Protein Kinase G Transmits the Cardioprotective Signal From Cytosol to Mitochondria

Alexandre D.T. Costa, Keith D. Garlid, Ian C. West, Thomas M. Lincoln, James M. Downey, Michael V. Cohen, Stuart D. Critz

Abstract—Ischemic and pharmacological preconditioning can be triggered by an intracellular signaling pathway in which G_i-coupled surface receptors activate a cascade including phosphatidylinositol 3-kinase, endothelial nitric oxide synthase, guanylyl cyclase, and protein kinase G (PKG). Activated PKG opens mitochondrial K_{ATP} channels (mitoK_{ATP}) which increase production of reactive oxygen species. Steps between PKG and mitoK_{ATP} opening are unknown. We describe effects of adding purified PKG and cGMP on K^+ transport in isolated mitochondria. Light scattering and respiration measurements indicate PKG induces opening of mitoK_{ATP} similar to K_{ATP} channel openers like diazoxide and cromakalim in heart, liver, and brain mitochondria. This effect was blocked by mitoK_{ATP} inhibitors 5-hydroxydecanoate, tetraphenylphosphonium, and glibenclamide, PKG-selective inhibitor KT5823, and protein kinase C (PKC) inhibitors chelerythrine, Ro318220, and PKC-ϵ peptide antagonist $\epsilon V_{1\text{-}2}$. MitoK_{ATP} are opened by the PKC activator 12-phorbol 13-myristate acetate. We conclude PKG is the terminal cytosolic component of the trigger pathway; it transmits the cardioprotective signal from cytosol to inner mitochondrial membrane by a pathway that includes PKC-ϵ. **(*Circ Res*. 2005;97:0-0.)**

Key Words: ATP-sensitive K^+ channel ■ cGMP ■ preconditioning ■ protein kinase C ■ protein kinase G

Ischemic (IPC) and pharmacological preconditioning by ligands such as acetylcholine and bradykinin initiates a signaling cascade that opens mitochondrial ATP-sensitive K^+ channels (mitoK_{ATP}). Many components of this signaling pathway have been identified.[1] Surface receptors activate phosphatidylinositol 3- (PI3-) kinase by transactivation of epidermal growth factor receptors (EGFRs). A signaling complex composed of transactivated EGFR, Src kinase, and PI3-kinase causes phosphorylation of phosphatidylinositol bisphosphate, which in turn activates the phosphatidylinositol-dependent kinases (PDKs).[2] The PDKs then phosphorylate Akt[3] which activates the remainder of the cytosolic signaling pathway (Figure 1) including phosphorylation of endothelial nitric oxide synthase (eNOS), production of NO, stimulation of guanylyl cyclase, generation of cGMP, and activation of protein kinase G (PKG).

cGMP and presumably PKG activation are important during preconditioning. Delayed preconditioning by diazoxide involves NO,[4] and cGMP accumulation after inhibition of cGMP-specific phosphodiesterase by sildenafil induces acute and delayed preconditioning.[5] Direct activation of PKG increases generation of reactive oxygen species (ROS) in cardiomyocytes, and this effect depends on mitoK_{ATP} opening.[3] Whereas PKG antagonists abort ROS generation by PKG activators, they have no effect on ROS triggered by diazoxide, a direct opener of mitoK_{ATP}, indicating PKG is upstream of mitoK_{ATP}. A direct activator of PKG mimics IPC in intact hearts.[6] We hypothesized that PKG opens mitoK_{ATP} by causing its phosphorylation,[7] and that the open channel increases generation of mitochondrial ROS secondary to matrix alkalinization produced by net potassium uptake.[8]

The problem with this hypothesis is that mitoK_{ATP} reside on the inner mitochondrial membrane which would be inaccessible to cytosolic PKG. Perhaps a pool of PKG resides within the mitochondrial intermembrane space and is activated by either NO or cGMP diffusing through the outer membrane. Another possibility is that cytosolic PKG phosphorylates some intermediate on the outer membrane, which initiates a signaling cascade terminating in mitoK_{ATP} opening.

To resolve the question of how the signal proceeds from PKG to mitoK_{ATP}, we assayed mitoK_{ATP} activity in isolated mitochondria using 2 independent techniques: light scattering and respiration. Addition of exogenous PKG and cGMP to mitochondria opened mitoK_{ATP} as seen with diazoxide and cromakalim. PKG-dependent mitoK_{ATP} opening was blocked by 5-hydroxydecanoate (5HD), glibenclamide, and tetraphenylphosphonium (TPP^+), known inhibitors of mitoK_{ATP},[9,10] and chelerythrine, a protein kinase C (PKC) inhibitor. We conclude PKG is the terminal cytosolic member of the protective trigger pathway in heart.

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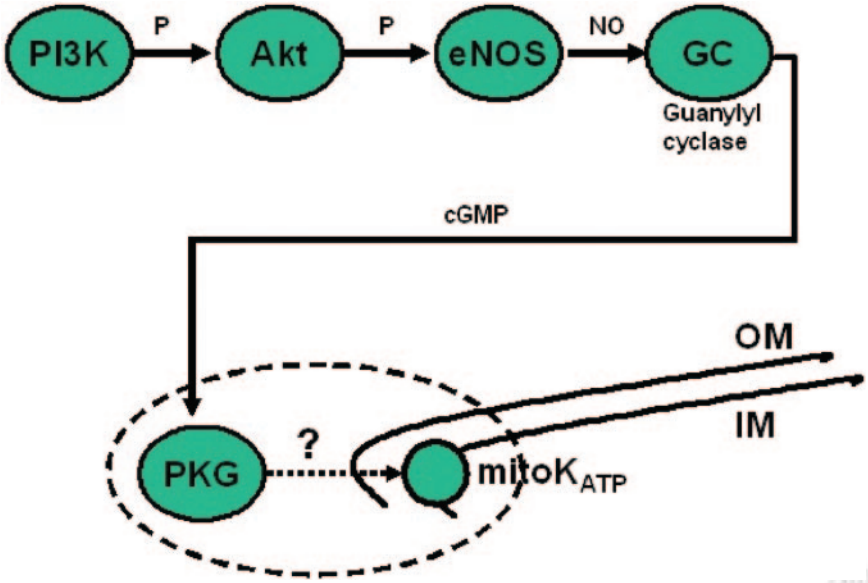

Figure 1. Activation of PI3-kinase leads to phosphorylation of Akt and eNOS. Phosphorylated eNOS produces NO, which stimulates guanylyl cyclase (GC) to produce cGMP. Higher levels of cGMP activate PKG, ultimately leading to opening of mitoK$_{ATP}$ in the mitochondrial inner membrane (IM). The signaling sequence from cytosolic PKG to inner membrane mitoK$_{ATP}$ (dashed oval) is the subject of this investigation. OM indicates outer membrane

Materials and Methods

Mitochondria Isolation

Mitochondria from heart, brain cortex, or liver were isolated by differential centrifugation from CO_2-anesthetized male Wistar rats (200 to 220 g). Time between induction of unconsciousness and beginning of the first centrifugation did not exceed 3 to 4 minutes.

Two hearts were excised, washed in buffer containing 250 mmol/L sucrose, 10 mmol/L HEPES (pH 7.2), and 5 mmol/L K-EGTA, and finely minced in the presence of 1 mg/mL protease (Nagarse, Sigma type XXIV). The minced suspension was then diluted with buffer supplemented with 0.5% fatty acid–free BSA (wt/vol) and homogenized with 4 up-and-down strokes using a rotating Teflon pestle. The homogenate was centrifuged at 1700*g* for 2 minutes, and the resulting supernatant was spun at 9000*g* for 5 minutes. The pellet was resuspended in the initial sucrose buffer devoid of BSA and centrifuged at 2300*g* for 2 minutes. The supernatant was finally spun at 9000*g* for 5 minutes to collect mitochondria. The final pellet was resuspended in the initial buffer devoid of K-EGTA. Percoll-purified rat heart mitochondria were prepared by resuspending the pellet from the first 9000*g* centrifugation in buffer devoid of BSA followed by centrifugation at 40 000*g* for 30 minutes in a self-generated 26% Percoll gradient. The yield was 4 to 6 mg protein/heart for non-Percoll purified and 2 to 3 mg protein/heart for Percoll-purified mitochondria.

Brain mitochondria were isolated from cerebral cortex of 2 rats, using slight modifications of the protocol described by Rosenthal et al.[11] Tissue was finely minced in buffer containing 220 mmol/L mannitol, 75 mmol/L sucrose, 10 mmol/L HEPES (pH 7.2), and 1 mmol/L K-EGTA supplemented with 0.5 mg/mL protease (Nagarse, Sigma type XXIV). The suspension was homogenized with 6 strokes of a loose and 6 strokes of a tight glass pestle. The homogenate was diluted 3× with buffer supplemented with 0.5% fatty acid–free BSA (wt/vol) and centrifuged at 2000*g* for 3 minutes. The supernatant was spun at 12 000*g* for 5 minutes and the pellet resuspended in the initial buffer devoid of BSA. To disrupt synaptosomal vesicles, digitonin was added to 10% and the suspension centrifuged for 2 minutes at 2500*g*. The supernatant was spun at 12 000*g* for 5 minutes and the pellet washed 2× with mannitol-sucrose buffer without BSA or EGTA. The yield was typically 6 to 9 mg protein per brain.

Liver mitochondria were isolated from 1 rat as previously described.[12] The yield was typically 60 to 80 mg protein per liver. Mitochondrial protein concentration was estimated using the Biuret reaction.[13]

Light Scattering and Oxygen Consumption Measurements

Changes in mitochondrial matrix volume were followed using a quantitative light-scattering technique. Within well defined limits, matrix volume is linearly related to the reciprocal of the apparent absorbance of mitochondrial suspensions, when corrected for the extrapolated value at infinite protein concentration.[12,14] Light scattering changes of mitochondrial suspensions (0.1 mg/mL) were followed at 520 nm and 30°C and reported as β defined as

$$\beta = P(1/A - 1/A4)$$

where P is the protein concentration in the assay, and 1/A4 is the reciprocal of the apparent absorbance at infinite protein concentration. Data are summarized in bar graphs as "% volume change at 120 sec" given by

$$100 \times [\beta(x) - \beta(ATP)]/[\beta(0) - \beta(ATP)]$$

where $\beta(x)$ is the observed value at 120 s under the given experimental condition, and $\beta(ATP)$ and $\beta(0)$ are observed values at 120 s in the presence and absence of ATP, respectively.

Oxygen consumption was measured with a Clark-type electrode (Yellow Springs Instruments) in a temperature-controlled chamber. Oxygen concentration was taken to be 440 ng atom O/mL at 30°C.

The assay medium for light scattering and respiration studies contained KCl (120 mmol/L), HEPES (pH 7.2; 10 mmol/L), EGTA (0.1 mmol/L), succinate (10 mmol/L), $MgCl_2$ (0.5 mmol/L), phosphate (5 mmol/L), rotenone (5 μmol/L), and oligomycin (0.67 μmol/L). Some experiments were conducted using K^+-free medium in which TEA-Cl was substituted for KCl. Osmolality ranged between 275 to 280 mOsm.

Chemicals and Reagents

Most chemicals were from Sigma Chemical Co. KT5823, TPP^+, and cpt-cGMP were from Calbiochem. HMR1098 was a gift of Dr Heinrich Englert of Sanofi Aventis. Cyclic GMP protein kinase 1α (PKG) was purified from bovine lung.[15] PKC isozyme-specific peptide antagonists $\epsilon V_{1\text{-}2}$ (EAVSLKPT)[16] and $\delta V_{1\text{-}1}$ (SFNSYELGSL)[17] were synthesized with a purity >98% by EZBiolab (Westfield, Ind).

Results

PKG-Induced Mitok$_{atp}$ Opening in Isolated Heart Mitochondria

Figure 2 contains representative traces from light-scattering experiments on rat heart mitochondria. Mitochondrial matrix

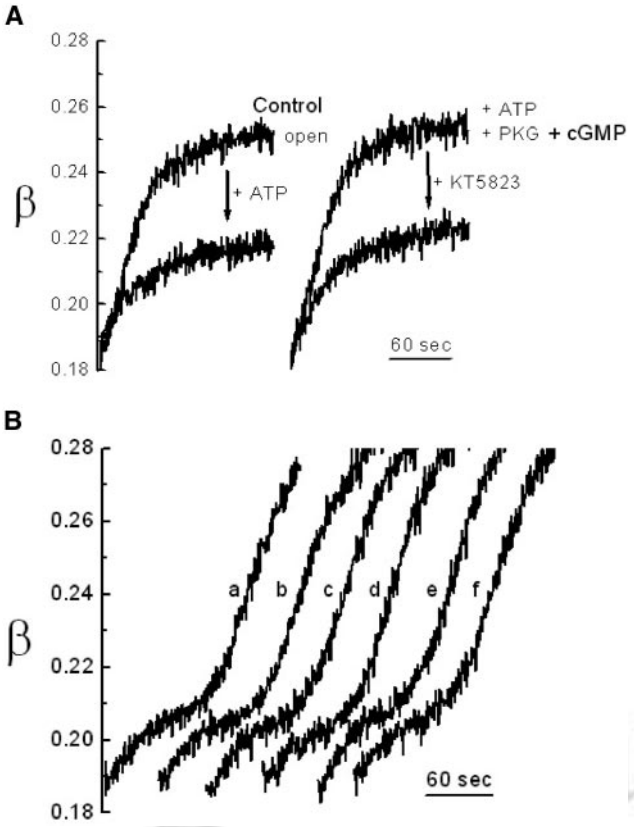

Figure 2. The light-scattering parameter "β," an index of matrix volume, is plotted vs time. Assay contained 0.1 mg/mL of rat heart mitochondria. A, K^+ medium. Medium contained 0.2 mmol/L ATP, except in "control," which contained no ATP. ATP alone (ATP) inhibited swelling, whereas PKG (50 ng/mL)+cGMP (10 μmol/L) (ATP+PKG+cGMP) reversed inhibition by ATP, and this effect was blocked by the PKG antagonist KT5823. The traces are representative of 15 independent experiments. B, TEA^+ medium. Each assay medium contained ATP (0.2 mmol/L), PKG (50 ng/mL), cGMP (10 μmol/L), and the following additions: a, no further addition; b, 0.3 mmol/L 5-hydroxydecanoate; c, 1 μmol/L KT5823; d, 0.1 μmol/L chelerythrine; e, 0.5 μmol/L Ro318220; f, 0.5 μmol/L Gö6983. The conditions were the same as those for K^+ medium, except that TEA^+ was substituted for K^+. The agents had no effect in the absence of K^+. The delayed swelling beginning at ≈100 s occurs when all matrix K^+ is lost via K^+/H^+ antiport, and TEA^+ uptake is uncompensated by K^+ efflux. The traces are representative of 4 independent experiments.

swelling in K^+ medium caused by respiration-driven uptake of K^+ and accompanying anions and water was inhibited by addition of ATP (0.2 mmol/L) as previously described[9,18–20] (Figure 2A). ATP inhibition of swelling was reversed by PKG (50 ng/mL)+cGMP (10 μmol/L), resulting in a light scattering curve essentially identical to that recorded in absence of ATP (Control). This amount of PKG used after initial experiments showed that doubling its concentration did not further increase mitochondrial swelling. PKG was highly purified with a specific activity of ≈5 U/μg (1 unit is the amount of enzyme required to transfer 1 pmol of phosphate from ATP to Histone II-A in 1 minute), comparable to the highest activity previously reported.[15] PKG in smooth muscle cells is $\approx 1\times10^{-7}$ mol/L and lower in cardiomyocytes.[21] Fifty ng/mL of exogenous PKG is $\approx 3\times10^{-10}$ mol/L, somewhat

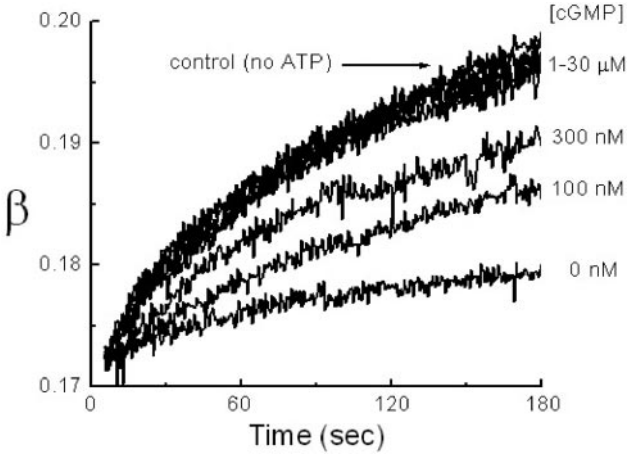

Figure 3. Light-scattering traces of isolated heart mitochondria (0.1 mg/mL) during incubation in K^+ medium containing 0.2 mmol/L ATP, 50 ng/mL PKG, and the indicated concentration of cGMP (0 to 30 μmol/L). The EC_{50} for cGMP-induced opening of $mitoK_{ATP}$ was ≈280 nM in this experiment and 240 nM in a second identical experiment.

less than that seen in whole cardiomyocytes and, therefore, physiologically relevant.

Closing $mitoK_{ATP}$ with 5HD (data not shown) or blocking PKG with KT5823 (1 μmol/L) (Figure 2A) prevented changes in mitochondrial volume induced by PKG+cGMP+ATP. Neither PKG nor cGMP alone nor KT5823 had any effect. Hence exposing mitochondria to activated PKG caused immediate opening of $mitoK_{ATP}$.

PKG+cGMP failed to affect mitochondrial matrix volume in K^+-free TEA^+ medium (Figure 2B, curve a) or in medium containing Li^+ (data not shown), confirming K^+ was the relevant transported ion. Moreover, addition of either 5HD (b), KT5823 (c), or PKC antagonists chelerythrine (d), Ro318220 (e), or Gö6983 (f) had no effect on light scattering in TEA^+ medium. These controls demonstrate K^+ specificity and exclude unwanted drug actions, such as inhibition or uncoupling of respiration. These effects can be readily detected in non-K^+ media, and these controls are essential to avoid misinterpretation of results obtained in K^+ medium. No drugs at the concentrations used in the present study affected mitochondrial respiration. Figure 2B illustrates an important point about regulation of mitochondrial volume.[22] Mitochondria placed in K^+-free TEA^+ medium initially maintain steady-state volume regulation because TEA^+ leakage into the matrix is compensated by K^+ efflux effected by the K^+/H^+ antiporter. After ≈100 s matrix potassium is exhausted, and TEA^+ uptake can no longer be balanced by K^+ extrusion. Rapid swelling then begins as water is osmotically drawn into the matrix by TEA^+.

We further characterized the effect of PKG on $mitoK_{ATP}$ opening by measuring changes in mitochondrial volume as the concentration of the cofactor cGMP of the enzyme was varied in the presence of ATP (Figure 3). PKG in the absence of cGMP had no effect on mitochondrial swelling, thus excluding any nonspecific effect of the protein itself. Additionally, heat-inactivated PKG had no effect on mitochondrial swelling (data not shown). Stepwise increases of cGMP concentration up to ≈1 μmol/L was accompanied by a

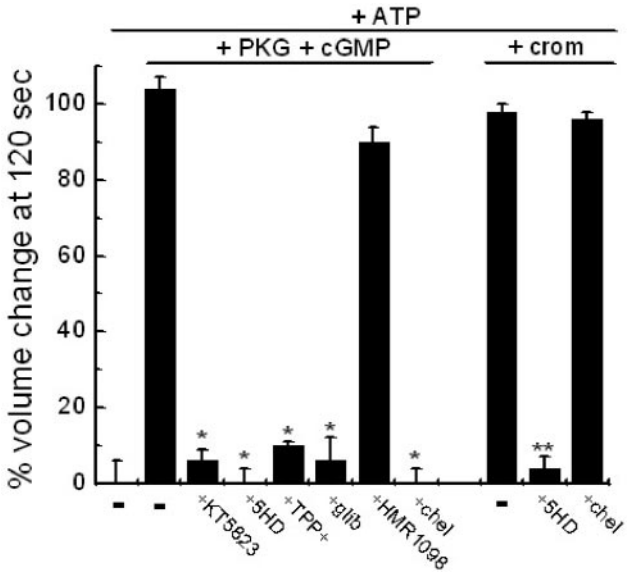

Figure 4. Percent volume change at 120 sec in K^+ medium in the presence of 0.2 mmol/L ATP (+ATP). One set of experiments was performed with 50 ng/mL PKG+10 μmol/L cGMP (+PKG+cGMP) and another with 100 μmol/L cromakalim (+crom). Means±SD of values were obtained from 8 independent experiments. Chel indicates chelerythrine; glib, glibenclamide; 5HD, 5-hydroxydecanoate; TPP^+, tetraphenylphosphonium. *P<0.001 vs PKG+cGMP; **P<0.001 vs crom

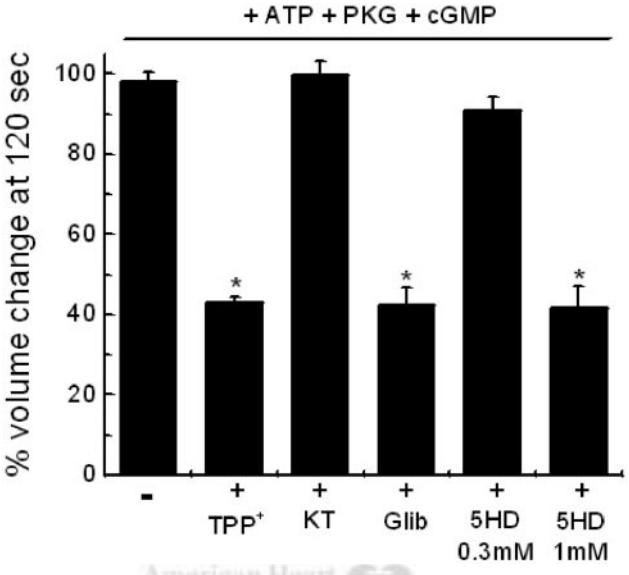

Figure 5. Percent volume change at 120 s as in Figure 4 except K_{ATP} channel openers and inhibitors were introduced 45 s after mitochondria were added to a solution containing PKG, cGMP, and ATP. 5HD (300 μmol/L, the dose in experiments of Figure 4) was unable to inhibit mitochondrial swelling under these conditions, but 1 mmol/L 5HD did. Mean±SD of data were obtained from 4 independent experiments. Chel indicates chelerythrine; glib, glibenclamide; 5HD, 5-hydroxydecanoate; TPP^+, tetraphenylphosphonium; KT, KT5823. *P<0.001 vs ATP+PKG+cGMP

progressive increase in rate and magnitude of mitochondrial swelling. The EC_{50} value for cGMP was 200 to 300 nM (2 experiments), which is in accord with the reported K_{act} of ≈200 nM for PKG1α.[23] We used 10 μmol/L cGMP in remaining experiments.

Inhibitors of PKG-Induced MitoK$_{ATP}$ Opening

PKG +cGMP-induced mitochondrial swelling, and hence mitoK$_{ATP}$ opening, was blocked by the specific PKG inhibitor KT5823 (1 μmol/L) (Figure 4). It was also blocked by 3 chemically diverse mitoK$_{ATP}$ blockers[9,10]: 5HD (300 μmol/L), glibenclamide (50 μmol/L), and TPP^+ (0.5 μmol/L). However, the PKG effect was not blocked by HMR1098 (15 μmol/L), which is a blocker of sarcoplasmic K_{ATP}.[24]

The PKC inhibitor chelerythrine (0.1 μmol/L) also blocked mitoK$_{ATP}$ opening by PKG, implying endogenous PKC is present in isolated mitochondria and PKC is an intermediate in the mitochondrial signaling pathway leading to mitoK$_{ATP}$ opening. 5HD inhibited direct mitoK$_{ATP}$ opening by cromakalim (100 μmol/L), whereas chelerythrine did not. The findings in Figure 4 were potassium-specific; no agent had any effect on light scattering when mitochondria were respiring in TEA^+ medium (data not shown). TPP^+, HMR 1098, and chelerythrine had no effect on K^+ flux when added in the absence of a mitoK$_{ATP}$ opener. Cromakalim had no effect in the absence of ATP. Furthermore, mitoK$_{ATP}$ are completely insensitive to 5HD and glibenclamide when the open state of the channel is induced by the absence of ATP and Mg^{++}, but are keenly sensitive when the channels are pharmacologically opened by diazoxide.[9] Nonspecific effects of glibenclamide on cation flux could only be demonstrated at either very high drug concentrations or under unphysiological conditions.

State-Dependent Inhibition of PKG-Induced MitoK$_{ATP}$ Opening

Data in Figure 4 were obtained by adding inhibitors to the assay medium before PKG. A different picture emerged when inhibitors were added after a brief (45 s) incubation with PKG+cGMP (Figure 5). As expected, KT5823 was unable to block mitochondrial swelling after PKG+cGMP had presumably exerted its effects. TPP^+, which acts on the potassium channel subunit of mitoK$_{ATP}$,[10] and glibenclamide, which targets the sulfonylurea receptor subunit,[20] inhibited the activated channel. However, delayed addition of 5HD did not block mitoK$_{ATP}$ opening until its concentration was increased to 1 mmol/L. This suggests a dependency of the inhibitory action of 5HD on the type of open state of mitoK$_{ATP}$.[9]

PKG+cGMP Stimulates Respiration by Opening MitoK$_{ATP}$

We assayed effects of PKG on respiration (Figure 6). ATP inhibits respiration in K^+ medium by blocking mitoK$_{ATP}$, which reduces futile cycling of potassium.[25] PKG+cGMP reversed inhibition of ATP, and this effect was blocked by 5HD and chelerythrine, confirming results of light-scattering experiments. Increased respiration attributable to diazoxide (30 μmol/L)-induced mitoK$_{ATP}$ opening was blocked by 5HD but not by chelerythrine. This finding, also observed in the light scattering assay (Figure 4), demonstrates that PKC is not an intermediate step in the direct opening of mitoK$_{ATP}$ by K_{ATP} channel openers. Increased respiration attributable to the K^+ ionophore valinomycin (1 pmol/mg) was not blocked by 5HD. From these data K^+ flux associated with PKG-induced mitoK$_{ATP}$ opening is calculated to be 30 to 40 nmol·min^{-1}·mg protein^{-1}, similar to results obtained with diazoxide.[25] None

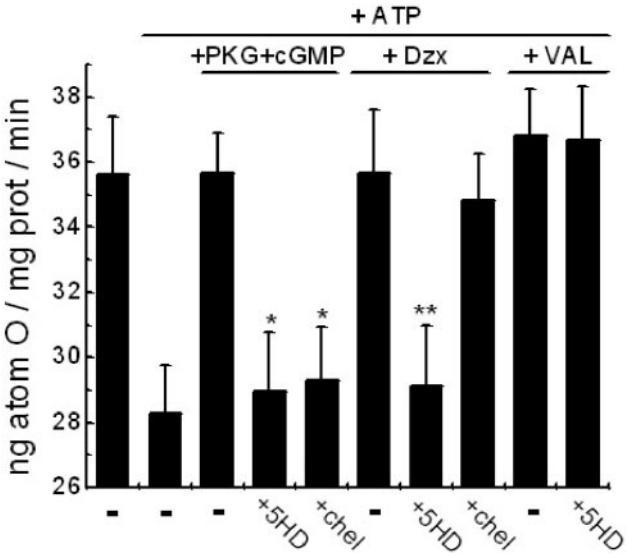

Figure 6. Rates of oxygen consumption by heart mitochondria (0.25 mg/mL) incubated in K^+ medium. The open-state rate was obtained in the presence of rotenone and oligomycin. ATP (0.2 mmol/L) and either PKG+cGMP, 5-hydroxydecanoate (5HD), diazoxide (Dzx), valinomycin (VAL), or chelerythrine (chel) were added at the beginning of each experiment. Mean±SD of data were obtained from 4 independent experiments for each condition. *$P<0.001$ vs PKG+cGMP; **$P<0.001$ vs Dzx

of these effects were observed when TEA^+ was substituted for K^+ (data not shown), similar to observations when Li^+ was exchanged for K^+.[25] In the absence of ATP, diazoxide had no effect on mitochondrial oxygen consumption.

Evidence That a Specific PKC Isoform Is Required to Open MitoK_{ATP}

Because chelerythrine blocked mitoK_{ATP} opening induced by PKG+cGMP (Figures 4 and 6), PKC must reside in the pathway transmitting the signal to inner membrane mitoK_{ATP}. In the presence of ATP the nonselective PKC activator 12-phorbol 13-myristate acetate (PMA; 0.3 μmol/L) was able to open mitoK_{ATP} in isolated heart mitochondria (Figure 7A and 7B) to the same extent observed with cromakalim or PKG (Figure 4). PMA-induced mitoK_{ATP} opening was inhibited by 5HD (Figure 7A), TPP^+ (Figure 7A), glibenclamide (Figure 7A), and chelerythrine (Figure 7B), but not by HMR1098 (Figure 7A). Thus PMA induces mitoK_{ATP} opening with the same inhibitor sensitivity as seen with PKG+cGMP or K_{ATP} channel openers.

We examined the effects of 2 other PKC inhibitors which have some selectivity for various PKC isozymes, namely Ro318220 and Gö6983.[26,27] Ro318220 (0.5 μmol/L) blocked PMA-induced mitoK_{ATP} opening, whereas Gö6983 (0.5 μmol/L) had no effect (Figure 7B). The tyrosine kinase inhibitor genistein (5 μmol/L) was also without effect. In view of these findings, we examined the effects of these agents on PKG-induced mitoK_{ATP} opening. Channel opening was also inhibited by Ro318220 but not by Gö6983 (Figure 7B).

Because neither Ro318220 nor Gö6983 is isozyme-specific, we also used 2 highly specific synthetic peptides developed by Mochly-Rosen and colleagues[16,17] that block the activity of individual PKC isozymes. We selected antagonists to the ε and δ isozymes because both are involved in the signal transduction pathway of IPC.[17,28,29] As seen in Figure 7B, $\epsilon V_{1\text{-}2}$ (0.5 μmol/L) blocked mitoK_{ATP} opening and mitochondrial swelling triggered by PMA+ATP, whereas the δ isozyme antagonist, $\delta V_{1\text{-}1}$ (0.2 μmol/L), had no effect. Identical observations were made when PKG+cGMP was substituted for PMA (Figure 7B). Of note, none of the PKC antagonists, $\epsilon V_{1\text{-}2}$, $\delta V_{1\text{-}1}$, Ro318220, or Gö6983, had any effect on light scattering in the absence of either PMA or PKG+cGMP.

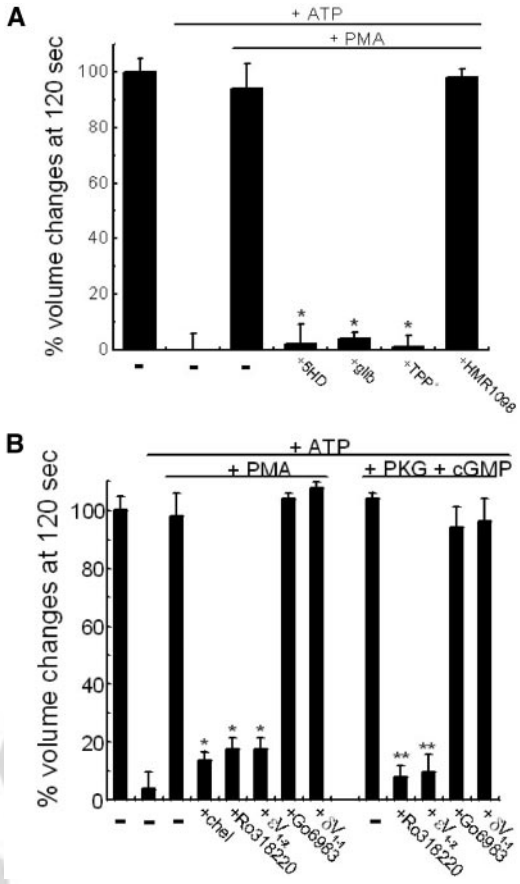

Figure 7. Percent volume change at 120 s in the presence of 0.2 mmol/L ATP (+ATP) and either 0.3 μmol/L 12-phorbol 13-myristate acetate (+PMA) or 50 ng/mL PKG+10 μmol/L cGMP (PKG+cGMP). A, Effects of K_{ATP} channel blockers. *$P<0.001$ vs PMA. B, Effects of protein kinase inhibitors. *$P<0.001$ vs PMA; **$P<0.001$ vs PKG+cGMP. Mean±SD of data were obtained from 4 independent experiments for each condition. Chel indicates chelerythrine; glib, glibenclamide; 5HD, 5-hydroxydecanoate; TPP^+, tetraphenylphosphonium.

These data demonstrate a critical role played by PKC in transmitting a signal from activated PKG to mitoK_{ATP}. However, they do not indicate where PKC is physically located. Baines et al[30] showed PKC-ε is constitutively present in heart mitochondria. However, the foregoing findings do not exclude the possibility that cytosolic PKC could be loosely docked on the outer mitochondrial membrane where it remained throughout the isolation procedure. Mitochondrial purification on a Percoll gradient would be expected to strip any loosely bound PKC from the outer membrane. We obtained identical results in Percoll-purified mitochondria,

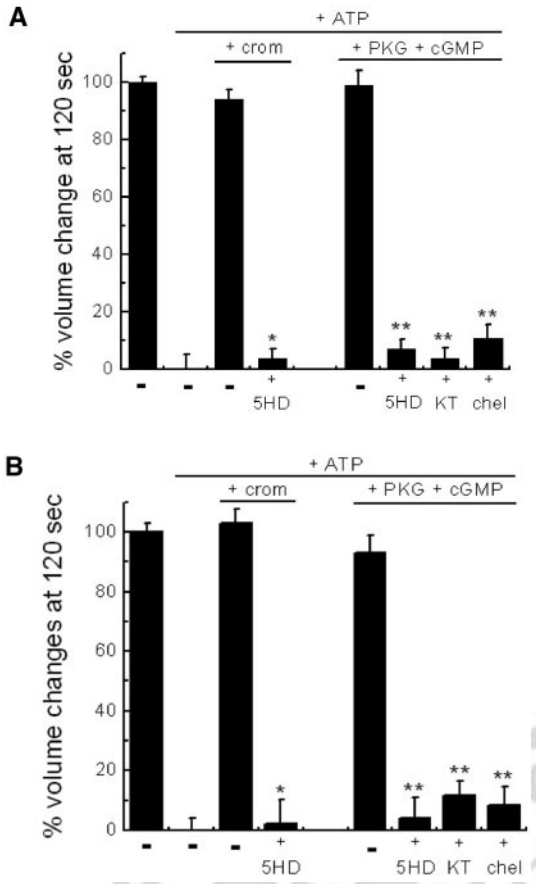

Figure 8. % Volume change at 120 sec. A, Rat brain mitochondria. B, Rat liver mitochondria. Experiments were performed in the presence of 0.2 mmol/L ATP (+ATP) and 100 μmol/L cromakalim (+crom) or 50 ng/mL PKG+10 μmol/L cGMP (PKG+cGMP). Mean±SD of values were obtained from 4 independent experiments for each panel. Chel indicates chelerythrine; glib, glibenclamide; 5HD, 5-hydroxydecanoate; TPP^+, tetraphenylphosphonium; KT, KT5823. *$P<0.001$ vs crom; **$P<0.001$ vs PKG+cGMP

indicating PKC was either very tightly bound or more likely was localized to the intermembrane space. Indeed, none of the effects reported here were affected by Percoll purification.

PKG-Induced MitoK_{ATP} Opening in Brain and Liver Mitochondria

We examined whether mitoK_{ATP} from other organs is also sensitive to PKG+cGMP. We observed mitoK_{ATP} opening by PKG+cGMP in rat brain (Figure 8A) and liver (Figure 8B) mitochondria. As seen in heart mitochondria (Figure 4), mitoK_{ATP} opening caused by PKG+cGMP was similar in magnitude as that induced by cromakalim. Moreover, KT5823, 5HD, and chelerythrine had identical inhibitory effects as those observed in heart mitochondria. TEA^+ controls confirmed that these effects in brain and liver mitochondria were specific for K^+ medium.

Discussion

Hearts exposed to brief ischemia become resistant to infarction during a subsequent period of ischemia (IPC). Activation of PKG triggers the preconditioned state by opening mitoK_{ATP}.[3] However, it remained unclear how PKG mediated mitoK_{ATP} opening (dashed oval, Figure 1).

We reconstituted the PKG–mitoK_{ATP} interaction in isolated mitochondria. Mitochondria suspended in K^+ medium swell in response to PKG+cGMP (Figure 2). Swelling is a robust indicator of opening of mitoK_{ATP}[9,18–20,25]: PKG+cGMP cause this channel to open. KT5823, a specific inhibitor of PKG, blocked mitochondrial swelling induced by PKG+cGMP, suggesting a phosphorylation-dependent process requiring active PKG is involved. Interestingly, the inhibition of matrix swelling by KT5823 could be reversed by addition of the direct mitoK_{ATP} opener diazoxide (data not shown).

Addition of both exogenous PKG and cGMP was required to trigger mitochondrial swelling. Exogenous PKG in the absence of cGMP caused no significant mitochondrial swelling. As cGMP levels were increased, however, a dose-dependent increase in mitochondrial swelling was observed up to ≈1 μmol/L cGMP, consistent with levels of cGMP required for activation in vitro.[15] Furthermore, maximal swelling was comparable to that seen in the absence of ATP (Figure 2A) or in the presence of cromakalim (Figure 4), an indication that mitoK_{ATP} channels were maximally activated by PKG+cGMP. In a previous study[15] the concentration of cGMP required to maximally activate purified PKG was 200 nM. That study, however, was performed with purified proteins in a test tube, whereas the present investigation examined the response of isolated mitochondria. Therefore, it is not surprising that higher concentrations of cGMP were needed to achieve maximal mitochondrial swelling. Maximal activation of PKG was observed at a dose of 10 μmol/L cGMP in rabbit cardiomyocytes.[31]

PKG-induced swelling was also inhibited by glibenclamide, a nonselective K_{ATP} antagonist, and 5HD and TPP^+, selective mitoK_{ATP} blockers, confirming PKG caused swelling by opening mitoK_{ATP}. In contrast, HMR1098, a surface K_{ATP} channel blocker, was ineffective, indicating the effect of PKG on mitochondrial swelling is specifically the result of its action on mitoK_{ATP}. Glibenclamide,[32] 5HD,[32] and TPP^+ (Garlid and Rissa, unpublished observation), also block protection by IPC, whereas HMR 1098 does not.[33,34]

Closing mitoK_{ATP} with ATP depressed mitochondrial respiration (Figure 6), whereas direct opening of mitoK_{ATP} with diazoxide reversed the inhibitory effect of ATP. 5HD blocked the rescue by diazoxide, although it had no effect on the stimulation of oxygen consumption by valinomycin. Thus, mitochondrial respiration was intimately influenced by the state of mitoK_{ATP}. PKG+cGMP reversed the inhibitory effect of ATP on respiration, and this rescue was blocked by 5HD. Hence, the 2 very different assays, swelling and oxygen consumption, produced concordant results.

The effects of PKG were blocked by chelerythrine (Figures 4 and 6), whereas chelerythrine had no effect on mitoK_{ATP} opening caused by cromakalim (Figure 4). Furthermore, PMA was equally effective in opening mitoK_{ATP} (Figure 7A and 7B) as cromakalim or PKG (Figure 4), and this effect was abolished by mitoK_{ATP} channel blockers (Figure 7A) as well as by chelerythrine (Figure 7B). Hence endogenous PKC mediates the effect of PKG on mitoK_{ATP}. An intimate connection between PKC and mitoK_{ATP} was suggested by Sato et

al[35] in cardiomyocytes and Wang et al[36,37] in Langendorff-perfused hearts. Korge et al[38] showed that PMA elicits mitoK$_{ATP}$ activity in isolated mitochondria, and Baines et al[30] demonstrated that PKC-ϵ is constitutively localized within the mitochondrial compartment. Our results are entirely consistent.

We tried to identify the specific PKC isozyme that was responsible for mitoK$_{ATP}$ opening using PKC antagonists which lack absolute specificity. Neither Ro318220 nor Gö6983 has been evaluated against all PKC isozymes. Both inhibit PKC-α, -β, and -γ.[26,27] Ro318220 also inhibits PKC-ϵ,[26] although its effect on PKC-δ has not been reported. Gö6983 inhibits PKC-δ as well as PKC-ζ, but not PKC-μ, and it has not been tested against PKC-ϵ.[27] PMA-induced opening of mitoK$_{ATP}$ was blocked by Ro318220, but not by Gö6983 (Figure 7B). That should exclude any isozyme that is sensitive to inhibition by Gö6983. Because Ro318220 did block mitoK$_{ATP}$ opening, the isozyme in question must be sensitive to this antagonist. Of the known isozymes antagonized by Ro318220 and present in mitochondria, PKC-ϵ is a good candidate.

Mochly-Rosen and colleagues[39] designed peptides with amino acid sequences duplicating portions of the PKC-binding site contained in the first variable binding region, the C2/V1 domain, of the PKC molecule. These peptides have high affinities for the docking proteins of PKC and are isozyme-specific.

The ϵV$_{1-2}$, but not the δV$_{1-1}$, peptide will block protection from ischemic and hypoxic preconditioning in isolated cardiomyocytes[40,41] and intact hearts.[17,29] Only the peptide derived from PKC-ϵ blocked mitochondrial swelling induced by either PMA or PKG+cGMP (Figure 7B). Because we did not investigate peptide antagonists to all PKC isozymes, we cannot conclude that there is no other PKC isozyme that also plays a role in opening mitoK$_{ATP}$. But failure of Gö6983 to block mitochondrial volume changes triggered by PKG+cGMP further excludes PKC-α, -β, -γ, and -ζ.

The effectiveness of mitoK$_{ATP}$ inhibition is known to depend on the manner in which the channel opens.[9] Phosphorylation of mitoK$_{ATP}$ reduces the affinity of the channel for 5HD, because higher doses were required to block the channel after addition of PKG (Figure 5). This state dependency could explain the observation of Wang et al[42] that less 5HD was needed to block protection of IPC when administered before the preconditioning ischemia than when given after the preconditioning ischemia.

The effect of PKG on mitoK$_{ATP}$ is not organ-specific. K^+ flux in mitochondria from rat brain (Figure 8A) and liver (Figure 8B) was also regulated by activation of PKG, as it was in the heart.

PKG1α, the predominant PKG isozyme found in cardiomyocytes and the one used in this study, and cGMP are compartmentalized.[43,44] It is unknown how PKG and cGMP are actually directed to interact with mitochondria. Identity of the PKG-sensitive shuttling signal and the possibility that mitochondrial membranes contain specific docking sites for PKG and/or PKC are yet to be elucidated.

In summary, we have described novel regulation of mitoK$_{ATP}$ by activated PKG. We propose cGMP activates PKG localized at the cytosolic surface of the mitochondrial outer membrane. Activated PKG presumably phosphorylates some target protein that shuttles the cardioprotective signal to PKC-ϵ residing in the intermembrane space of mitochondria. The basis for this compartmentalization model is that exogenous PKG+cGMP is capable of activating mitoK$_{ATP}$ in isolated mitochondria whereas cGMP alone cannot. Also, addition of phorbol ester elicits the same response.

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