Characterization of human cardiac mitochondrial ATP-sensitive potassium channel and its regulation by phorbol ester in vitro

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The mitochondrial ATP-sensitive potassium channel was first discovered in the inner membrane (IMM) of rat liver mitochondria (21). Subsequent studies have confirmed its presence by patch-clamp studies of the mitoplasts (11, 13) or IMM reconstituted in lipid bilayers (1, 5, 27, 28, 31, 45). However, the unitary conductance of the putative mitoK\textsubscript{ATP} channel reported in these studies differed significantly, varying between ~10 and 100 pS. Equally controversial is the proposed molecular composition of the mitoK\textsubscript{ATP} channel (1, 27). Several groups have even questioned the very existence of mitoK\textsubscript{ATP}, based on the measurement of mitochondrial K\textsuperscript{+} fluxes or volume change (7, 12). In humans, the mitoK\textsubscript{ATP} channel was detected in a T-lymphocyte cell line (11), but the existence and properties of human cardiac mitoK\textsubscript{ATP} channel have not been reported.

Numerous pharmacological studies have shown that the mitoK\textsubscript{ATP} channel plays a critical role in myocardial protection, induced by ischemic and pharmacological preconditioning (14, 39, 44). Ischemic preconditioning (IPC) triggers the translocation of PKC isoforms, such as δ or ε, from the cytosolic to the particulate fraction containing the mitochondria (33, 42, 43). The translocated PKC has been proposed to regulate the mitoK\textsubscript{ATP} channel, and blockade of the PKC transfer prevents IPC in both animals and humans (20, 38, 42, 43). Activation of the mitoK\textsubscript{ATP} channel by phorbol 12-myristate 13-acetate (PMA), a PKC activator, was found to protect the integrity of isolated mitochondria by preventing the opening of permeability transition pore and cytochrome-c release to simulated ischemia (24). Furthermore, there is evidence that PKC activation by PMA potentiates the effect of diazoxide on flavoprotein oxidation, an indirect indicator of mitoK\textsubscript{ATP} channel activity (35). Although these studies suggest a role of PKC in preconditioning, there is no evidence that the mitoK\textsubscript{ATP} channel is directly regulated by cytosolic PKC or other protein kinases due to the physical barrier of outer mitochondrial membrane (OMM). Costa et al. (9) proposed that phosphorylation of a target protein on OMM by protein kinase G (PKG) transmits the cardioprotective signals to PKC-ε located in the intermembrane space during preconditioning. On the other hand, it is possible that the mitoK\textsubscript{ATP} is regulated by local protein kinase(s) associated with IMM or intermembrane space.

The purpose of our study was to characterize human cardiac mitoK\textsubscript{ATP} channel and to investigate its potential regulation by a local PKC control mechanism. Reconstitution of the human mitoK\textsubscript{ATP} channel in lipid bilayers, away from the cytosolic environment, allowed us to examine whether the mitoK\textsubscript{ATP} channel is directly regulated by PKC located within the IMM.

METHODS

Mitochondrial isolation. The Institutional Review Board for Clinical Studies at the Medical College of Wisconsin approved this study. The investigation conforms to the principles outlined in the Declaration of Helsinki. Human ventricular muscles were obtained from two nonfailing donor hearts from brain-dead patients. Human atria were obtained during cardiac surgery with informed consent. Cardiac mitochondria were isolated according to the procedure of Solem and
Wallace (37), with modifications as described previously (28). Briefly, tissues from the left ventricles or atria were cut into small pieces and homogenized in an ice-cold isolation buffer containing (in mM) 30 MOPS (pH 7.2), 200 mannitol, 50 sucrose, 5 K$_2$HPO$_4$, and 1 EGTA and 0.1% BSA in the presence of a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). After centrifugation at 3,000 g for 20 min, the supernatant was centrifuged at 8,000 g to obtain mitochondria (the pellet). Mitochondria were suspended in 3% Percoll in 10% human serum albumin (HSA) and centrifuged at 20,000 g for 10 min. The pellet was resuspended with the isolation medium without EGTA and then stored on ice for preparation of IMM.

**Preparation of IMM.** The submitochondrial fraction enriched with IMM was prepared as reported (6, 29). The mitochondrial pellet was osmotically shocked by incubation in 10 mM phosphate buffer (pH 7.4) for 20 min and then in 20% sucrose for another 15 min. Membranes were sonicated (Dual Horn for model 550, Fisher Scientific, Hanover Park, IL) three times for 30 s and centrifuged at 8,000 g for 10 min. The supernatant, containing submitochondrial particles, was filtered by using a continuous sucrose gradient (30% to 60%) and then centrifuged at 80,000 g overnight. The heavy fraction was resuspended with the isolation medium without EGTA and centrifuged at 380,000 g for 30 min. The final pellet, enriched in IMM, was resuspended in the isolation medium without EGTA and BSA and then stored at −80°C in small aliquots until use.

**Reconstitution of mitoK$_{ATP}$ channels into lipid bilayers.** The vesicles of IMM were reconstituted into lipid bilayers as reported previously (28). Briefly, the IMM vesicles were added to the cis chamber and fused into the lipid bilayers in a symmetrical solution containing (in mM) 30 MOPS (pH 7.4), 150 potassium glutamate, 1 EGTA, 1.03 CaCl$_2$ (free Ca$^{2+}$ = 10 μM), 0.05 K$_2$ATP, and 0.5 MgCl$_2$. Ag/AgCl electrodes were placed into each chamber via agar salt (0.5 M KCl) bridges, and the trans chamber was connected to the head stage of a bilayer clamp amplifier (BC-525C, Warner Instrument, Hamden, CT). The cis chamber was held at virtual ground. Experiments were performed at room temperature. After incorporation of cation channel(s), single channel currents at a holding potential of +30 or +40 mV (trans/cis, −30 or −40 mV by convention) or as indicated were collected by using an Axon Digidata 1332 AD/DA interface (Axon Instruments, Union City, CA) with pClamp software (version 8.01, Axon Instruments). The currents were filtered at 0.5 kHz with an eight-pole Bessel filter and digitized at 2.5 kHz. The channel activity was abolished by 5-HD, an inhibitor cocktail, and calculated slope conductance is 24 pS. We did not observe significant rectification within the range of voltages used, which probably can be better evaluated at higher voltages closer to a physiological range of matrix potential (100–180 mV).

**Western blot analysis.** Western blot analysis assays for sarcolemmal K$_{ATP}$ (sarK$_{ATP}$) channel subunits in mitochondria were conducted as described previously (22) using a 4–20% gradient Criterion Precast Gel (Bio-Rad), and that for PKC isoforms were done by using 7.5% Criterion Precast Gel (Bio-Rad) as described elsewhere (36). The antibodies were obtained from Santa Cruz Biotechnology, including inward rectifying K$^+$ channel (Kir6.2 (G-16), Kir6.1 (C-16), sulfonylurea receptor (SUR)2 (C-15), SUR1 (C-16), PKC-ε, PKC-δ, and PKC-β. The secondary antibodies were conjugated with horseradish peroxidase. Detected proteins were visualized by using chemiluminescence. To ensure detection specificity, negative controls were done by using primary antibodies preabsorbed with immunogen peptides in a one-to-five ratio.

**Characterization of mitoK$_{ATP}$ channel reconstituted in lipid bilayers.** MitoK$_{ATP}$ channels were identified by their inhibition with ATP and 5-hydroxydecanoic acid (5-HD) and their activation by diazoxide, a mitoK$_{ATP}$ channel opener. All modulators were added to the cis chamber and stirred for 30 s.

**Effect of PMA on mitoK$_{ATP}$ channel opening.** After the appearance of K$^+$–conducting current in lipid bilayers, 0.5 mM ATP was used to screen ATP-sensitive channels. PMA (2 μM) was added to the cis chamber while being stirred. The inactive phorbol ester 4a-phorbol 12,13-didecanoate (PDD) was used as the negative control. Channel activities were monitored for up to 15 min, and the identity of mitoK$_{ATP}$ channels was confirmed at the conclusion of the experiment by their inhibition with 5-HD.

**Chemicals.** The following drugs and chemicals were used: protease inhibitor cocktail, n-decane, MOPS, diazoxide, 5-HD, and ATP (Sigma-Aldrich); BSA (Serologicals, Milwaukee, WI); 1-α-phosphatidylethanolamine and 1-α-phosphatidyl-serine (Avanti Polar Lipid, Alabaster, AL); and PMA and PDD (Calbiochem). PMA, PDD, and diazoxide were dissolved in DMSO before adding to the experimental solution. The final concentration of DMSO (<0.1%) alone did not exhibit any effect on channel current.

**RESULTS**

Identification of human cardiac mitoK$_{ATP}$ channel. The human IMM vesicles were fused into the lipid bilayers, and K$_{ATP}$ channels were identified based on their inhibition by ATP and/or 5-HD (15, 16, 32), as well as on their activation by diazoxide, a putative agonist selective for the mitoK$_{ATP}$ channel (15, 18, 19).

In the presence of 150 mM symmetrical potassium glutamate and at the holding potential of +40 mV, recorded chord conductance of single mitoK$_{ATP}$ channels was mostly below 80 pS (n = number of observations): −20 pS (n = 8), −40 pS (n = 8), −60 pS (n = 7), although the channel conductance >80 pS was also recorded (n = 9). Figure 1A shows a K$^+$ channel with a cord conductance of 65 pS at a holding potential of +40 mV. The channel activity was abolished by 5-HD, confirming its identity as a mitoK$_{ATP}$. Figure 1B shows original traces of another mitoK$_{ATP}$ recorded at varying holding potentials, and Fig. 1C illustrates its corresponding current-voltage relationship (I-V). Linear regression analysis revealed a slope conductance of 57 pS, similar to that observed for mitoK$_{ATP}$ from bovine (45) or rat hearts (28). Figure 2A shows original traces of a mitoK$_{ATP}$ channel with a small conductance at various holding potentials. Its I-V curve is presented in Fig. 2B, and calculated slope conductance is 24 pS. We did not observe significant rectification within the range of voltages used, which probably can be better evaluated at higher voltages closer to a physiological range of matrix potential (~180 mV). However, we were limited by the poor stability of artificial bilayers beyond −60 or +60 mV, especially in patches with multiple channels.

The single channel conductance from patches with multiple mitoK$_{ATP}$ channels was often better appreciated in the presence of mitoK$_{ATP}$ inhibitors ATP or 5-HD. As shown in Fig. 3, at baseline (control, Fig. 3A), the observed peak current was −6 pA at +30 mV, with a small conductance channel opening at or above 0.75 pA (25 pS). An addition of 0.5 mM ATP (Fig. 3B) inhibited most of the larger flickering channels, but the small channel remained mostly open. Increasing ATP to 1 mM (Fig. 3C) gradually inhibited this channel until complete closure. Fig. 3C, inset, shows the segment of recordings with transition of the small conductance mitoK$_{ATP}$ from opening (at two levels, O$_1$ and O$_2$) to closing on an expanded scale. The
corresponding amplitude histogram clearly shows a single channel conductance of 25 and 75 pS, with the latter likely representing simultaneous opening of three channels of the 25 pS conductance. These data show that ATP induced both concentration- and time-dependent inhibition of mitoKATP channels, which was frequently observed.

This transition can also be observed in the presence of another blocker, 5-HD. Figure 4 shows regulation of mitoKATP channels by ATP, GTP, and 5-HD from a different patch of IMM fused in bilayers. At baseline (Fig. 4A), mitoKATP channel openings were active; an addition of 0.5 mM ATP (Fig. 4B) reduced the peak current by ∼75% to give a smaller conductance of ∼20 pS. An addition of 0.5 mM GTP (Fig. 4C), a mitoKATP channel agonist (32), markedly increased the peak current that exceeded that seen at baseline. 5-HD (200 μM, Fig. 4D) turned the sustained opening into a burst, with steplike conductance levels of ∼40, 80, and 120 pS, as shown on an expanded scale (Fig. 4D, inset). This was followed by complete closure (Fig. 4E). Thus similar to ATP, 5-HD also caused a steplike closure of the channels, revealing multiple conductance states. These smaller conductance levels could arise from clusters of smaller channels, or they could represent subconductance states of the larger channels. Similar steplike behavior was also reported for sarcKATP inhibited by glibenclamide (23).

We also tested the effect of the mitoKATP channel opener diazoxide on some mitoKATP channels with low initial activities (NPₒ < 0.1). Data from multiple experiments are summarized in Fig. 5. An addition of diazoxide (either 50 or 100 μM) significantly increased NPₒ from the control levels (P < 0.05),...
and this current was subsequently blocked by 5-HD (200 μM, P < 0.05 vs. diazoxide). These results confirmed the presence of mitoK<sub>ATP</sub> channels in native human IMM. The effects of diazoxide and 5-HD were similar to those reported in reconstituted bovine or rat cardiac K<sub>ATP</sub> channels (28, 45). These concentrations were chosen because they produced consistent activation (diazoxide) or inhibition (5-HD) in preliminary studies. Similar concentrations were found to be effective for bovine (45) or rat mitoK<sub>ATP</sub> (28) channels.

Because the exact molecular composition of the mitoK<sub>ATP</sub> channel is not known, we explored its potential similarity to the sarcK<sub>ATP</sub> channel. We probed the isolated IMMs with antibodies targeting the subunits of sarcK<sub>ATP</sub>: the SUR (SUR1 and SUR2) and the Kir channel (Kir6.1 and Kir6.2). As shown in Fig. 6 (left), Kir6.2 antibody detected a 56-kDa peptide in human ventricular IMM and in two atrial mitochondrial preparations (Fig. 6, M). The specificity was confirmed by preincubation of the antibody with its antigen peptide (Fig. 6, right). Interestingly, this 56-kDa peptide is similar to the ethanol-extracted 55-kDa mitochondrial protein reported by Miranova et al. (27), which was proposed to be the mitochondrial inward rectifier subunit. However, we were not able to detect Kir6.1, as reported by Lacza et al. (25), or SUR subunits with specificity (not shown).

**Regulation of human cardiac mitoK<sub>ATP</sub> by local PKC.** To explore whether human mitoK<sub>ATP</sub> channels are regulated by PKC associated with the IMM, we tested the effect of PKC activator PMA on mitoK<sub>ATP</sub> channels reconstituted into the lipid bilayers. Figure 7A shows the original recording of mitoK<sub>ATP</sub> openings at baseline (control), followed by inhibition with 0.5 mM ATP. An addition of PMA (2 μM) in the cis chamber reactivated the channels that were eventually inhibited by 5-HD (400 μM). In contrast to PMA, its inactive analog PDD had no effect (Fig. 7B). Cumulative data from six separate observations are summarized in Fig. 7C. These results confirmed that PMA significantly increases the NP<sub>o</sub> of mitoK<sub>ATP</sub> channels previously inhibited by ATP (P < 0.05).

The effects of PMA on mitoK<sub>ATP</sub> obtained at the single-channel level are in agreement with the effect of PMA on the mitoK<sub>ATP</sub> activity in studies (9, 24) done on isolated mitochondria. Because the mitoK<sub>ATP</sub> channels reconstituted in lipid bilayers are in cytosol-free environment, no cytosolic PKC

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**Fig. 3.** ATP-induced steplike inhibition of mitoK<sub>ATP</sub> conductance. At control (A), a cluster of channels was seen open at and above 0.75 pA (25 pS), with peak current of ~6 pA due to the spikes of openings. Subsequent addition of ATP (0.5 mM) into cis (B) inhibited most of the spiking channels, with sustained opening at 0.75 pA. When ATP was raised to 1 mM (C), channels underwent steplike transition to smaller conductance states before complete closure (C); this is clearly shown on an expanded scale (C, left inset) (O<sub>2</sub> and O<sub>1</sub>, open states). Histogram analysis (C, right inset) showed a transition from 75 pS (i.e., O<sub>2</sub>) to 25 pS (i.e., O<sub>1</sub>) before closure (C).

**Fig. 4.** Effects of ATP, GTP, and 5-HD on mitoK<sub>ATP</sub> channels reconstituted in lipid bilayers. Original recordings of mitoK<sub>ATP</sub> channel activities are shown, and upward deflection represents channel opening at +30 mV (transciss). A cluster of mitoK<sub>ATP</sub> channels was active at baseline (control, A), inhibited by 0.5 mM ATP (B), and then reactivated by 0.5 mM GTP (C). Addition of 5-HD inhibited channels, which initially appeared in bursts (D), with conductance levels seen at ~40 (O<sub>1</sub>), 80 (O<sub>2</sub>), and 120 (O<sub>3</sub>) pS (D, inset) before complete closure (E). Data represent 5 observations.
translocation was thus possible; the effect of PMA was likely mediated by PKC associated with the IMM. To confirm this hypothesis, we screened the IMM for PKC isozymes. As shown in Fig. 7C, inset, we were able to detect PKC-δ/H9254 but not PKC-ε/H9280 (or H9252, not shown) in human IMM.

**DISCUSSION**

To our knowledge, this is the first study characterizing the human cardiac mitoK<sub>ATP</sub> channel and its modulation by PMA. Our results demonstrate that the mitoK<sub>ATP</sub> channels are present in human cardiac mitochondria, activated by diazoxide, and inhibited by ATP and 5-HD. Moreover, the mitoK<sub>ATP</sub> channels reconstituted in lipid bilayers can be directly stimulated by the PKC activator PMA without translocation of cytosolic kinases. Western blot analysis demonstrated that Kir6.2 immunoreactive protein and PKC-δ are present in human IMM. These data suggest that the mitoK<sub>ATP</sub> channel is regulated locally by PKC associated with the IMM. This local control mechanism may serve as an alternative signal transduction pathway for the mitoK<sub>ATP</sub> channel activation during preconditioning to that proposed for PKC translocated from the cytosol to the mitochondria (33, 34, 41, 42).

The existence of the mitoK<sub>ATP</sub> channel was first reported by Inoue et al. (21) in a patch-clamp study on liver mitoplasts. In the present study, we observed multiple conductances of the mitoK<sub>ATP</sub> channels (mostly below 80 pS), similar to previous observations in the rat heart (28). The conductance levels of these channels, however, are modulated by 5-HD as well as ATP. In mitochondria isolated from human lymphocyte cell line, Dahlem et al. (11) observed an outwardly rectifying K<sup>+</sup> channel with a slope conductance of 82 pS at positive test potentials and 15 pS at negative test potentials. This channel, however, responded poorly to ATP inhibition: it was only partially inhibited by 12.5 mM ATP, whereas a higher ATP concentration had no effect. In our experiments, 0.5 mM or 1 mM ATP was effective in blocking most of the mitoK<sub>ATP</sub> channels.

A variety of mitoK<sub>ATP</sub> channel conductances have been reported since its initial discovery. In mitochondria from bovine hearts reconstituted in lipid bilayers, recorded mitoK<sub>ATP</sub> conductance was 30 pS (31) and 56 pS (45). In mitoplasts prepared from rat cardiomyocytes, Er et al. (13) observed a 13-pS channel that was voltage dependent with no rectification. Ardehali et al. (1) observed a mitoK<sub>ATP</sub> channel at 200 pS in 500 mM K<sup>+</sup> and <100 pS in 100 mM K<sup>+</sup>. Szewczyk and
coworkers (4, 5) recorded the mitoKATP channel with 103-pS conductance that was reduced by over 50% in the presence of 1 mM Mg$^{2+}$. Different observations may result from differences in preparations, concentrations of charge carriers, as well as the presence of modulators such as Mg$^{2+}$ and ATP. They also reflect the complexity of studying the mitoKATP channels and raise the possibility that there may be more than one type of channel that respond to known activators and inhibitors of mitoKATP channels. Although we detected mitoKATP channels at $\sim$20 pS, channels with smaller conductance at $\sim$10 pS could not be resolved under holding voltages used in our observations. Nevertheless, the results from our study revealed multiple conductance states of the human mitoKATP channel. Whether they represent clusters of mitoKATP channels or multiple-conducting states of a single channel has yet to be determined.

MitoKATP channel has not yet been identified at the molecular level. Several studies (10, 25) that attempted to reveal its molecular structure relied on probing the mitochondria with antibodies against sarcKATP subunits. However, this approach is often criticized due to lack of proper negative controls with antigen peptide competition, which may show false positive findings (7). In the present study, anti-Kir6.2 antibody detected a 56-kDa protein in human cardiac IMM, but we failed to detect Kir6.1 and SUR subunits in the same sample with specificity. Garlid and coworkers (3, 27) have extracted the putative mitochondrial KIR and mitochondrial SUR from mitochondria and proposed a molecular structure similar to sarcKATP (3, 27). An alternative molecular complex, which consists of five mitochondrial proteins within the IMM [the ATP binding cassette protein, a phosphate carrier, adenosine nucleotide translocator (ANT), ATP synthase, and succinate dehydrogenase], has been recently proposed (1). Thus the definition of the mitoKATP channel molecular nature remains far from resolved. Moreover, the very existence of the mitoKATP channel has been challenged, based on measurements of mitochondrial volume change, K$^+$ fluxes, and respiration in the presence of diazoxide or 5-HD (7, 12).

Activation of the mitoKATP channel by PKC has been considered a crucial event in cardiac preconditioning (17, 30). Although it has been reported that PKC-ε translocates to the particulate fraction during IPC (33, 42, 43), there is no evidence that cytosolic PKC isoforms can permeate the OMM and interact directly with the mitoKATP in the IMM. The role of mitochondrial PKC in the mitoKATP channel regulation was suggested in the study (24) on isolated mitochondria, which are devoid of cytosolic components. The authors of that study observed that PKC activation by PMA mimics the effect of diazoxide in preventing permeability pore transition, which was abolished by the mitoKATP inhibitor 5-HD. Costa et al. (9) proposed that PKG may phosphorylate some target protein on the OMM and transmit the cardioprotective signals from cytosol to IMM via PKC-ε located in the intermembrane space. The evidence for this proposal, however, was based only on pharmacological studies in isolated mitochondria, using changes in mitochondrial volume and respiration as indirect indexes of the mitoKATP channel activity.

In our experiments, PMA-activated mitoKATP channels constituted in lipid bilayers, and we were able to detect PKC-δ in IMM. These data suggest that PKC is associated with the IMM where it can directly activate mitoKATP channels, probably by forming a signaling module. Further study is needed to investigate whether Kir6.2 is physically bound with PKC-δ in IMM. PKC was previously shown to phosphorylate the Kir subunits in sarcKATP (26), and a similar mechanism may apply to mitoKATP. This phosphorylation likely reduced the sensitivity of mitoKATP to ATP inhibition, as well as 5-HD inhibition (Fig. 7).

In the mitochondria, the role of PKC is not limited to regulating the mitoKATP channels. PKC-δ was also shown recently to bind specifically with pyruvate dehydrogenase and to prevent its reactivation during reperfusion (8), although the source (cytosolic vs. mitochondrial) of the PKC remains elusive. PKC-ε, although not detected in the present study with human IMM, has been shown to form a functional module with components of the permeability transition pore from mouse mitochondria, including the voltage-dependent anion channel (VDAC) located on the OMM and the ANT located on the IMM (2, 40). The VDAC, located on the OMM, is obviously a better target for cytosolic kinases, such as PKCs, than the mitoKATP located in the IMM. On the other hand, ANT may also form a part of the proposed mitoKATP complex (1); its interaction with PKC-ε may also regulate mitoKATP activity.

PKC-ε was also shown recently to interact with cytochrome-c oxidase (19) and with a scaffold protein cypher and ANT on the IMM (40). This evidence suggests that PKC (and probably other kinases), located within the cardiac mitochondria, may play an important role in regulating respiration as well as ionic homeostasis. In the intact cell, a soluble second messenger, such as the diacylglycerol or reactive oxygen species, may activate mitochondrial PKC during IPC.

In summary, we have provided the first characterization of the human cardiac mitoKATP channel and its regulation by PMA in vitro. Our data suggest that the mitoKATP channel is activated by PKC associated with the IMM. This local control mechanism may represent an alternative regulatory pathway to that proposed for cytosolic PKC translocated to mitochondria during preconditioning. Further studies are needed to validate this local control mechanism.

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