The Mechanism by Which the Mitochondrial ATP-sensitive K⁺ Channel Opening and H₂O₂ Inhibit the Mitochondrial Permeability Transition

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Myocardial infarction is a manifestation of necrotic cell death as a result of opening of the mitochondrial permeability transition (MPT). Receptor-mediated cardioprotection is triggered by an intracellular signaling pathway that includes phosphatidylinositol 3-kinase, endothelial nitric-oxide synthase, guanylyl cyclase, protein kinase G (PKG), and the mitochondrial K⁺ ATP channel (mitoK⁺ATP). In this study, we explored the pathway that links mitoK⁺ATP with the MPT. We confirmed previous findings that diazoxide and activators of PKG or protein kinase C (PKC) inhibited MPT opening. We extended these results and showed that other K⁺ channel openers as well as the K⁺ ionophore valinomycin also inhibited MPT opening and that this inhibition required reactive oxygen species. By using isoform-specific peptides, we found that the effects of K⁺ ATP channel openers, PKG, or valinomycin were mediated by a PKCε. Activation of PKCε by phorbol 12-myristate 13-acetate or H₂O₂ resulted in mitoK⁺ATP-independent inhibition of MPT opening, whereas activation of PKCε by PKG or the specific PKCε agonist 8e receptor for activated C kinase caused mitoK⁺ATP-dependent inhibition of MPT opening. Exogenous H₂O₂ inhibited MPT, because of its activation of PKCε, with an IC₅₀ of 0.4 (±0.1) μM. On the basis of these results, we propose that two different PKCε pools regulate this signaling pathway, one in association with mitoK⁺ATP and the other in association with MPT.

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2 The abbreviations used are: MPT, mitochondrial permeability transition; mitoK⁺ATP, the mitochondrial ATP-sensitive K⁺ channel; CCCP, carbonyl cyanide m-chlorophenylhydrazone; TEA⁺, tetraethylammonium cation; 5-HD, 5-hydroxydecanoic acid; PMA, 12-phorbol 13-myristate acetate; PKC, protein kinase C; PKG, protein kinase G isoform 1α; TPP⁺, tetraphosphonylphosphonium ion; ROS, reactive oxygen species; RACK, receptor for activated C kinase; RR, ruthenium red; Dzx, diazoxide; MPG, N-2-mercaptopropionylglycine.

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Mitochondrial functions. These and other recent findings from our group lead to a preliminary model of the mitochondrial segment of the cardioprotective signaling pathway that inhibits MPT opening and cell necrosis.

MATERIALS AND METHODS

Mitochondrial Isolation—Heart, liver, and brain mitochondria were isolated by differential centrifugation from male Sprague-Dawley rats (220–240 g) exactly as described previously (9). Mitoplasts were prepared by digitonin permeabilization of the outer membrane (10).

Measurement of Light Scattering—Matrix swelling is the standard technique for assaying the MPT. As described previously (11, 12), light scattering changes of mitochondrial suspensions (0.1 mg/ml) were followed at 520 nm and 30 °C and are reported as \( \beta \), which is inverse absorbance normalized for protein concentration, \( Ps \), as shown in Equation 1,

\[
\beta = Ps \times \left( \frac{1}{A} - \frac{1}{A_{in}} \right) 
\]

(Rates of light scattering change, reflecting rates of matrix swelling, were obtained by taking the linear term of a second-order polynomial fit of the light scattering trace, calculated over the initial 2 min following MPT induction by the uncoupler (from 60–180 s after mitochondrial addition). Data in this paper are summarized in bar graphs as “Ca\textsuperscript{2+}-induced swelling rate (%),” which is calculated by taking Ca\textsuperscript{2+}-induced swelling rates in the absence and the presence of 1 \( \mu \)m cyclosporin A as 100 and 0%, respectively. Statistical significance of the difference of the means was assessed using unpaired Student’s t test. A value of \( p < 0.05 \) was considered significant and is indicated in each figure by an asterisk. (All asterisks indicate comparison of that particular mean with Fig. 1B, column Ca\textsuperscript{2+}.)

Assay of MPT—In vitro studies of MPT have traditionally been performed in media containing sucrose, to support irreversible swelling, and lacking Mg\textsuperscript{2+} and ATP, which inhibit MPT (13). However, in order to study interactions among mitoK\textsubscript{ATP}, PKC, and MPT, it was necessary to employ salt media containing Mg\textsuperscript{2+} and ATP. By using these media, we observed that high [Ca\textsuperscript{2+}] caused a robust MPT opening after a variable time lag of 1–3 min duration. Bernardi and co-workers (14) found that the time lag is primarily a population effect, reflecting the fraction of mitochondria that have been recruited at a given time, and they showed that it is possible to synchronize MPT opening by sequential additions of Ca\textsuperscript{2+}, ruthenium red (RR), and CCCP. This protocol was followed in all of the experiments reported here. Mitochondria (0.1 mg of mitochondrial protein/ml) were added to medium at 30 °C (t = 0). CaCl\textsubscript{2} was added at 20 s; RR (0.5 \( \mu \)M, to block further Ca\textsuperscript{2+} uptake) was added at 40 s; and CCCP (250 NM, to initiate MPT) was added at 60 s. Free Ca\textsuperscript{2+} concentrations were calculated using the Free-ware computer program BAD4 (15).

Immunodetection of PKCe—Samples (80 \( \mu \)g) from crude mitochondria, Percoll-purified mitochondria, and mitoplasts made from Percoll-purified mitochondria were precipitated using methanol/chloroform (Ref. 16 with minor modifications) and resolved on 10% SDS-polyacrylamide gels. The gels were electrophoretically transferred onto polyvinylidene difluoride membranes. After blocking with 3% gelatin in Tris-buffered saline, immunoblots were exposed to anti-PKCe antibody (BD Transduction Laboratories) in a 1:500 dilution followed by alkaline phosphatase-conjugated secondary antibody (immunoblot assay kit goat anti-mouse IgG alkaline phosphatase; Bio-Rad). A colorimetric assay was used to visualize antigen-antibody reactions following the manufacturer’s instructions.

Chemicals—Protein kinase G isoform Iα, cGMP, KT5823, Gö6983, and Ro318220 were purchased from Calbiochem. PKC isoform–specific peptides were synthesized by EZ Biosyn (Westfield, IN), according to the published amino acid sequences (17). All other chemicals were from Sigma. The PKG1α concentration and activity used in this study was comparable with that used in our previous paper, and the concentration present in cells (see Ref. 9 and references therein). In the present experiments the enzyme had a specific activity of 10 units/\( \mu \)g (1 unit is the amount of enzyme required to transfer 1 pmol of phosphate from ATP to the synthetic substrate GRT-GRNNSI per min at 30 °C). We used 25 ng/ml, corresponding to 1.5 x 10\textsuperscript{-10} mol/liter. PKG in smooth muscle cells is ~1 x 10\textsuperscript{-7} mol/liter and slightly lower in cardiomyocytes (9).

RESULTS

Regulation of MPT by mitoK\textsubscript{ATP}—A typical set of experiments carried out in K\textsuperscript{+} medium (see “Materials and Methods”) is shown in Fig. 1A. That the Ca\textsuperscript{2+}-dependent swelling (Fig. 1A, trace marked none) is because of MPT opening is confirmed by the fact that it was inhibited by cyclosporin A. The K\textsubscript{ATP} channel opener diazoxide (Dzx) also inhibited MPT opening, and 5-HD (Fig. 1A, Dzx + 5HD) blocked this inhibition. In this and the experiments that follow, we added mitoK\textsubscript{ATP} openers and blockers either before or immediately after the mitochondria and before adding Ca\textsuperscript{2+}.

Fig. 1B summarizes the results of experiments carried out as in Fig. 1A. It can be seen that both selective (diazoxide) and nonselective (cromakalim and nicorandil) mitoK\textsubscript{ATP} channel blockers inhibited MPT opening and that this inhibition was blocked by the mitoK\textsubscript{ATP} blocker 5-HD (18) as well as by the nonselective mitoK\textsubscript{ATP} blocker tetraphenylphosphonium (TPP\textsuperscript{+}) (19). Importantly, valinomycin also inhibited MPT opening at a concentration that increases K\textsuperscript{+} flux to the same extent as a K\textsubscript{ATP} channel opener (20). As expected, this effect of valinomycin was not blocked by 5-HD. We conclude from these results that mitoK\textsubscript{ATP} opening inhibits MPT in isolated heart mitochondria and that the inhibition is a consequence of mitoK\textsubscript{ATP}\textsuperscript{+-}dependent K\textsuperscript{+} influx.

mitoK\textsubscript{ATP} Opening Inhibits MPT via ROS Activation of PKCe—The preceding results raised the following question: How does increased K\textsuperscript{+} uptake into the matrix cause inhibition of MPT? We have shown that net K\textsuperscript{+} influx leads to matrix
alkalinization and thereby to increased ROS production (20–22). It is known that increased ROS activates protein kinases (23); and it has been reported that PKCe inhibits MPT opening in heart mitochondria (5,7). Accordingly, we hypothesized that a PKC may be an intermediate in this process. We investigated the effects of ROS scavengers and PKC inhibitors on mitoK$_{\text{ATP}}$-mediated MPT inhibition, with the results shown in Fig. 2. These data show that diazoxide-mediated inhibition of MPT opening was blocked by the free radical scavenger MPG, by the PKC inhibitors chelerythrine and Ro318220, and by the PKCe-specific inhibitor peptide e$_{V1–2}$ (17). Moreover, MPT inhibition mediated by valinomycin was also inhibited by MPG or e$_{V1–2}$. MPT inhibition by diazoxide was not blocked by the PKCδ-specific inhibitor Gö6983, by the PKCδ-specific inhibitor peptide e$_{V1–2}$ or the scrambled peptide analogue of e$_{V1–2}$ (not shown). On the basis of these findings, we conclude that mitoK$_{\text{ATP}}$-opening inhibits MPT via PKCe which is activated by valinomycin- or mitoK$_{\text{ATP}}$-dependent ROS production.

PKG-mediated MPT Inhibition Occurs via mitoK$_{\text{ATP}}$, ROS, and PKCe—We showed recently that addition of PKG + cGMP to isolated rat heart mitochondria causes PKCe-dependent opening of mitoK$_{\text{ATP}}$ (9). This finding, together with the results contained in Figs. 1B and 2, suggests that PKG + cGMP should inhibit MPT in a mitoK$_{\text{ATP}}$- and ROS-dependent manner. This is indeed the case, as shown in Fig. 3. Inhibition of MPT by PKG + cGMP was blocked by the PKC-specific inhibitor KT85283, by the mitoK$_{\text{ATP}}$ blockers 5-HD, glibenclamide, and TPP$^+$, by the ROS scavenger MPG, and by the PKCe inhibitors chelerythrine and peptide e$_{V1–2}$. PKG-induced MPT inhibition was not blocked by the PKCδ inhibitor Gö6983 or the scrambled peptide analogue of e$_{V1–2}$. Moreover, neither heat-inactivated PKG nor PKG in the absence of cGMP had any effect on MPT (data not shown). From these experiments, we conclude that PKG inhibits MPT via mitoK$_{\text{ATP}}$ opening and that this effect is mediated by PKCe and ROS.

Evidence for Two PKCe, One Upstream and the Other Downstream of mitoK$_{\text{ATP}}$—The finding in Fig. 2 that the peptide e$_{V1–2}$ blocks mitoK$_{\text{ATP}}$ inhibition of MPT leads us to conclude that PKCe is downstream of mitoK$_{\text{ATP}}$; however, the finding that PKCe is required for mitoK$_{\text{ATP}}$ opening by PKG + cGMP...
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(9) implies that PKCe is upstream of mitoK<sub>ATP</sub>. Fig. 4 contains data that address the question of how PKCe can be both upstream and downstream of mitoK<sub>ATP</sub>. In both Fig. 4A (K<sup>+</sup> medium) and Fig. 4B (TEA<sup>+</sup> medium), we see that MPT was inhibited by the PKCe activators PMA and H<sub>2</sub>O<sub>2</sub>. MPT was not inhibited by the inactive αPMA (data not shown). Inhibition by PMA and H<sub>2</sub>O<sub>2</sub> was prevented by the PKCe-specific peptide inhibitor eV<sub>1,2</sub>- but not by glibenclamide (Fig. 4, A and B) or 5-HD (data not shown). Thus, PKCe activation by PMA or H<sub>2</sub>O<sub>2</sub> can inhibit MPT directly, without intervention of mitoK<sub>ATP</sub>. This conclusion is further supported by the fact that PKCe and H<sub>2</sub>O<sub>2</sub> have identical effects in K<sup>+</sup>-based medium and TEA<sup>+</sup>-free medium (Fig. 4A) and TEA<sup>+</sup> medium (Fig. 4B). Note in particular that the peptide eV<sub>1,2</sub>-is still able to block PMA or H<sub>2</sub>O<sub>2</sub> inhibition of MPT in K<sup>+</sup>-free medium, showing that a PKCe exists that can modulate MPT without the intervention of mitoK<sub>ATP</sub>.

The data in Fig. 4A are in apparent disagreement with the findings of Korge et al. (5), who found that 5-HD blocked PMA protection just as it did diazoxide protection. A possible explanation for this result is that mitoK<sub>ATP</sub> was not sufficiently blocked before addition of PMA, and therefore MPT was blocked via the mitoK<sub>ATP</sub>-dependent pathway. We can obtain the same result if we add PMA simultaneously with 5-HD. In our protocols, PMA was added 4 s after 5-HD. Under these conditions, glibenclamide (or 5-HD; data not shown) consistently fails to inhibit the protective effect of PMA or H<sub>2</sub>O<sub>2</sub>, as shown in Fig. 4A. Again, this ability of PMA or H<sub>2</sub>O<sub>2</sub> to inhibit MPT by a mitoK<sub>ATP</sub>-dependent pathway is confirmed by the findings in TEA<sup>+</sup>-medium (Fig. 4B).

In contrast to the results with PMA or H<sub>2</sub>O<sub>2</sub>, PKCe activation by PKG + cGMP (9) or by the pseudo-RACK peptide ψeRACK cannot inhibit MPT directly and requires mitoK<sub>ATP</sub> opening as an intermediate. This is evidenced by the fact that the effects of these agents were abolished by glibenclamide (Fig. 4A) or 5-HD (not shown). (The K<sub>1/2</sub> for ψeRACK in opening mitoK<sub>ATP</sub> was about 0.2 μM, and 2 mM ψeRACK was also ineffective in blocking MPT in the presence of glibenclamide.) These findings suggest that two different PKCe pools are involved in this part of the mitochondrial signaling pathway, one in association with mitoK<sub>ATP</sub> and the other in association with MPT. This conclusion is confirmed by experiments performed in K<sup>+</sup>-free medium (Fig. 4B), in which diazoxide, or PKG + cGMP, or ψeRACK was ineffective in inhibiting MPT.

H<sub>2</sub>O<sub>2</sub>-induced Inhibition of H<sub>2</sub>O<sub>2</sub>-induced MPT Opening—MPT onset in vivo is thought to be an interplay between Ca<sup>2+</sup>, ROS, and the anti-oxidant system (glutathione peroxidase in
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![Graph showing Ca\textsuperscript{2+}-induced swelling rate](image)

FIGURE 5. H\textsubscript{2}O\textsubscript{2} inhibition of H\textsubscript{2}O\textsubscript{2}-induced MPT via PKCe. The effects of low concentrations of H\textsubscript{2}O\textsubscript{2} and other compounds upon MPT induced by two different procedures are shown: (a) our standard procedure involving high concentrations of Ca\textsuperscript{2+} (250 \textmu M total, 100 \textmu M free), and (b) by a combination of low concentrations of Ca\textsuperscript{2+} (83.3 \textmu M total, 2 \textmu M free) + high concentrations of H\textsubscript{2}O\textsubscript{2} (100 \textmu M). Other conditions were as described under "Materials and Methods" and for Fig. 1A. The low "conditioning" dose of H\textsubscript{2}O\textsubscript{2} (2 \mu M) was added immediately before the mitochondria. Other compounds tested were 5-HD (0.3 mM) and the PKCe-specific inhibitor peptide eV\textsubscript{1-2} (0.5 \textmu M), added as indicated. Note that the low conditioning dose of H\textsubscript{2}O\textsubscript{2} inhibited MPT-dependent swelling in both high Ca\textsuperscript{2+} and low Ca\textsuperscript{2+} + H\textsubscript{2}O\textsubscript{2} conditions. Ca\textsuperscript{2+}-induced swelling rate is expressed as % of MPT-dependent rate as described in Fig. 1A. The data are shown as average ± S.D. of at least five independent experiments.

heart mitochondria) (24, 25). In the presence of phosphate, MPT can be elicited with high concentrations of Ca\textsuperscript{2+} and the low amounts of H\textsubscript{2}O\textsubscript{2} that are normally produced by the respiratory chain. In the first two bars of Fig. 5, 100 \textmu M free Ca\textsuperscript{2+} caused MPT opening; however, when 2 \textmu M H\textsubscript{2}O\textsubscript{2} was added prior to the Ca\textsuperscript{2+}, MPT opening was inhibited. MPT can also be induced by a combination of low Ca\textsuperscript{2+} and high H\textsubscript{2}O\textsubscript{2} (24). Fig. 5 shows that 2 \textmu M free Ca\textsuperscript{2+} was insufficient to open MPT, whereas 2 \textmu M Ca\textsuperscript{2+} plus 100 \textmu M H\textsubscript{2}O\textsubscript{2} caused MPT opening. Interestingly, pretreatment with a low concentration (2 \textmu M) of H\textsubscript{2}O\textsubscript{2} inhibited the onset of MPT. The protection afforded by low preconditioning concentrations of H\textsubscript{2}O\textsubscript{2} is mediated by the inhibition by the specific inhibitor peptide eV\textsubscript{1-2} (0.5 \textmu M). Our standard procedure involving high concentrations of Ca\textsuperscript{2+} and low concentrations of H\textsubscript{2}O\textsubscript{2} (100 \textmu M). Other conditions were as described under "Materials and Methods" and for Fig. 1A. The low "conditioning" dose of H\textsubscript{2}O\textsubscript{2} (2 \mu M) was added immediately before the mitochondria. Other compounds tested were 5-HD (0.3 mM) and the PKCe-specific inhibitor peptide eV\textsubscript{1-2} (0.5 \textmu M), added as indicated. Note that the low conditioning dose of H\textsubscript{2}O\textsubscript{2} inhibited MPT-dependent swelling in both high Ca\textsuperscript{2+} and low Ca\textsuperscript{2+} + H\textsubscript{2}O\textsubscript{2} conditions. Ca\textsuperscript{2+}-induced swelling rate is expressed as % of MPT-dependent rate as described in Fig. 1A. The data are shown as average ± S.D. of at least five independent experiments.
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**FIGURE 8.** Reaction sequence leading to inhibition of MPT. The diagram summarizes the biochemical and pathological evidence, presented in this report and in other work (9, 20, 22) (A. D. T. Costa and K. D. Garlid, unpublished work), for the reaction sequence diagrammed in Fig. 9. CHEL, chelerythrine; glib, glibenclamide; VAL, valinomycin.

PKC is subject to complex regulation. In the presence of an anionic phospholipid, such as cardiolipin or phosphatidyl-L-serine, diacylglycerol or PMA is thought to activate the enzyme by promoting flexure at the hinge region causing removal of the pseudosubstrate from the catalytic site (27). ROS stimulate PKCe by causing thiol oxidation to disulfide and the loss of Zn$^{2+}$ (28). Korichnova et al. (23) presented evidence that Zn$^{2+}$ is bound to two zinc fingers in PKC and that PMA/diacylglycerol binding to one or oxidation of the other leads to Zn$^{2+}$ release and activation.

PKC activation may also be associated with translocation to a site where it is anchored to a specific RACK (29). Mochly-Rosen and co-workers (30–32) have identified isozyme-specific activator and inhibitor peptides and hypothesized specific binding sites and consequences to explain their function. We have used $\varepsilon$V$_{1-2}$, a specific inhibitor of PKCe translocation activation, and $\psi$eRACK, a specific activator of PKCe translocation and kinase function (30). These peptides were designed to interact with specific regions of PKCe and alter their function. Thus, the $\psi$eRACK peptide acts as an allosteric agonist by preventing intramolecular autoinhibitory interaction within PKCe, and the $\varepsilon$V$_{1-2}$ peptide blocks substrate access to the catalytic site (31, 32).

**FIGURE 9.** The cardioprotective signaling pathway in mitochondria. In step 1, activated PKG, which cannot cross the outer mitochondrial membrane, phosphorylates a protein at the external surface of the outer membrane. This leads by an unknown mechanism to activation of PKCe1, which is presumably bound to the outer surface of the inner mitochondrial membrane. In step 2, PKCe1 phosphorylates mitoK$_{ATP}$, causing it to open and catalyze net K$^+$ influx (step 3) (9). This leads to matrix alkalinization (20), which causes a modest increase in matrix H$_2$O$_2$ (steps 4 and 5) (22). Increased H$_2$O$_2$ activates PKCe2 (step 6), causing inhibition of MPT opening (step 7) (this paper). We have found that increased H$_2$O$_2$ also activates PKCe1, causing mitoK$_{ATP}$ opening (step 8) and constituting a positive feedback loop. Because this work is not yet published, step 8 is labeled 2. Finally, it is generally accepted that H$_2$O$_2$ acts as a second messenger in cell signaling by activating a variety of kinases (step 9) (33–38).

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**The Role of H$_2$O$_2$—**Our data show for the first time that H$_2$O$_2$ inhibits MPT and that it does so via activation of PKCe (Figs. 4 and 5). H$_2$O$_2$ has been shown to be a signaling molecule (33–38), but its relationship with MPT has previously been that of an inducer, rather than an inhibitor, of MPT opening (24, 25, 39). The important physiological (signaling) role of H$_2$O$_2$ has always been difficult to distinguish from its pathological role. Here we show H$_2$O$_2$ acting as a signaling molecule, protecting against MPT pore opening in isolated mitochondria, at a concentration

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3. M. Jaburek and K. D. Garlid, unpublished data.
at least 50× smaller than that shown to induce MPT pore opening (Fig. 5). The demonstration that prior exposure to a low concentration of H$_2$O$_2$ protects against MPT opening caused by higher levels of H$_2$O$_2$ may be of pathophysiological importance for cardioprotection. Indeed, cardiomyocytes were shown to be protected from simulated ischemia via a H$_2$O$_2$ induction of a pathway that contains PKC$_e$ and mitoK$_{ATP}$ (37).

**Evidence for Two Distinct Mitochondrial PKCes—**PKCe participates in cardioprotection (40–42), and it is known that signaling by this PKCe depends strongly on its location (43–46). In the model of Fig. 8, PKCe1 is associated with and regulates mitoK$_{ATP}$, whereas PKCe2 is associated with and regulates MPT. The hypothesis that two separate PKCs are involved is suggested by the following studies. 1) Activation of PKCe by either PMA or H$_2$O$_2$ opens mitoK$_{ATP}$ and inhibits MPT. $\Delta$$V_{1-2}$ blocks both of these effects, whereas 5-HD and glibenclamide block only mitoK$_{ATP}$ opening and have no effect on MPT inhibition by PMA or H$_2$O$_2$ (Fig. 4A). This shows that PKCe2 can inhibit MPT directly, without the intervention of mitoK$_{ATP}$ (see Fig. 8). 2) The demonstration that PMA or H$_2$O$_2$ inhibits MPT in the complete absence of mitoK$_{ATP}$ activity (TEA$^+$ medium, see Fig. 4B) further demonstrates that PKCe2 acts independently of mitoK$_{ATP}$. 3) Conversely, PKG inhibition of MPT requires mitoK$_{ATP}$ opening (Fig. 4, A and B). Because it is known that PKG opens mitoK$_{ATP}$ by activating a mitochondrial PKCe (9), it follows that this PKC (PKCe1) cannot open MPT directly, i.e. if there were only one PKCe in this pathway, PKG should be able to inhibit MPT without intervention of mitoK$_{ATP}$. These findings are consistent with the conclusion of Ping (46) that PKCe does not function in isolation during cardioprotection, but rather forms close alliances with a variety of other proteins.

**Differential Behavior of $\Psi$eRACK—**Based on the proposed mechanism by which $\Psi$eRACK activates PKCe (29, 30), both PKCs are expected to respond to this peptide. Indeed, both PKCs respond in the predicted manner to the peptide inhibitor $\Delta$$V_{1-2}$. Why then is $\Psi$eRACK able to activate PKCe1 but not PKCe2? A plausible explanation is based on the chemical structure of the two peptides as follows: the ionic state of the $\Psi$eRACK is negative (−1), whereas $\Delta$$V_{1-2}$ is electroneutral. Therefore, it is likely that $\Delta$$V_{1-2}$ can cross the inner membrane and interact with a PKCe located on its inner face, whereas it is virtually impossible for negatively charged $\Psi$eRACK to diffuse across the inner membrane in the face of a large negative membrane potential. Thus, the findings are consistent with the possibility that PKCe1 is located on the exterior face of the inner membrane, whereas PKCe2 is located on the interior face of the inner membrane. The validity of this explanation must await further experiments.

**mitoK$_{ATP}$ Effect on Ca$^{2+}$ Uptake—**The cardioprotective effect of mitoK$_{ATP}$ opening has been shown to be upstream of the onset of MPT (47). The inhibitory effect of mitoK$_{ATP}$ activity on MPT has usually been attributed to decreased Ca$^{2+}$ uptake derived from the slight uncoupling induced by K$^+$ flux into mitochondria (48–50). However, this conclusion has been controversial, and the results of such studies have been variable, perhaps due to the different experimental conditions used. Under the conditions in which we study isolated mitochondria, we showed that pharmacological concentrations of diazoxide induced opening of mitoK$_{ATP}$ without affecting Ca$^{2+}$ uptake or membrane potential (20, 51). Therefore, the results shown herein cannot be attributed to decreased Ca$^{2+}$ uptake into the mitochondrial matrix.

**Summary—**We show that mitoK$_{ATP}$ opening, either by intracellular signaling (9) or by K$_{ATP}$ channel openers, causes inhibition of MPT opening. This may be an in vitro manifestation of a cardioprotective effect, because MPT opening is considered to be the primary cause of necrotic cell death after ischemia-reperfusion (1, 2). Identification of PKCe within these pathways was inferred from the actions of a variety of agonists and antagonists, including peptides that are specific for PKCe. An interesting outcome of these studies is the suggestion of two separate pools of mitoPKCe, emphasizing the additional point that the same PKC isoform may exert effects at multiple locations in the cytosolic and mitochondrial compartments.

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