPT induction by CCCP. MPT inhibition was calculated by taking the $Ca^{2+}$-induced swelling rates in the presence and absence of 1 μM cyclosporin A (CsA) as 100% and 0%, respectively.

*$H_2O_2$ production.* Hydrogen peroxide production was measured by deesterified 2,7-dichlorofluorescein diacetate (DCF-DA) or Amplex Red, exactly as described previously (1).

*Chemicals.* Protein kinase G isoform 1α, cGMP, (±)-*S*-nitroso-*N*-acetylpenicillamine (SNAP), KT-5823 and the PKCε scrambled peptide (negative control for $\varepsilon V_{1\text{-}2}$) were from Calbiochem (San Diego, CA). PKCε-specific peptides antagonist $\varepsilon V_{1\text{-}2}$ (EAVSLKPT) or agonist ψεRACK (HDAPIGYD) and PKCδ-specific peptide antagonist $\delta V_{1\text{-}1}$ (SFNSYELGSL) were synthesized with a purity >98% by EZBiolab (Westfield, IN) according to published amino acid sequences (14, 27). All other chemicals were from Sigma (St Louis, MO). The PKG1α concentration (25 ng/ml, corresponding to $1.5 \times 10^{-10}$ M) and activity used in this study were comparable with those used in our previous study and with the concentration present in cells (see Ref. 6 and references therein).

*Data analysis.* All data were analyzed by unpaired Student's *t*-test. *P* values <0.05 were considered significant.

## RESULTS

*PKCε-dependent regulation of mitoK$_{ATP}$ in isolated heart mitochondria.* The experiments in Figs. 1 and 2 were designed to confirm that activation of PKCε opens mitoK$_{ATP}$. Figure 1 contains light scattering traces from heart mitochondria respiring in $K^+$ medium. The addition of the PKCε activator peptide ψεRACK or $H_2O_2$ to mitochondria incubated in the presence of ATP caused identical increases in steady-state matrix volume to an extent comparable to that obtained in the absence of ATP or in the presence of ATP + $K_{ATP}$ channel opener (not shown). The addition of either 5-hydroxydecanoate (5-HD) or the PKCε inhibitor peptide $\varepsilon V_{1\text{-}2}$ inhibited mitoK$_{ATP}$-dependent $K^+$ influx and prevented the increase in matrix volume. It should be noted that ψεRACK and $\varepsilon V_{1\text{-}2}$ are small peptides (mol wt 888.5 and 845.5, respectively) that readily diffuse across the MOM. In experiments not shown, we estimated that the $EC_{50}$ for $H_2O_2$-induced mitoK$_{ATP}$ opening was 0.4 μM (±0.1 μM; Hill coefficient = 1) and the $EC_{50}$ for the specific PKCε peptide agonist ψεRACK was 72 nM (±30 nM; Hill coefficient = 1).

To confirm that the effects of $H_2O_2$ and ψεRACK were specific for PKCε and mitoK$_{ATP}$, we investigated their effects in the presence of various regulators of PKC and mitoK$_{ATP}$ and also in the presence of the MPT inhibitor CsA. The results obtained from five or more independent experiments are summarized in Fig. 2. $H_2O_2$-dependent mitoK$_{ATP}$ opening was inhibited by the mitoK$_{ATP}$ blockers 5-HD and glibenclamide

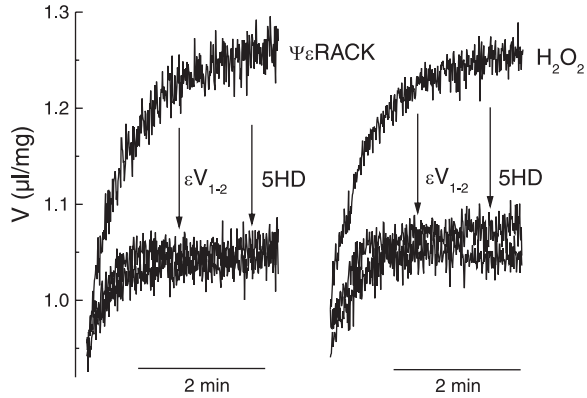


Fig. 1. PKCε-mediated mitochondrial ATP-sensitive $K^+$ channel (mitoK$_{ATP}$) opening. Changes in mitochondrial matrix volume (V) are plotted vs. time. Rat heart mitochondria (0.1 mg/ml) were suspended in assay medium described in METHODS. $H_2O_2$ (2 μM) or ψε receptor for activated C kinase (RACK) (0.5 μM) was added to medium in the presence of ATP (0.2 mM) ~1 s after the mitochondria. Other additions to the assay medium were 5-hydroxydecanoate (5-HD, 0.3 mM) and $\varepsilon V_{1\text{-}2}$ (0.5 μM). Traces are representative of at least 5 independent experiments.

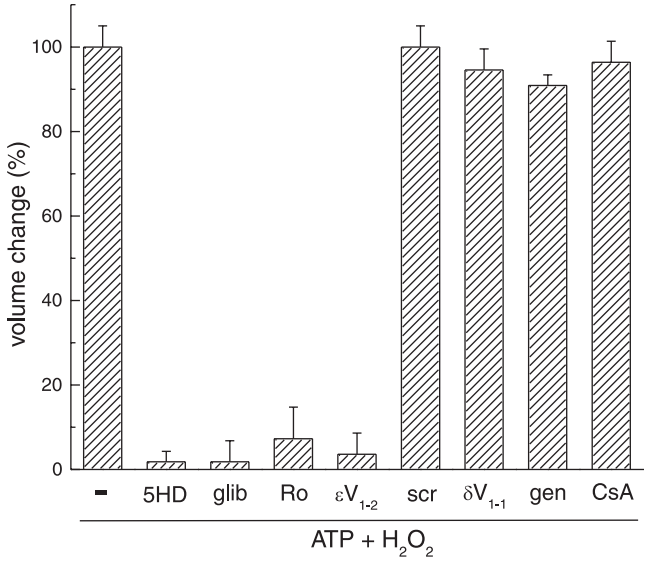


Fig. 2. $H_2O_2$-induced mitoK$_{ATP}$ opening is blocked by PKCε inhibitors. Shown are the effects of several compounds on mitoK$_{ATP}$ opening induced by $H_2O_2$ plotted as volume change (%). Indicated additions to the assay were ATP (0.2 mM), $H_2O_2$ (2 μM), 5-HD (0.3 mM), glibenclamide (glib, 10 μM), Ro-318220 (Ro, 0.5 μM), εV$_{1-2}$ (0.5 μM), PKCε scrambled peptide (scr, 1 μM), δV$_{1-1}$ (0.2 μM), genistein (gen, 5 μM), and cyclosporin A (CsA, 1 μM). Data are means ± SD of at least 4 independent experiments.

and by the PKCε blockers εV$_{1-2}$ and Ro-318220. A scrambled peptide with the same amino acid composition as εV$_{1-2}$ was without effect. CsA had no effect on steady-state volume in the presence of $H_2O_2$, indicating that its effects were not caused by opening of MPT. Other tested compounds that had no effect on $H_2O_2$-dependent mitoK$_{ATP}$ opening included the PKCδ inhibitors δV$_{1-1}$ (mol wt 1,117.8), Gö-6983 (not shown), and the tyrosine kinase inhibitor genistein. Identical results were obtained with the same agents when mitoK$_{ATP}$ was opened by ψεRACK or PMA (data not shown).

*Superoxide does not open mitoK$_{ATP}$.* Superoxide anion has been suggested to induce mitoK$_{ATP}$ opening in cardiomyocytes and perfused hearts (11, 34, 39, 43, 46, 61). Superoxide was generated with hypoxanthine plus xanthine oxidase (XOx). As shown in Fig. 3, 60 mU of XOx opened mitoK$_{ATP}$, but this effect was blocked by catalase, indicating that $H_2O_2$ from spontaneous dismutation of superoxide is the species responsible for the observed effect. We then lowered the XOx concentration to 6 mU, which was not effective in opening mitoK$_{ATP}$. However, 6 mU of XOx plus 30 U of superoxide dismutase (SOD), to convert the superoxide anion to $H_2O_2$, did cause mitoK$_{ATP}$ opening, and this effect was abolished by subsequent addition of catalase, to remove $H_2O_2$. The effects of hypoxanthine + XOx + SOD on mitoK$_{ATP}$ were inhibited by 5-HD or εV$_{1-2}$ (Fig. 3).

In experiments not shown, we measured $H_2O_2$ production under these experimental conditions, using Amplex Red or deesterified carboxy-DCF (1) in the absence of mitochondria. Addition of 60 mU of XOx produced ~30 times more $H_2O_2$ by spontaneous dismutation than addition of 6 mU, which led to barely detectable levels of $H_2O_2$. Addition of SOD to medium containing 6 mU of XOx resulted in a 60 ± 10% increase in $H_2O_2$ detected by both fluorescent probes, and addition of catalase resulted in barely detectable levels of $H_2O_2$. 5-HD and εV$_{1-2}$ did not affect $H_2O_2$ production under these in vitro experimental conditions. We conclude from these studies that superoxide anion does not activate mitoK$_{ATP}$ directly, but rather through its dismutation products.

*NO opens mitoK$_{ATP}$ via PKCε.* We next examined the effects of NO, which has been suggested to induce mitoK$_{ATP}$ opening in cardiomyocytes and perfused hearts (55, 64, 65). Using mitochondrial membranes incubated in KCl medium, Dahm et al. (10) found that 1 mM SNAP generates 1 μM NO within 3 min, at an approximate rate of 0.3 μM NO/min. As shown in Fig. 4, SNAP reversed the ATP inhibition of mitoK$_{ATP}$ with an apparent $K_m$ of 2 mM, corresponding to ~2 μM NO. SNAP inhibits mitochondrial swelling at concentrations above 50 mM, probably due to inhibition of cytochrome-*c* oxidase (12, 51). Therefore, we used 10 mM SNAP in our studies. SNAP-induced mitoK$_{ATP}$ opening in mitochondria was inhibited by 5-HD, *N*-(2-mercaptopropionyl)glycine (MPG) (not shown), and εV$_{1-2}$, but not by catalase (Fig. 4). SNAP-induced mitoK$_{ATP}$ opening in mitoplasts was blocked by MPG and protein phosphatase 2A (PP2A). From these results, we conclude that NO opens mitoK$_{ATP}$ indirectly, through activation of PKCε.

*Role of mitochondrial outer membrane in PKG- and PKCε-dependent mitoK$_{ATP}$ opening.* PKCε copurifies and coreconstitutes with mitoK$_{ATP}$ in a fully functional manner (24), and we refer to this mitoK$_{ATP}$-associated enzyme as PKCε1 (7). PKCε1 is retained in heart mitoplasts and appears to be tightly bound to the inner membrane (7). The experiments summarized in Fig. 5 were performed in intact mitochondria and in mitoplasts lacking the MOM. PMA, which can readily diffuse across the MOM to activate PKCε1, opened mitoK$_{ATP}$ in both intact mitochondria and mitoplasts (Fig. 5). Exogenous PKG + cGMP opens mitoK$_{ATP}$ in a PKCε-dependent process (6, 7). PKG, which cannot cross the MOM, opens

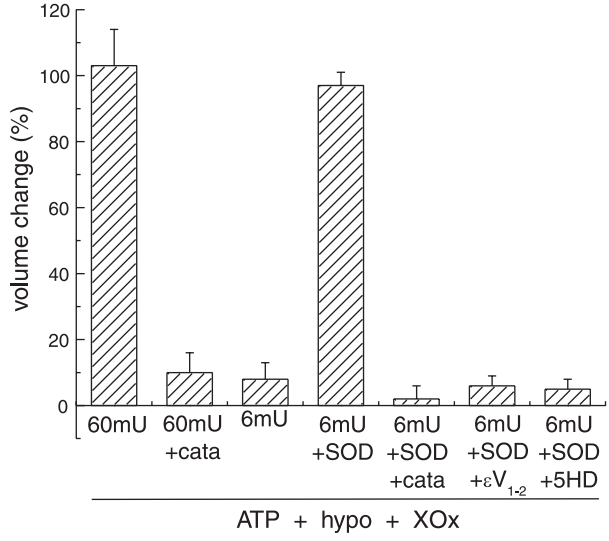


Fig. 3. $H_2O_2$, but not superoxide, opens mitoK$_{ATP}$ via PKCε. Shown are the effects of xanthine oxidase (XOx) + hypoxanthine on mitoK$_{ATP}$ activity, plotted as volume change (%). Rat heart mitochondria (0.1 mg/ml) were suspended in assay medium containing ATP (0.2 mM) and hypoxanthine (hypo, 0.2 μM). XOx (6 or 60 U/ml) was added to medium ~1 s after mitochondria. As indicated, medium also contained catalase (cata, 10 U/ml), superoxide dismutase (SOD, 30 U), 5-HD (0.3 mM), and εV$_{1-2}$ (0.5 μM). Data are means ± SD of at least 4 independent experiments.

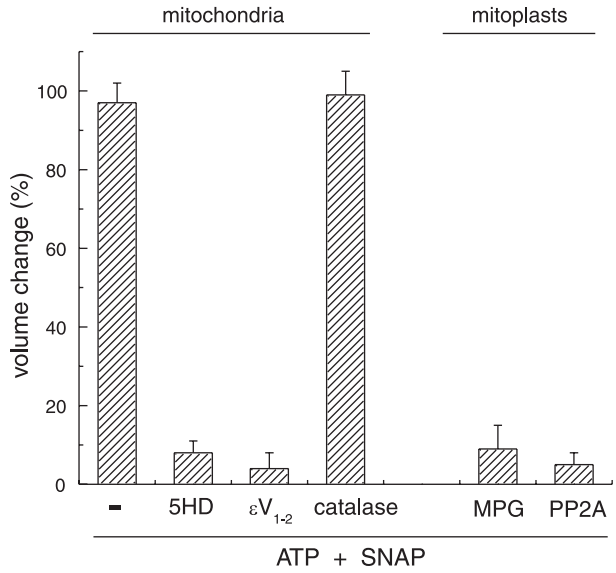


Fig. 4. Nitric oxide (NO) opens mitoK$_{ATP}$ via PKCε. Shown are the effects of the NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP) on mitoK$_{ATP}$ activity, plotted as volume change (%). Rat heart mitochondria (0.1 mg/ml) were suspended in assay medium containing ATP (0.2 mM). SNAP (10 mM) was added 2 min before mitochondria to allow generation of NO. As indicated, medium also contained 5-HD (0.3 mM), εV$_{1-2}$ (0.5 μM), and catalase (10 U/ml). Columns on *right* demonstrate the results of experiments with mitoplasts lacking the mitochondrial outer membrane (MOM). *N*-(2-mercaptopropionyl)glycine (MPG, 0.3 mM) and protein phosphatase 2A (PP2A, 11 ng/ml) blocked mitoK$_{ATP}$ opening by SNAP in mitoplasts. Data are means ± SD of at least 4 independent experiments.

mitoK$_{ATP}$ in intact mitochondria but not in mitoplasts. Thus PKG-dependent mitoK$_{ATP}$ opening requires an intact MOM. The Ser/Thr phosphatase PP2A, which cannot cross the MOM, had no effect on PMA-induced mitoK$_{ATP}$ opening in intact mitochondria, but it negated the effects of PMA in mitoplasts, indicating that mitoK$_{ATP}$ is opened by PKCε-dependent phosphorylation at the level of the inner membrane. PP2A blocked the effect of PKG in intact mitochondria, showing that PKG-dependent mitoK$_{ATP}$ opening depends on phosphorylation of a MOM protein necessary for signal transmission to mitoK$_{ATP}$.

*ROS are not involved in mitoK$_{ATP}$ opening by diazoxide, PMA, or PKG.* The data in Fig. 6 show that ROS are not involved in mitoK$_{ATP}$ opening by diazoxide or by PKCε-dependent opening mediated by PMA or PKG. 5-HD, which acts directly on the channel, inhibited the effects of all three mitoK$_{ATP}$ agonists. The PKCε inhibitor chelerythrine inhibited the effect of PMA, and the PKG-specific inhibitor KT-5823 inhibited the effect of PKG + cGMP. However, the ROS scavenger MPG had no effect on mitoK$_{ATP}$ opening by any of these mechanisms.

*mitoK$_{ATP}$-induced ROS-generation activates PKCε and maintains mitoK$_{ATP}$ in the open state.* mitoK$_{ATP}$ opening in isolated heart mitochondria causes an increase in ROS production (1). As shown in Figs. 1–3, $H_2O_2$ induces mitoK$_{ATP}$ opening via activation of PKCε1 (24). These results led us to hypothesize that the ROS produced by mitoK$_{ATP}$ opening could activate mitochondrial PKCε1 and maintain mitoK$_{ATP}$ in the open state for prolonged periods. To test this hypothesis, we performed experiments on mitoplasts. They were preincubated in 150-μl aliquots of assay medium with ATP and diazoxide (30 μM), diluted 10-fold in sucrose buffer containing 0.5% fatty acid-free BSA, and centrifuged at 15,000 *g* for 30 s. The pellet was resuspended and added to assay medium in the presence of ATP. After this treatment, the mitoplasts lost their sensitivity to ATP (Fig. 7). Moreover, they became insensitive to diazoxide, suggesting that the channel was already open. This was confirmed by the findings that PP2A + ATP restored the closed state and the sensitivity to diazoxide (Fig. 7). This was presumably due to dephosphorylation of mitoK$_{ATP}$. We interpret these results to mean that preincubation with ATP + diazoxide opened mitoK$_{ATP}$, leading to increased ROS that activated mitochondrial PKCε1, which in turn phosphorylated mitoK$_{ATP}$, leading to a sustained open

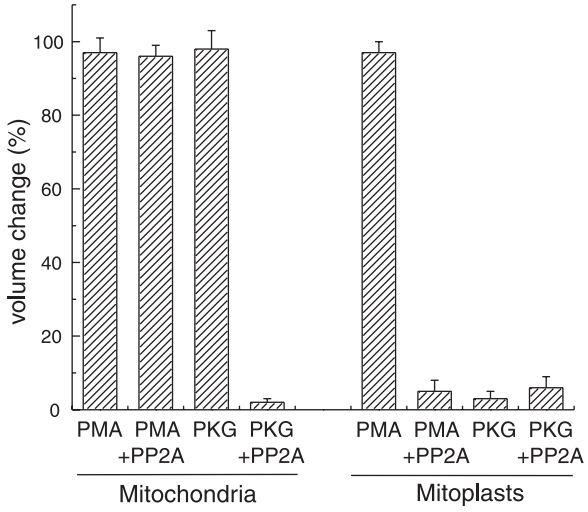


Fig. 5. The MOM is essential for PKG-induced, but not PKCε-induced, mitoK$_{ATP}$ activity. Shown are the effects of PKG or PMA, and PP2A, on the matrix volume of heart mitochondria or mitoplasts. Data are plotted as volume change (%). Indicated additions to the assay were PKG (25 ng/ml), PMA (0.2 μM), and PP2A (11 ng/ml). Data are means ± SD of at least 4 independent experiments.

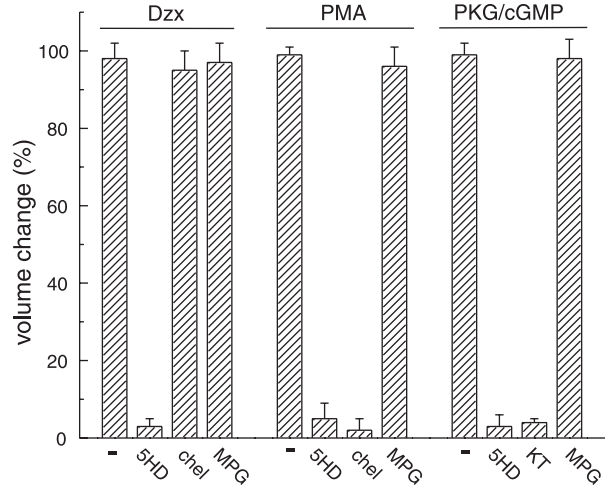


Fig. 6. mitoK$_{ATP}$ opening by diazoxide, PKG, or PMA does not require reactive oxygen species (ROS). Shown are the effects of MPG on diazoxide (Dzx)-, PMA-, or PKG/cGMP-induced mitoK$_{ATP}$ opening, plotted as volume change (%). Rat heart mitochondria (0.1 mg/ml) were suspended in assay medium as described in METHODS. Additions to the assay were ATP (0.2 mM), Dzx (30 μM), PKG (25 ng/ml), cGMP (10 μM), PMA (0.2 μM), 5-HD (0.3 mM), chelerythrine (chel, 0.1 μM), MPG (0.3 mM), and KT-5823 (KT, 0.5 μM). Data are means ± SD of at least 4 independent experiments.

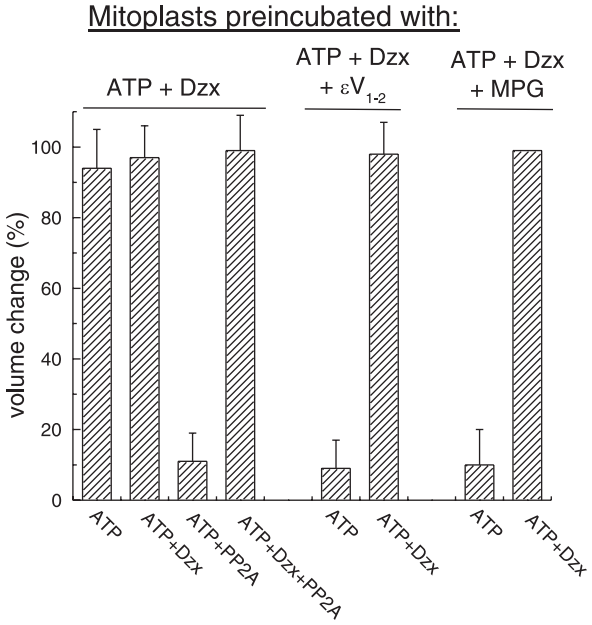


Fig. 7. Phosphorylation-dependent mitoK$_{ATP}$-induced mitoK$_{ATP}$ opening in vitro. Shown are the effects of preincubating heart mitoplasts (0.3 mg) with ATP (0.2 mM) + Dzx (30 μM), plotted as volume change (%). Preincubations were carried out in assay medium containing phosphatase inhibitors and, where indicated, εV$_{1-2}$ (0.5 μM) or MPG (0.3 mM, 0.5%) at 30°C. After 3 min, the mitoplasts were washed and diluted 100-fold into the assay medium in order to avoid effects of Dzx, εV$_{1-2}$, or MPG during the assay. Indicated additions to the assay were ATP (0.2 mM), Dzx (30 μM), and PP2A (11 ng/ml). Data are means ± SD of at least 3 independent experiments.

state. This interpretation is supported by the further finding that preincubation of mitoplasts with ATP, diazoxide, and εV$_{1-2}$, to inhibit PKCε1, resulted in a normal response of mitoK$_{ATP}$ to ATP and diazoxide in the assay medium (Fig. 7). A similar effect was observed when mitoplasts were preincubated with ATP, diazoxide, and the ROS scavenger MPG (Fig. 7), which restored the normal response to ATP and diazoxide in the assay medium (Fig. 7).

*Persistence of open state in mitochondria from diazoxide-treated hearts.* We next investigated whether a similar effect could be observed in mitoplasts prepared from diazoxide-perfused hearts. Figure 8 shows that mitoplasts from sham-perfused hearts displayed normal sensitivity to diazoxide and PP2A and, further, that the diazoxide response was not modified by PP2A. However, mitoplasts from hearts perfused with diazoxide displayed an open mitoK$_{ATP}$, and ATP and diazoxide had no effect unless the diazoxide-dependent phosphorylation was removed by PP2A. These findings agree with those in Fig. 7. The data in Figs. 7 and 8 support the hypothesis that mitoK$_{ATP}$ activity induced by a K$_{ATP}$ channel agonist or by preconditioning will maintain mitoK$_{ATP}$ in an open state, even after the K$_{ATP}$ channel opener is washed away (19, 20).

*NO inhibits MPT via PKCε.* $H_2O_2$ inhibits MPT opening by oxidizing thiols in PKCε and causing its activation (7). We reasoned that NO should have a similar effect. The results in Fig. 9 show that the NO donor SNAP inhibited MPT opening in isolated heart mitochondria to the same extent as diazoxide. This result agrees with that reported by Brookes et al. (5), who found that 0.7 μM NO inhibited MPT in liver mitochondria. Figure 9 also shows that inhibition of mitoK$_{ATP}$ by 5-HD had no effect on SNAP inhibition of MPT, and that εV$_{1-2}$ completely abolished the protective effects of SNAP. No further inhibition was observed when mitochondria were incubated with 5-HD and εV$_{1-2}$ at the same time. These effects of NO on MPT are similar to those previously observed with $H_2O_2$ (7). We conclude, as before, that NO is activating a second PKCε, PKCε2, that regulates MPT (7). The added NO acts directly on PKCε2, thereby bypassing mitoK$_{ATP}$; hence, 5-HD has no effect.

*Inhibition of GSK-3β has no effect on MPT in isolated mitochondria.* It has been proposed that GSK-3β, a negative modulator of preconditioning (62), is "immediately proximal to the permeability transition pore complex" (29). Given that mitoK$_{ATP}$ opening by a variety of mechanisms has been shown to inhibit MPT in isolated mitochondria (Ref. 7 and present study), it should be possible to demonstrate inhibition of MPT under similar conditions by inhibiting GSK-3β. However, the inhibitor SB-415286 had no effect on MPT opening in isolated mitochondria (not shown), indicating that the GSK-3β that interferes with cardioprotection is extramitochondrial.

## DISCUSSION

The interactions among mitoK$_{ATP}$, PKCε, ROS, and MPT constitute a well-regulated intramitochondrial signaling pathway. The diagram in Fig. 10 summarizes several years of work on this pathway (1, 6–8, 18, 24), including the present findings. The primary function of the pathway is to inhibit MPT opening, which is widely considered to be the cause of cell death after ischemia-reperfusion (3, 9, 13). The sequence begins with mitoK$_{ATP}$ opening, which may occur by three distinct mechanisms: direct, by administration of a K$_{ATP}$ channel opener (KCO) (18); indirect, by activation of PKCε1 (24); and physiological, by cytosolic signaling kinases such as PKG (6). Each of these methods has its counterpart in cardioprotec-

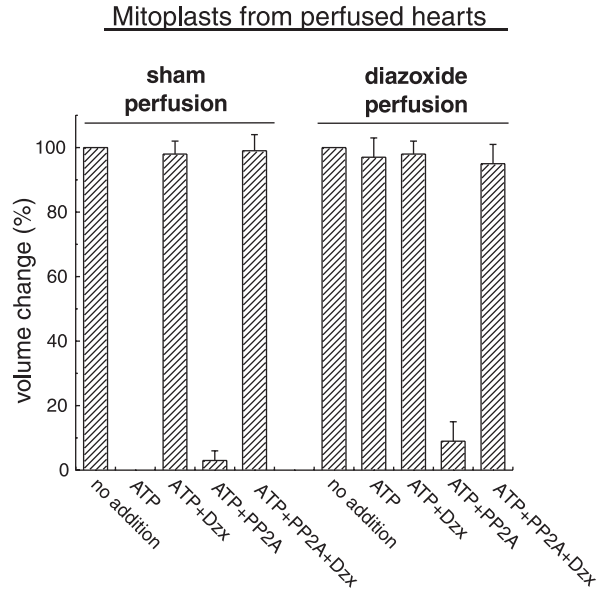


Fig. 8. Phosphorylation-dependent persistence of mitoK$_{ATP}$ opening in diazoxide-perfused hearts. Shown are the effects of sham perfusion or Dzx perfusion on mitoK$_{ATP}$ activity in mitoplasts isolated from perfused rat hearts. mitoK$_{ATP}$ activity is plotted as volume change (%). Indicated additions to the assay were ATP (0.2 mM), Dzx (30 μM), and PP2A (11 ng/ml). Data are means ± SD of at least 3 independent experiments.

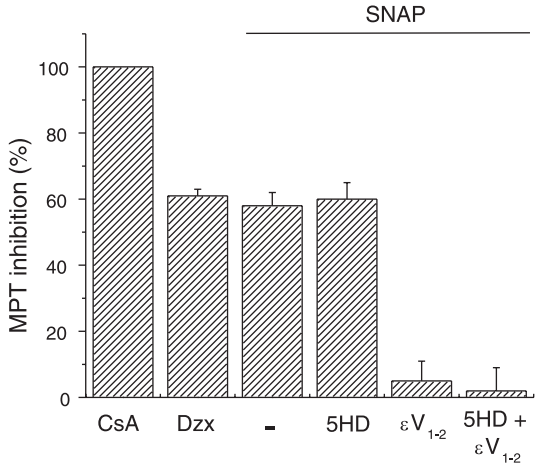


Fig. 9. NO inhibits mitochondrial permeability transition (MPT) via PKCε and independently of mitoK$_{ATP}$. Shown are the effects of Dzx or the NO donor SNAP on MPT-induced swelling, expressed as MPT inhibition (%). Synchronized MPT opening in rat heart mitochondria (0.1 mg/ml) was elicited by $Ca^{2+}$ and the uncoupler CCCP, as described in METHODS. Rates of matrix swelling in the presence and absence of 1 μM CsA were taken as 0% and 100%, respectively. SNAP (10 mM) was added 2 min before mitochondria to allow generation of NO. 5-HD (0.3 mM) and εV$_{1-2}$ (0.5 μM) were added immediately before mitochondria. Data are means ± SD of at least 3 independent experiments.

tion of the perfused heart. Thus diazoxide and other KCOs are protective (21), PKCε activation is protective (42, 66), and PKG activation, arising for example from perfusion of the heart with bradykinin, is protective (44).

Direct mitoK$_{ATP}$ opening by KCOs has been described previously (22). KCOs act on the regulatory sulfonylurea receptors (SUR) of K$_{ATP}$ channels. Pinacidil, cromakalim, and nicorandil are effective openers of cardiac K$_{ATP}$ through their action on SUR2A, but ineffective on the pancreatic β-cell K$_{ATP}$, which uses SUR1. Conversely, diazoxide is an effective opener of β-cell K$_{ATP}$ but ineffective on the cardiac channel (40). Interestingly, all KCOs we have examined, including those listed above, open mitoK$_{ATP}$ (8, 21–23, 25, 38, 45, 52).

Indirect mitoK$_{ATP}$ opening by activation of PKCε1 was demonstrated by the effects of the PKCε-specific peptide agonist ψεRACK (Figs. 1 and 2) and the PKCε agonists $H_2O_2$ (Figs. 1–3), NO (Fig. 4), and PMA (Figs. 5 and 6). That these agents were acting via PKCε1 was verified by the finding that the PKCε-specific binding antagonist εV$_{1-2}$ blocked all four modes of PKCε activation of mitoK$_{ATP}$ but did not block mitoK$_{ATP}$ opening by diazoxide (Ref. 24 and present study). Importantly, superoxide cannot activate PKCε1 to open mitoK$_{ATP}$, as shown in Fig. 3. Jaburek et al. (24) observed similar effects of ψεRACK and εV$_{1-2}$ in liposomes reconstituted with partially purified mitoK$_{ATP}$ and PKCε1. The PKCδ-specific peptide antagonist δV$_{1-1}$, or a scrambled analog of εV$_{1-2}$, had no effect on $H_2O_2$-dependent mitoK$_{ATP}$ opening, and we conclude that this effect is mediated specifically by an intrinsic mitochondrial PKCε. PKCε1 effect requires phosphorylation, perhaps of mitoK$_{ATP}$ itself. Thus, when given access in mitoplasts to the MIM, PP2A prevented mitoK$_{ATP}$-dependent swelling induced by PKCε agonists (Fig. 4).

PKCε requires anionic phospholipids for activity and is activated physiologically by one of two second messengers—diacylglycerol (or phorbol ester) and a sulfhydryl oxidizing agent, such as $H_2O_2$ (60) or NO (present study). Addition of PMA or $H_2O_2$ has been shown to open up one of the two zinc fingers in PKCε (30, 37). The phospholipid requirement is met by cardiolipin, which is abundant in mitochondria and enhances PKCε activity three- to fourfold compared with phosphatidylserine (36). ψεRACK, PMA, $H_2O_2$, and NO each open mitoK$_{ATP}$. These agents cause conformational changes that expose the substrate domain on PKCε and cause its binding to its RACK (54). ψεRACK is a PKCε-specific peptide agonist that acts by regulating intramolecular PKCε binding, and εV$_{1-2}$ is a PKCε-specific peptide antagonist that acts by preventing protein-protein interactions between PKCε and its binding protein, RACK (27, 54, 59). Murriel and Mochly-Rosen (42) found that ψεRACK protected cardiac cells from ischemic damage, whereas εV$_{1-2}$ caused a loss of protection.

Physiological mitoK$_{ATP}$ opening is mediated by cytosolic signaling kinases, such as PKG, that act on the MOM. The data in Figs. 5 and 6 show that PKG + cGMP induce mitoK$_{ATP}$

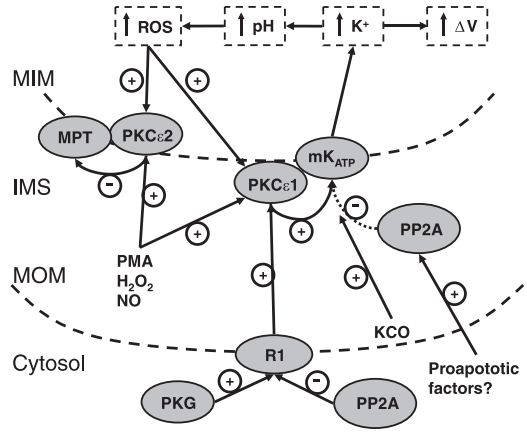


Fig. 10. The intramitochondrial signaling pathways. There are 3 distinct ways of opening mitoK$_{ATP}$ and initiating the signaling sequence described. *1*) Direct mitoK$_{ATP}$ opening by K$_{ATP}$ channel openers (KCO) has been demonstrated in mitochondria and in liposomes containing reconstituted mitoK$_{ATP}$ (22). *2*) Indirect mitoK$_{ATP}$ opening by activation of PKCε1 was demonstrated by the effects of the PKCε-specific peptide agonist ψεRACK, PMA, $H_2O_2$, and NO. That these agents were acting via PKCε1 was verified by the finding that the PKCε-specific binding antagonist εV$_{1-2}$ blocked all 4 modes of PKCε activation of mitoK$_{ATP}$ but did not block mitoK$_{ATP}$ opening by diazoxide (Ref. 24 and present study). PKCε1 effect requires phosphorylation, perhaps of mitoK$_{ATP}$ itself. Thus, when given access in mitoplasts to the mitochondrial inner membrane (MIM), PP2A prevented mitoK$_{ATP}$-dependent swelling induced by PKCε agonists. *3*) Physiological mitoK$_{ATP}$ opening by signals arriving at the MOM from the cytosol, such as PKG (6). PKG + cGMP open mitoK$_{ATP}$ by phosphorylating a MOM receptor (labeled R1). Thus PKG-dependent mitoK$_{ATP}$ opening is blocked either if PP2A is added to the assay or if the MOM is removed. Phosphorylation of R1 leads by an unknown mechanism to activation of PKCε1 and opening of mitoK$_{ATP}$. Once mitoK$_{ATP}$ is opened, the increase in $K^+$ uptake leads to increased matrix volume (ΔV), which is the basis of the light scattering assay for mitoK$_{ATP}$ activity (8). More $K^+$ than phosphate will be taken up into the matrix, because the cytosolic concentration of $K^+$ is much higher than that of phosphate. This imbalance leads to matrix alkalinization (8). Matrix alkalinization, in turn, inhibits complex I, leading to increased production of superoxide and its products, $H_2O_2$ and hydroxyl anion radical (1). The increase in ROS plays 2 roles. It activates a second PKCε, PKCε2, that then inhibits the MPT in a phosphorylation-dependent reaction (7). We hypothesize that this effect is the primary means by which preconditioning and ischemic postconditioning prevent cardiac cell death. The increase in ROS also activates PKCε1, which is bypassed when KCOs are administered to the heart, to cause a persistent phosphorylation-dependent open state of mitoK$_{ATP}$ (present study). We hypothesize that this positive feedback loop for mitoK$_{ATP}$ opening is the mechanism of memory, which is seen with all preconditioning triggers (15, 47).

opening that is blocked by the specific PKG inhibitor KT-5823, the mitoK$_{ATP}$ inhibitor 5-HD, and the PKC inhibitor chelerythrine. The latter finding shows that activation of PKCε (PKCε1 in Fig. 10) is an essential step in PKG-dependent mitoK$_{ATP}$ opening (6). Activation of PKCε1 by this mechanism is not prevented by MPG (Fig. 6); therefore it does not involve ROS. In mitochondria with an intact MOM, PKG-dependent mitoK$_{ATP}$ opening is blocked by PP2A (Fig. 5). In mitoplasts with the MOM disrupted, PKG is no longer able to induce mitoK$_{ATP}$ opening (Fig. 5). These findings show that the MOM is required for transmission of cytosolic signals to mitoK$_{ATP}$ and that PKG phosphorylates a MOM receptor protein (R1 in Fig. 10), whose molecular identity is not yet known. The mechanism of signal transmission from MOM to PKCε1 on the inner membrane is also not known but may involve a pseudo-RACK mechanism.

Once mitoK$_{ATP}$ is opened, the increase in $K^+$ uptake leads to increased matrix volume (ΔV in Fig. 10), which is the basis of the light scattering assay for mitoK$_{ATP}$ activity (8). The cytosolic concentration difference between $K^+$ and phosphate means that more $K^+$ than phosphate will be taken up, leading to matrix alkalinization (8). Matrix alkalinization, in turn, inhibits complex I, leading to increased production of superoxide and its products, $H_2O_2$ and hydroxyl anion radical (1).

The increase in ROS now activates a second mitochondrial PKCε (PKCε2 in Fig. 10). We showed previously (7) that activation of PKCε2 inhibits the MPT in a phosphorylation-dependent reaction. The evidence for two distinct mitochondrial PKCε, one acting on mitoK$_{ATP}$ and the other on MPT, is given in Ref. 7. $H_2O_2$ activates PKCε2 and inhibits MPT (7). The results in Fig. 9 show that NO, but not superoxide, also inhibits MPT in a PKCε-dependent manner. Thus the redundant modes of cardioprotective mitoK$_{ATP}$ opening lead by these pathways to inhibition of MPT, and presumably to reduction of cell death after ischemia-reperfusion injury (3, 9, 13).

The mitoK$_{ATP}$-dependent increase in ROS plays an additional role in cardioprotection. It should be noted in Fig. 10 that PKCε1 is bypassed when KCOs are administered to the heart; however, as shown in Figs. 7 and 8, PKCε1 is soon activated by mitoK$_{ATP}$-dependent ROS, leading to a persistent phosphorylation-dependent open state of mitoK$_{ATP}$. These data define a new, positive feedback loop for mitoK$_{ATP}$ opening, whose existence, which has been suggested by a number of authors (29, 31, 39), means that mitoK$_{ATP}$ may be either upstream or downstream of PKCε, depending on the triggering stimulus. We suggest that feedback phosphorylation of mitoK$_{ATP}$ is the mechanism of memory, which is seen with all preconditioning triggers (15, 47). Thus cardioprotective stimuli can be washed away from the system, and the perfused heart remains protected from a major ischemic assault due to phosphorylation of mitoK$_{ATP}$. We infer, but have not demonstrated, that mitoK$_{ATP}$ opening is eventually reversed by an endogenous phosphatase (PP2A in Fig. 10) within the intermembrane space. For example, PP2A has been found in mitochondria, where it is activated by proapoptotic factors (35).

The model in Fig. 10 and the findings reported here help to clarify and extend results of previous studies. Several reports have correlated mitoK$_{ATP}$ opening, ROS, and PKCε activity, but none in isolated mitochondria. Jiang et al. (26) observed PKC and 5-HD regulation of the human cardiac mitoK$_{ATP}$ in lipid bilayers. Garg and Hu (17) showed that PKCε modulates mitoK$_{ATP}$ activity in cardiomyocytes and COS-7 cells. Penna et al. (49) demonstrated that postconditioning protection involves a redox-sensitive mechanism and persistent activation of mitoK$_{ATP}$ and PKC. Our results are fully consistent with these studies. Sasaki et al. (55) suggested that NO may open mitoK$_{ATP}$ directly; however, mitoK$_{ATP}$ opening by NO is blocked by εV$_{1-2}$ (Fig. 4), indicating that NO opens mitoK$_{ATP}$ indirectly through PKCε1. Several authors have shown that exogenous and endogenous NO are cardioprotective and have attributed this effect to MPT inhibition (5, 28, 33, 63). Brookes et al. (5) showed that NO inhibited MPT and cytochrome *c* release in isolated liver mitochondria. Here, we confirm that NO inhibits MPT in heart mitochondria and show that this effect is independent of mitoK$_{ATP}$ activity and occurs via activation of PKCε2 (Fig. 9). Forbes et al. (16) and Pain et al. (47) found that *N*-acetylcysteine or MPG reversed the protective effect of diazoxide in perfused hearts. Our data suggest that block of protection occurred because mitoK$_{ATP}$-dependent ROS was scavenged and unavailable to activate PKCε2 and inhibit MPT. Lebuffe et al. (39) found that PMA-induced protection was blocked by 5-HD and that this block was reversed by coadministration of $H_2O_2$ and NO. This is also consistent with the model of Fig. 10 in that $H_2O_2$ and NO can bypass the blocked mitoK$_{ATP}$ and act directly on PKCε2, thereby inhibiting MPT and protecting the heart. Some effects of mitoK$_{ATP}$-dependent ROS signaling appear to result from a second messenger effect of the ROS on extramitochondrial pathways. Thus diazoxide and other cardioprotective signals cause phosphorylation of GSK-3β in cardiomyocytes and isolated hearts (29, 41, 48, 62); however, inhibition of GSK-3β has no effect on MPT opening in isolated mitochondria (present study), suggesting that the GSK isoform that interferes with cardioprotection resides outside of mitochondria.

*Limitations.* In these experiments, mitochondria were respiring on the nonphysiological substrate succinate. However, we showed previously (1) that mitoK$_{ATP}$ activity is also observed when mitochondria respire on pyruvate/malate. $K^+$ flux via mitoK$_{ATP}$ depends on membrane potential and does not appear to be influenced directly by the mechanism of producing this driving force, so we do not anticipate different behavior in vivo. The results of Fig. 8, in which mitoK$_{ATP}$ was opened ex vivo by diazoxide, are at least consistent with this view. Also, for practical reasons, this study was based solely on light scattering measurements. As described in METHODS, this assay has been validated quantitatively by five independent techniques.

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