Mitochondrial PKCϵ and Mitochondrial ATP-Sensitive K^+ Channel Copurify and Coreconstitute to Form a Functioning Signaling Module in Proteoliposomes

Martin Jabůrek, Alexandre D.T. Costa, Jana R. Burton, Cinthia L. Costa, Keith D. Garlid

Abstract—Mitochondria are key mediators of the cardioprotective signal and the mitochondrial ATP-sensitive K^+ channel (mitoK$_{ATP}$) plays a crucial role in originating and transmitting that signal. Recently, protein kinase C ϵ (PKCε) has been identified as a component of the mitoK$_{ATP}$ signaling cascade. We hypothesized that PKCε and mitoK$_{ATP}$ interact directly to form functional signaling modules in the inner mitochondria membrane. To examine this possibility, we studied K^+ flux in liposomes containing partially purified mitoK$_{ATP}$. The reconstituted proteins were obtained after detergent extraction of isolated mitochondria, 200-fold purification by ion exchange chromatography, and reconstitution into lipid vesicles. Immunoblot analysis revealed the presence of PKCε in the reconstitutively active fraction. Addition of the PKC activators 12-phorbol 13-myristate acetate, hydrogen peroxide, and the specific PKCε peptide agonist, ψεRACK, each activated mitoK$_{ATP}$-dependent K^+ flux in the reconstituted system. This effect of PKCε was prevented by chelerythrine, by the specific PKCε peptide antagonist, εV$_{1-2}$, and by the specific mitoK$_{ATP}$ inhibitor 5-hydroxydecanoate. In addition, the activating effect of PKC agonists was reversed by exogenous protein phosphatase 2A. These results demonstrate persistent, functional association of mitochondrial PKCε and mitoK$_{ATP}$. **(*Circ Res*. 2006;99:878-883.)**

Key Words: ATP-sensitive K^+ channel ■ protein kinase Cϵ ■ protein phosphatase ■ reactive oxygen species ■ reconstitution ■ cardioprotection ■ mitochondria

Ischemic preconditioning is a powerful method for reducing ischemia/reperfusion injury in the heart, and it is widely considered that opening of the mitochondrial ATP-sensitive K^+ channel (mitoK$_{ATP}$) plays a crucial role in this process. Cardioprotection by ischemic preconditioning can be mimicked by administering G_i-coupled surface receptor agonists, such as bradykinin, and there has been progress in understanding the mechanisms by which receptor agonists open mitoK$_{ATP}$. Recent findings by Oldenburg et al[1] led those authors to conclude that protein kinase G (PKG) is the terminal cytosolic kinase in this pathway, with the next step being opening of mitoK$_{ATP}$. We then showed that mitoK$_{ATP}$ opening could be induced by adding exogenous PKG+cGMP to isolated heart mitochondria.[2] We also showed that an endogenous, mitochondrial protein kinase C ϵ (PKCε) is an obligatory intermediate between PKG and mitoK$_{ATP}$ in this process.[2] These findings raise the question of PKCε localization in mitochondria. It seemed unlikely that PKCε would be required to probe the entire inner membrane surface area to find its substrate, given that mitoK$_{ATP}$ is a very low abundance protein. Accordingly, we hypothesized that these 2 proteins are joined in a complex.

To examine this possibility, we studied K^+ flux in proteoliposomes containing partially purified mitoK$_{ATP}$. We found that the reconstitutively active fraction contains PKCε, as revealed by immunoblot. We observed that addition of the PKC activators, 12-phorbol 13-myristate acetate (PMA) and H_2O_2, and the specific PKCε peptide agonist, ψεRACK, reversed ATP inhibition of K^+ flux in proteoliposomes reconstituted with mitoK$_{ATP}$. PKCε-dependent mitoK$_{ATP}$ opening was inhibited by the PKC inhibitor chelerythrine, by the specific PKCε peptide antagonist, εV$_{1-2}$, and by the mitoK$_{ATP}$ inhibitor 5-hydroxydecanoate (5-HD). Moreover, the activating effect of PKCε agonists was reversed by exogenous protein phosphatase 2A (PP2A). We conclude that activation of PKCε leads to phosphorylation of mitoK$_{ATP}$, that PKCε is constitutively expressed in mitochondria, and that there is a functional association of mitochondrial PKCε and mitoK$_{ATP}$.

Materials and Methods

Chemicals and Reagents

The PKCε-specific peptides εV$_{1-2}$ (EAVSLKPT) and ψεRACK (HDAPIGYD) were synthesized with stated purity >98% by EZBiolab (Westfield, Ind.), according to the amino acid sequences described by Dorn et al.[3] The PKCε inhibitor peptide control, a scrambled peptide to εV$_{1-2}$ (LSETKPAV), was from Calbiochem (San Diego, Calif.), and the anti-PKCε antibody (clone 21) was from

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From the Department of Biology (M.J., A.D.T.C., J.R.B., C.L.C., K.D.G.), Portland State University, Portland, Ore; and the Department of Membrane Transport Biophysics (M.J.), Institute of Physiology, Academy of Sciences of the Czech Republic, Prague.

Correspondence to Keith D. Garlid, Department of Biology, Portland State University, PO Box 751, Portland, Oregon 97207. E-mail garlid@pdx.edu

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BD Biosciences (San Jose, Calif). PP2A was from Calbiochem (San Diego, Calif) and exhibited a specific activity of 1800 U/mg as supplied. BODIPY-FL-glibenclamide and the potassium binding fluorescent indicator potassium-binding benzofuran isophthalate (PBFI) were from Molecular Probes (Eugene, Ore). All other chemicals were from Sigma-Aldrich Chemical Co (St. Louis, Mo).

Isolation of Mitochondria and Fractionation of Mitochondrial Proteins

Rat liver mitochondria (RLM) were used for these studies. RLM possess the same PKCε -dependent mitoK$_{ATP}$ opening as heart and brain mitochondria,[2] and the yield of RLM is much greater because of the greater amount of starting material. Mitochondria were isolated and purified on a Percoll gradient exactly as previously described.[2] The mitochondrial protein extraction and fractionation followed previously described protocols,[4,5] with modifications. Mitochondria were solubilized at a protein concentration of 5 mg/mL in column buffer containing Triton X-100 (46.5 mmol/L), EDTA (1 mmol/L), β-mercaptoethanol (13 mmol/L), and Tris (50 mmol/L), pH 7.2 adjusted by HCl. Approximately 600 mg of the extracted proteins was loaded on 10 mL of DEAE-cellulose ion exchange column at a flow rate of 0.4 mL/min. The loading of the first 300 mg of protein was followed by washing the column with 2 column bed volumes of column buffer, containing Triton X-100 (3 mmol/L), EDTA (1 mmol/L), β-mercaptoethanol (13 mmol/L), and Tris (50 mmol/L), pH 7.2 adjusted by HCl. The remaining 300 mg of the protein was loaded and the column was washed for 10 hours at a flow rate of 0.1 mL/min with column buffer containing 100 mmol/L KCl. The column was further washed sequentially with column buffer containing 150, 250, and 500 mmol/L KCl, at least 2 column bed volumes each, at a flow rate of 0.4 mL/min. MitoK$_{ATP}$ activity was found in the 250 mmol/L KCl fraction,[4] referred to here as the "active fraction".

Reconstitution and K$^+$ Flux Measurements

Reconstitution of mitoK$_{ATP}$ activity into PBFI-loaded liposomes was performed as described previously.[5] Intraliposomal buffer contained PBFI (150 μmol/L) and tetraethylammonium (TEA$^+$) salts of SO_4 (100 mmol/L), EDTA (1 mmol/L), and Hepes (25 mmol/L), pH 6.8. Kinetic studies were performed in external medium containing $MgCl_2$ (0.5 mmol/L) and K$^+$ salts of SO_4 (80 mmol/L), and Hepes (25 mmol/L), pH 7.2. Electrophoretic K$^+$ flux through mitoK$_{ATP}$ was initiated by FCCP (0.25 μmol/L), which provides charge compensation. Fluorescence changes of the K$^+$-sensitive probe PBFI were monitored using an SLM/Aminco 8000C fluorescence spectrophotometer ($\lambda_{ex}/\lambda_{em}$=345/485 nm), with fluorescence signals calibrated to K$^+$ flux as previously described.[6] Control experiments were performed using an ion exchange chromatography fraction that did not exhibit specific mitoK$_{ATP}$ activity. None of the tested compounds had any effect on these K$^+$ fluxes (data not shown). As is usual, we observed K$^+$ flux in the presence of ATP (see Figure 3), which was about twice as high as background diffusive K$^+$ flux (data not shown). As this level of flux is only observed in the fraction containing mitoK$_{ATP}$ activity, we speculate that it is attributable to insertion of incomplete and unregulatable mitoK$_{ATP}$ channels.

Western Blot Studies

Proteins from RLM (85 μg) and from the active fraction (3 μg to 6 μg) were precipitated using methanol-chloroform ref[7] (with minor modifications) and resolved on 10% SDS-PAGE gels. Proteins separated on the gels were silver stained (Silver Stain Plus, Bio-Rad) or transferred onto polyvinylidene fluoride (PVDF) membranes (Immobilon-P transfer membrane, Millipore). After blocking with gelatin (25 g/L) in Tris-buffered saline, the PVDF mebranes were exposed to anti-PKCε antibody 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 manufacturer's instructions.

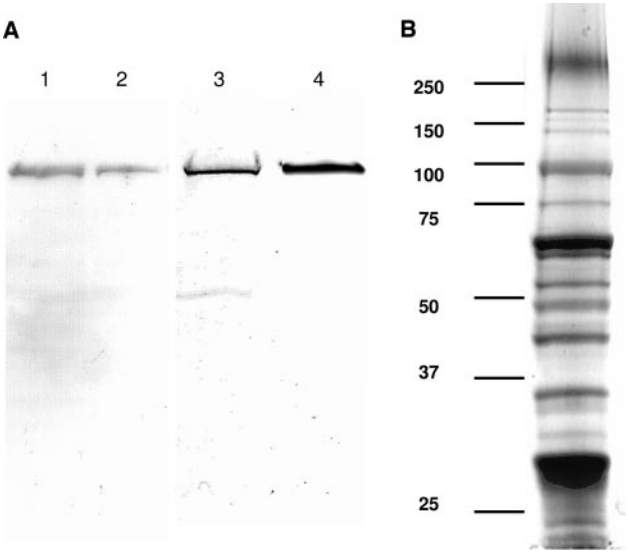

Figure 1. Panel A, Immunoblot using anti-PKCε antibodies on rat liver mitochondria (lane 1); Percoll-purified mitochondria (lane 2); the reconstitutively active fraction (lane 3); and recombinant PKCε (lane 4). Lanes 1 and 2 contained 85 μg proteins, and lanes 3 and 4 contained 6 μg of proteins. Each result is representative of 3 to 4 independent experiments. Panel B, Silver-stained SDS-polyacrylamide gel containing 3 μg of proteins from the reconstitutively active fraction from rat liver mitochondria.

BODIPY-FL-glibenclamide Labeling of the DEAE-Cellulose Fractions

Aliquots of each fraction eluted from the DEAE-cellulose column were incubated with BODIPY-FL-glibenclamide (100 nmol/L) in the presence or absence of unlabeled glibenclamide (1 μmol/L) for 10 minutes at room temperature. Adding a 10-fold excess of unlabeled glibenclamide is not expected to affect nonspecific (low-affinity) labeling, but it is expected to compete at specific (high-affinity) binding sites and to lower BODIPY-FL-glibenclamide labeling at those sites at least 10-fold. The samples were transferred to Costar 24-well ultra-low attachment cluster plates and UV-irradiated for 6 minutes on ice in a UVP CL-1000 Ultraviolet Crosslinker at 200 mJ/cm^2. To remove unbound BODIPY-FL-glibenclamide, the samples were precipitated using trichloroacetic acid and sodium deoxycholate followed by two 5-minute 400 μL acetone washes. The samples were dried and resuspended in TEA-EDTA (1 mmol/L), SDS (17.3 mmol/L), and Tris-HCl (50 mmol/L), pH 7.2. The fluorescence emission spectrum for each sample from 495 to 565 nm was collected using an SLM/Aminco 8000C spectrofluorometer, and analyzed for BODIPY-FL-glibenclamide fluorescence ($\lambda_{ex}/\lambda_{em}$=493/515 nm). For each fraction, the 2 emission spectra, with and without unlabeled glibenclamide, were integrated between 510 and 550 nm. Subtracting the former from the latter and expressing the difference as a ratio over the unlabeled glibenclamide control corrected for small differences in the protein concentration.

Results

Copurification of Mitochondrial PKCε With MitoK$_{ATP}$

Our previous finding that activation of PKCε causes mitoK$_{ATP}$ opening in heart, liver, and brain mitochondria[2] indicated that PKCε is constitutively localized in mitochondria. This was confirmed by immunodetection, as shown in Figure 1A. A clear signal at ≈90 kDa was observed in RLM (lane 1), which persisted after Percoll purification (lane 2). The abundance of mitoK$_{ATP}$ is very low, and efficient regulation would seem to

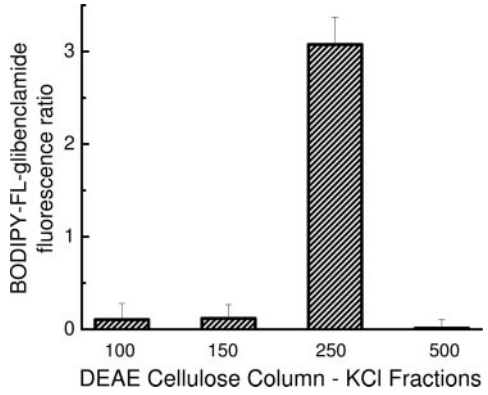

Figure 2. The bar graph is representing high affinity BODIPY-FL-glibenclamide binding in the 100, 150, 250, and 500 mmol/L KCl fractions from DEAE-cellulose ion exchange column. For each fraction, the fluorescence emission spectra in the presence or absence of unlabeled glibenclamide (1 μmol/L) were collected and integrated from 510 to 550 nm. The former was subtracted from the latter and the result is expressed as a ratio of the difference over the unlabeled glibenclamide control. These data depict the means±S.D. for 4 or more independent labeling experiments.

require colocalization of mitoK$_{ATP}$ and PKCε. Accordingly, we investigated whether PKCε and mitoK$_{ATP}$ copurify following detergent extraction and subsequent fractionation using ion exchange column chromatography. The immunoblot shown in lane 3 of Figure 1A was performed on the reconstitutively active fraction containing mitoK$_{ATP}$ and demonstrates the presence of PKCε in this fraction. None of the other fractions from the ion exchange column exhibited a positive reaction with anti-PKCε antibodies (not shown). Essentially identical results were obtained using rat heart mitochondria (n=4, data not shown).

Figure 1B contains an SDS-PAGE gel of the reconstitutively active fraction. Numerous bands were observed on silver staining, as is typical of rat liver mitochondria. Figure 1B shows the band pattern we currently find in the fraction used for reconstitution experiments. The band pattern of the reconstitutively active fraction is similar but not identical to the patterns shown previously by Paucek et al,[4] who used RLM, and by Bajgar et al,[5] who used rat brain mitochondria. We attribute the differences in the observed protein band pattern to the differences in selected tissue and staining procedure, to the modifications in the protein fractionation protocol, which included broader salt step gradient, to a different flow rate of the ion exchange column, and to the fact that a much smaller aliquot was used for the gel in Figure 1B, so that minor bands were emphasized (see Materials and Methods).

To further confirm colocalization of PKCε and mitoK$_{ATP}$, we performed BODIPY-FL-glibenclamide labeling experiments[5] on the column fractions, with the results shown in Figure 2. It can be seen that the fraction containing PKCε ("250" fraction) also exhibited specific labeling with BODIPY-FL-glibenclamide. High affinity glibenclamide binding is one of the ascribed properties of mitoK$_{ATP}$ and these data provide strong evidence that PKCε copurifies with mitoK$_{ATP}$. We shall refer to this pool of PKCε as mitoPKCε.

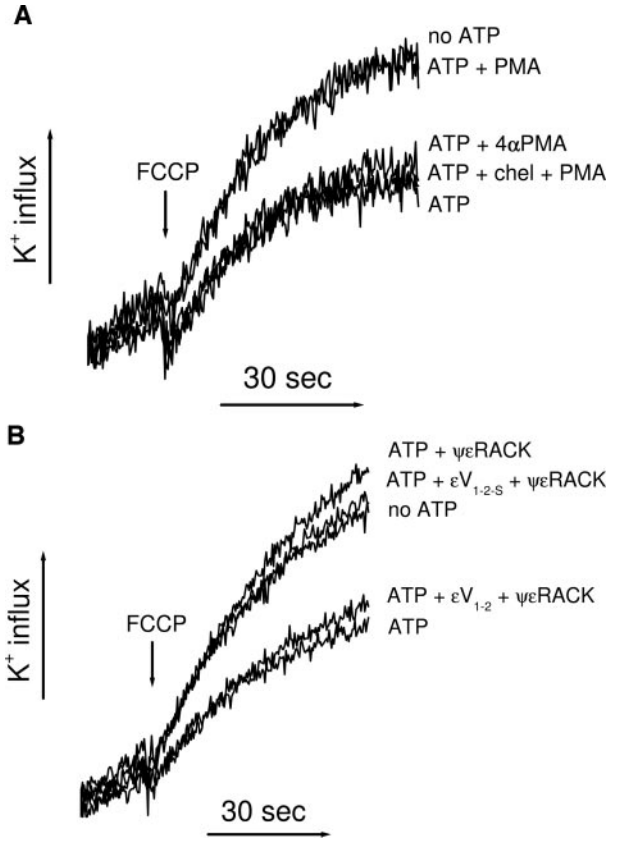

Figure 3. Shown are traces reflecting electrophoretic K^+ influx in proteoliposomes containing reconstituted mitoK$_{ATP}$ as monitored by PBFI fluorescence. Panel A, Addition of 250 nmol/L FCCP caused electrophoretic K^+ influx (no ATP) which was inhibited by 0.2 mmol/L ATP (ATP). The addition of 0.1 μmol/L PMA reversed the ATP inhibition (ATP+PMA). The effect of PMA was inhibited by 50 nmol/L chelerythrine (ATP+chel+PMA). Addition of 0.1 μmol/L of inactive phorbol ester was without effect (ATP+4αPMA). Panel B, Addition of 250 nmol/L FCCP caused electrophoretic K^+ influx (no ATP) which was inhibited by 0.2 mmol/L ATP (ATP). The addition of 1 μmol/L $\psi\varepsilon$RACK reversed the ATP inhibition (ATP+$\psi\varepsilon$RACK). The effect of $\psi\varepsilon$RACK was prevented by 1 μmol/L $\varepsilon V_{1\text{-}2}$ (ATP+$\varepsilon V_{1\text{-}2}$+$\psi\varepsilon$RACK). Addition of 1 μmol/L of scrambled peptide analog was without effect (ATP+$\varepsilon V_{1\text{-}2\text{-}S}$+$\psi\varepsilon$RACK).

Functional Association of MitoPKCε With MitoK$_{ATP}$

We next performed experiments to examine whether the colocalized mitoPKCε and mitoK$_{ATP}$ retained a functional association through detergent extraction, partial purification and reconstitution in liposomes. If this were so, the activation of endogenous PKCε should reverse ATP inhibition of K^+ flux in proteoliposomes as it does in isolated mitochondria.[2] Indeed, this was found to be the case, as shown in Figure 3. In these experiments, K^+ flux in the open state (Figure 3, no ATP) was inhibited by ATP (Figure 3, ATP), as previously shown.[4] Addition of the phorbol ester PMA reversed ATP inhibition so that K^+ flux returned to the control value (Figure 3A, ATP+PMA). Addition of the inactive 4α analogue of PMA did not reverse ATP inhibition (Figure 3A, ATP +4αPMA). The PKC inhibitor, chelerythrine, blocked the activating effect of PMA (Figure 3A, ATP+chel+PMA).

In a similar experiment, addition of the PKCε peptide agonist $\psi\varepsilon$RACK reversed the ATP inhibition (Figure 3B,

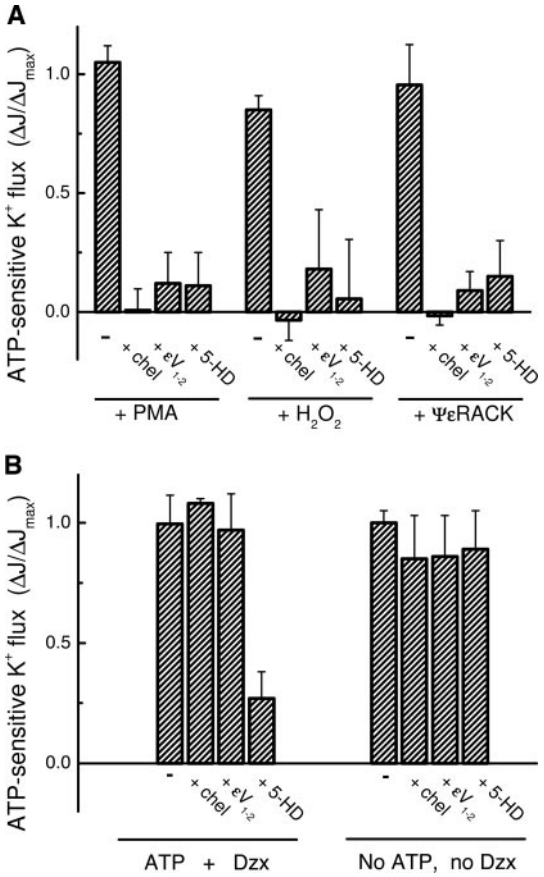

Figure 4. Panel A, The bars summarize experiments examining the effects of PKCε agonists on reconstituted mitoK$_{ATP}$ in the presence of 0.2 mmol/L ATP. The agonists used were PMA (0.1 μmol/L), H_2O_2 (10 μmol/L), and ψεRACK (1 μmol/L). The half-maximal activation because of ψεRACK was ≈0.25 μmol/L (data not shown). Panel B, The bars summarize experiments comparing the effect of PKCε antagonists on K^+ fluxes in the presence of 30 μmol/L diazoxide (Dzx) and 0.2 mmol/L ATP, and the FCCP-induced K^+ fluxes in the absence of ATP. Experiments were performed as described in the legend to Figure 3. The concentrations of chelerythrine, εV$_{1-2}$ and 5-HD were 50 nmol/L, 1 μmol/L, and 0.5 mmol/L, respectively. The half-maximal inhibition by εV$_{1-2}$ was ≈0.3 μmol/L (data not shown). The data±S.D. were obtained from 3 or more independent reconstitutions. ATP-sensitive K^+ flux was calculated as a ratio of rates corrected for the flux in the presence of ATP (ΔJ) and normalized for the maximal ATP-sensitive control rate (ΔJ$_{max}$).

ATP+ψεRACK). Addition of the PKCε-specific peptide antagonist εV$_{1-2}$ blocked the activating effect of ψεRACK (Figure 3B, ATP+εV$_{1-2}$+ψεRACK). The scrambled peptide analog to εV$_{1-2}$ did not block the effect of ψεRACK (Figure 3B, ATP+εV$_{1-2-S}$+ψεRACK) or the effect of PMA (data not shown). Ten-fold higher concentrations of the scrambled peptide analog were also without an effect (data not shown).

The results obtained from 3 or more independent reconstitutions examining the effects on K^+ flux of various PKC agonists and antagonists are summarized in Figure 4A. The addition of the general PKC activators PMA and H_2O_2, as well as the specific PKCε peptide agonist ψεRACK, increased K^+ fluxes in the presence of ATP nearly to the level of control. MitoK$_{ATP}$ opening by PKCε activators was inhibited by both chelerythrine and the PKCε-specific peptide antagonist εV$_{1-2}$. MitoK$_{ATP}$ opening by PKCε activators was also inhibited by the mitoK$_{ATP}$ inhibitor 5-HD. These results show that PKCε and mitoK$_{ATP}$ copurified and remained functionally and physically associated through purification and reconstitution.

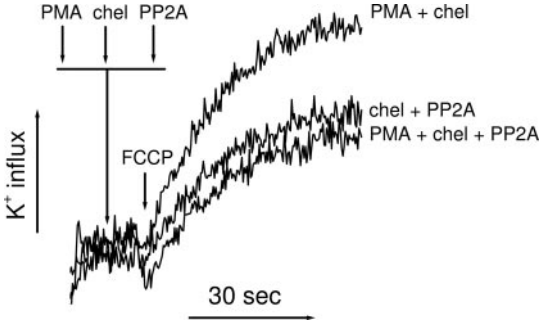

Figure 5. Shown are traces reflecting electrophoretic K^+ influx in proteoliposomes containing reconstituted mitoK$_{ATP}$ as monitored by PBFI fluorescence. The electrophoretic K^+ influx was initiated by the addition of 250 nmol/L FCCP. Addition of 0.2 mmol/L ATP was followed by the addition of 50 nmol/L PMA and, after 10 seconds, the addition of 50 nmol/L chelerythrine (ATP+PMA+chel). The addition of 22 ng/mL exogenous PP2A following the addition of chelerythrine 10 seconds before the addition of FCCP reversed the effect of PMA (ATP+PMA+chel+PP2A). In the absence of PMA, chelerythrine and PP2A had no effect on the ATP-inhibited K^+ flux (ATP+chel+PP2A).

We performed analogous experiments using the cardioprotective pharmacological opener diazoxide.[8] The results of 3 independent reconstitutions are summarized in Figure 4B. As shown previously,[9] ATP inhibition of K^+ flux was reversed by diazoxide, and the diazoxide-opened channel was inhibited by 5-HD. An important point to note in Figure 4B is that the PKC antagonists, chelerythrine and εV$_{1-2}$, had no effect on the K^+ flux induced by diazoxide. Additional control experiments were performed on the proteoliposomes, using the open state of mitoK$_{ATP}$ in the absence of ATP, which should be insensitive to regulation. Figure 4B shows that none of the tested compounds had any significant effect on the FCCP-induced K^+ fluxes observed in the absence of ATP.

Reversal of PKCε-Dependent MitoK$_{ATP}$ Opening By PP2A

Our results are consistent with a mitoPKCε-dependent phosphorylation of mitoK$_{ATP}$ or an intermediate protein that activates mitoK$_{ATP}$. The results in Figure 5 confirm that PKCε-dependent mitoK$_{ATP}$ opening was mediated by a Ser/Thr kinase by demonstrating its reversal with PP2A, a Ser/Thr protein phosphatase. These experiments were performed by adding PMA, chelerythrine, and PP2A sequentially at 10s intervals. PMA reversed ATP inhibition of K^+ flux, as shown in Figure 3. Addition of chelerythrine after PMA had no effect (Figure 5, PMA+chel), showing that chererythrine cannot block the effect of PKCε after phosphorylation has occurred. The subsequent addition of PP2A, 10 seconds before the addition of FCCP, reversed the activating effect of PMA (Figure 5, PMA+chel+PP2A). In the absence of PMA, PP2A had no effect on K^+ flux (Figure 5, chel+PP2A), demonstrating that PP2A had no further effect

on the ATP-inhibited K^+ flux and implying that mitoK$_{ATP}$ as purified is not significantly phosphorylated.

Discussion

The remarkable outcome of these studies is that they show a persistent association of mitoPKCε and mitoK$_{ATP}$ through detergent extraction, ≈200-fold purification by ion exchange chromatography, and reconstitution into proteoliposomes. Specific antibodies recognized PKCε in the same ion exchange column fraction that contains mitoK$_{ATP}$ activity and BODIPY-FL-glibenclamide binding (Figure 1 and 2). The amount of mitoPKCε is small; the immunoblot in Figure 1A was obtained using 1/3 of the entire active fraction from 4 rat livers. These findings indicate that PKCε is constitutively expressed in mitochondria and tightly bound to the inner mitochondrial membrane.

The PKC agonists PMA, H_2O_2, and $\psi\varepsilon$RACK caused mitoK$_{ATP}$ opening in the presence of ATP, and this activation of mitoK$_{ATP}$ was blocked by chelerythrine, εV_{1-2}, and 5-HD (Figure 3 and 4). PKCε-dependent mitoK$_{ATP}$ opening was reversed by the Ser/Thr protein phosphatase 2A (Figure 5). These results support and extend previous studies showing that PKCε-dependent signaling targets mitochondria[10,11] and that PKCε is a part of the mitoK$_{ATP}$ signaling cascade.[2,12–14] Our data demonstrate a functional association between mitoK$_{ATP}$ and PKCε, and suggest their coexistence in a multiprotein signaling complex.

Mochly-Rosen and coworkers[15–17] generated a PKCε-specific peptide agonist, $\psi\varepsilon$RACK, that acts by regulating intramolecular binding, and a PKCε-specific peptide antagonist, εV_{1-2}, that acts by preventing protein-protein interactions between PKCε and its binding protein, called a RACK (receptor for activated C kinase). $\psi\varepsilon$RACK was found to protect cardiac cells from ischemic damage, whereas εV_{1-2} caused a loss of protection. Based on those results, Murriel and Mochly-Rosen[11] concluded that PKCε activation is necessary and sufficient to mediate cardioprotection from ischemic damage.

PKCε requires anionic phospholipids for activity and is activated physiologically by 1 of 2 second messengers, diacylglycerol (DAG) or H_2O_2, or by phosphorylation.[18] The phospholipid requirement for most PKCs is usually met by phosphotidylserine; however cardiolipin, which is very abundant in mitochondria, enhances PKCε activity three- to four-fold compared with phosphatidylserine.[19] To date, most, but not all,[20] PKCs have been found to be translocated from 1 location to another. Indeed, translocation, rather than functional consequence, has been used most commonly as the indicator of PKC activation.[18,21] The mitoPKCε described here is not translocated from location to location, but rather is tightly bound to inner membrane proteins and phospholipids. Nevertheless, our findings can readily be interpreted in the context of the model described by Ron and Mochly-Rosen.[15] In this model, inactive mitoPKCε is bound to a RICK (receptor for inactive C kinase). Addition of $\psi\varepsilon$RACK, DAG (or PMA), or H_2O_2 causes conformational changes that expose the substrate domain on PKCε and cause its binding to its RACK. This step corresponds to translocation from RICK to RACK, which may reside on the same binding protein, and is followed by phosphorylation of the PKC substrate. Addition of PMA or H_2O_2 opens up 1 of the 2 zinc fingers in PKCε.[22,23] It has been suggested that H_2O_2 activates PKCε indirectly, via a protein tyrosine kinase (PTK), and indeed, this has been demonstrated in vitro by Konishi et al.[24] However, the latter work used 5 mmol/L H_2O_2, whereas our activation occurred with a $K_{1/2}$ of ≈0.5 μmol/L. Moreover, mitoK$_{ATP}$ opening via PKCε is insensitive to the PTK inhibitor genistein,[2] arguing against a role for PTK. Accordingly, we suggest that mitoPKCε is activated directly by H_2O_2. Because all 3 agonists cause binding of activated PKCε to its RACK,[17] the model predicts that the binding antagonist εV_{1-2} should block all 3 modes of PKCε activation of mitoK$_{ATP}$,[15] and this indeed was found to be the case in our experiments.

Our data indicate that there is a specific association between mitoK$_{ATP}$ and its regulatory kinase, mitoPKCε, that persists through several steps of purification. Although a functional mitoK$_{ATP}$-PKCε association is demonstrated by this work, the question whether this reflects existence of a structural signaling complex must await identification of the molecular entities responsible for mitoK$_{ATP}$ activity.

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Disclosures

None.

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