Modulation of Mitochondrial ATP-Dependent K⁺ Channels by Protein Kinase C

Toshiaki Sato, Brian O’Rourke, Eduardo Marbán

Abstract—Pharmacological openers of mitochondrial ATP-dependent K⁺ (mitoK ATP) channels mimic ischemic preconditioning, and such cardioprotection can be prevented by mitoK ATP channel blockers. It is also known that protein kinase C (PKC) plays a key role in the induction and maintenance of preconditioning. To look for possible mechanistic links between these 2 sets of observations, we measured mitochondrial matrix redox potential as an index of mitoK ATP channel activity in rabbit ventricular myocytes. The mitoK ATP channel opener diazoxide (100 μmol/L) partially oxidized the matrix redox potential. Exposure to phorbol 12-myristate 13-acetate (PMA, 100 nmol/L) potentiated and accelerated the effect of diazoxide. These effects of PMA were blocked by the mitoK ATP channel blocker 5-hydroxydecanoate, which we verified to be a selective blocker of the mitoK ATP channel in simultaneous recordings of membrane current and flavoprotein fluorescence. The inactive control compound 4α-phorbol (100 nmol/L) did not alter the effects of diazoxide. We conclude that the activity of mitoK ATP channels can be regulated by PKC in intact heart cells. Potentiation of mitoK ATP channel opening by PKC provides a direct mechanistic link between the signal transduction of ischemic preconditioning and pharmacological cardioprotection targeted at ATP-dependent K⁺ channels. (Circ Res. 1998;83:110-114.)

Key Words: diazoxide ■ preconditioning ■ protein kinase C ■ mitochondria ■ 5-hydroxydecanoate

Ischemic preconditioning is the well-known phenomenon in which brief periods of “conditioning” ischemia paradoxically protect the myocardium against subsequent lethal ischemia. The precise mechanism of IPC remains elusive, but at least 2 sets of relevant observations have been broadly accepted. First, activation of several kinases, most notably PKC, figures prominently in the signal transduction cascade of IPC. Second, extensive pharmacological evidence implicates K ATP channels as the effectors. Agonists of K ATP channels mimic IPC in the absence of conditioning ischemia, whereas K ATP channel blockers prevent IPC. Although such effects were initially attributed to classic surface K ATP channels, it now seems much more likely that mitoK ATP channels are the dominant players. Diazoxide, an agonist that opens mitoK ATP channels >1000-fold more potently than their surface counterparts in heart cells, cardioprotects at concentrations that open only the mitoK ATP channels, and can block IPC. Conversely, the mitoK ATP channel blocker 5HD can prevent diazoxide cardioprotection and can block IPC.

Despite compelling evidence supporting roles for both PKC and mitoK ATP channels, it is not clear how the 2 observations are linked mechanistically. Therefore, we investigated the effects of PKC activators on diazoxide-induced changes of mitochondrial redox potential in rabbit ventricular myocytes. The PKC activator PMA potentiated the effects of diazoxide and abbreviated the latency to mitoK ATP channel opening on application of diazoxide. These effects could be blocked by 5HD but were not reproduced by the inactive compound 4α-phorbol. The results indicate that mitoK ATP channels are upregulated by PKC and thus provide a specific link between the signal transduction of IPC and its likely effector.

Materials and Methods

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication No. 85-23, revised 1985).

Materials

Collagenase (type II) was purchased from Worthington. Diazoxide, DNP, PMA, and 4α-phorbol were obtained from Sigma Chemical Co. 5HD and pinacidil were purchased from Research Biochemicals International. Diazoxide, PMA, 4α-phorbol, and pinacidil were dissolved in DMSO before added into experimental solutions. The final concentration of DMSO was <0.1%.

Preparation of Rabbit Myocytes

Isolated ventricular myocytes were obtained from adult rabbit hearts by conventional enzymatic dissociation methods. In brief, hearts were excised from anesthetized (30 mg/kg IV pentobarbital) New Zealand White rabbits (weighing 1 to 2 kg) and mounted on a Langendorff apparatus. The hearts were perfused with modified Krebs-Henseleit solution composed of (mmol/L) NaCl 119, KCl 5, MgSO4 1, NaHCO3 25, KH2PO4 1, CaCl2 1, and glucose 10. The perfusate was bubbled with 95% O2/5% CO2 and maintained at 37°C.
After 5 minutes of perfusion, the hearts were perfused without Ca²⁺ for another 5 minutes, after which the perfusion solution was switched to one containing collagenase (0.8 mg/mL, Worthington type II). The perfusion pressure was monitored, and the flow rate was adjusted to maintain perfusion pressure at ~75 mm Hg. After 25 to 30 minutes of collagenase perfusion, hearts were removed from the perfusion apparatus, and the atria were trimmed away. The ventricles were minced and incubated in a shaking bath for another 5 minutes in collagenase-containing solution. Cells were then filtered through nylon mesh and washed several times with Ca²⁺-free solution. Ca²⁺ concentration was gradually brought back to 1 mmol/L. Cells were then cultured on laminin-coated coverslips in medium 199 with 5% FBS at 37°C. Experiments were performed over the next 2 days.

Flavoprotein Fluorescence

Opening of mitoKₐ₅ᵢ₆ channels dissipates the inner mitochondrial membrane potential established by the proton pump. This dissipation accelerates electron transfer by the respiratory chain and, if uncompensated by increased production of electron donors, leads to net oxidation of the mitochondria. We therefore monitored the mitochondrial redox state by recording the fluorescence of FAD-linked enzymes in the mitochondria as described by Liu et al. Cells were superfused with external solution containing (mmol/L) NaCl 140, KCl 25, MgCl₂ 0.5, potassium glutamate 120, KCl 25, MgCl₂ 0.5, potassium EGTA 10, HEPES 10, and MgATP 1 (pH 7.4 with KOH). Whole-cell currents were elicited every 6 seconds from a holding potential of ~80 mV by 2 consecutive steps to ~40 mV (for 100 milliseconds) and 0 mV (for 380 milliseconds), and flavoprotein fluorescence was excited during the 100-millisecond step to ~40 mV. Currents at 0 mV were measured 200 milliseconds into the pulse.

Data Analysis

Data are presented as mean±SEM, and the number of cells or experiments is shown as n. ANOVA combined with the Fisher post hoc test was used to test for differences among groups for electrophysiological and fluorescence data. A value of P<0.05 was considered significant.

Results

Effect of 5HD on Mitochondrial Kₐ₅ᵢ₆ and Surface Kₐ₅ᵢ₆ Channels

We first tested whether 5HD is a selective blocker of mitoKₐ₅ᵢ₆ channels in our system, given the controversy regarding this issue.10,11 Our system has the unique advantage that it enables us to assay mitoKₐ₅ᵢ₆ and surfaceKₐ₅ᵢ₆ channels simultaneously in intact cells.11 To test the specificity of 5HD, we examined the effects of 5HD on pinacidil-induced flavoprotein oxidation and Iₚₐ₅ᵢ₆. Unlike diazoxide, pinacidil targets both mitoKₐ₅ᵢ₆ and surfaceKₐ₅ᵢ₆ channels.10,11 Panels A and B of Figure 1 show the effects of pinacidil and 5HD in a representative experiment. Pinacidil at 100 μmol/L reversibly increased both flavoprotein oxidation and Iₚₐ₅ᵢ₆. In the presence of 5HD (500 μmol/L), a second exposure to pinacidil...
failed to increase flavoprotein oxidation, whereas \( I_{\text{K,ATP}} \), turned on without impediment after exposure to pinacidil. In fact, the second exposure to pinacidil elicited a larger response than the first, consistent with previous reports of pinacidil potentiation of \( I_{\text{K,ATP}} \). As summarized in Figure 1C and 1D, 5HD (500 \( \mu \)mol/L) virtually abolished the pinacidil-induced flavoprotein oxidation from 34±4% to 5±2% of the DNP value (n=5, \( P<0.01 \)) (Figure 1C) but did not inhibit the \( I_{\text{K,ATP}} \) induced by pinacidil (Figure 1D). These results indicate that 5HD selectively inhibits mitoK\(_{\text{ATP}}\) channels without affecting surfaceK\(_{\text{ATP}}\) channels.

Effect of PKC on Diazoxide-Activated MitoK\(_{\text{ATP}}\) Channels

We next turned our attention to the issue of modulation by PKC. Figure 2A shows the time course of flavoprotein fluorescence in a cell exposed twice to diazoxide. In the first application, diazoxide (100 \( \mu \)mol/L) reversibly oxidized the flavoproteins. Subsequent exposure to PMA (100 nmol/L) alone had no effects on flavoprotein fluorescence (as confirmed in 4 other cells), but a second application of diazoxide in the continued presence of PMA increased flavoprotein fluorescence above and beyond the levels reached in the first application. This augmentation is particularly notable given that 100 \( \mu \)mol/L diazoxide is a maximally effective concentration under basal conditions. Note also that the second application of diazoxide appears to act more quickly than the first: it begins to elicit oxidation within 3 minutes, whereas the latency exceeded 10 minutes during the initial exposure. In another cell (Figure 2B), PMA was equally effective in potentiating diazoxide-induced oxidation when applied after the effect of diazoxide had reached steady state. In contrast, the inactive compound 4\( \alpha \)-phorbol (100 nmol/L) did not augment the oxidation produced by diazoxide (Figure 2C). Figure 2D shows that 5HD (2 mmol/L) was able to prevent the diazoxide-induced oxidation, even with a concomitant application of PMA. These findings indicate that mitoK\(_{\text{ATP}}\) channels are upregulated by PMA: the effect of diazoxide is larger, and faster, when the PKC activator is applied.

The pooled data in Figure 3A confirm that the results in Figure 2 are indeed representative. Diazoxide (100 \( \mu \)mol/L) reversibly increased flavoprotein oxidation to 42±4% of the DNP value (n=17, DIAZO group). In the absence of PMA, the degree of oxidation is identical during a second exposure to diazoxide (as shown by Liu et al and as we verified in 2 new experiments [not shown]). PMA (100 nmol/L) significantly increased the diazoxide-induced flavoprotein oxidation to 68±4% of the DNP value (n=9, \( P<0.01 \) versus DIAZO group), whereas 4\( \alpha \)-phorbol (100 nmol/L) did not alter the effect of diazoxide (40±6% of the DNP value, n=4). 5HD (0.5 to 2 mmol/L) significantly blocked the oxidative effects of diazoxide in the presence of PMA (5±3% of the DNP value, n=4, \( P<0.01 \) versus DIAZO and DIAZO+PMA groups). Figure 3B summarizes the latency to mitoK\(_{\text{ATP}}\) activation, measured as the time required to increase the flavoprotein oxidation to 20% of maximal after washing in diazoxide. Consistent with our earlier findings, the oxidative effect of diazoxide was reversible and reproducible. There was no significant difference between the latency times of the
first [DIAZO(1)] and second [DIAZO(2)] exposures to diazoxide in the absence of PMA. In contrast, in the presence of PMA, the second exposures to diazoxide significantly abbreviated the latency time from 9.0 ± 2.0 minutes [DIAZO(1)] to 3.6 ± 0.3 minutes [PMA + DIAZO(2)] (n=5, P<0.05).

Effects of Diazoxide and PKC on I_{K,ATP}

By measuring membrane current and fluorescence in the same cells, we previously concluded that diazoxide targets mitoK_{ATP} but not surfaceK_{ATP} channels. These experiments were performed in the basal state, without stimulation of PKC. To verify that the diazoxide-induced flavoprotein oxidation observed in the presence of PMA reflects the selective activation of mitoK_{ATP} channels, we measured flavoprotein fluorescence and membrane current simultaneously in an additional group of myocytes. Figure 4A shows the effects of diazoxide on flavoprotein fluorescence and membrane current in a representative cell. Diazoxide (100 μmol/L) induced reversible flavoprotein oxidation in the presence of PMA (100 nmol/L) but did not affect membrane current. Nevertheless, I_{K,ATP} eventually turned on after prolonged exposure to DNP (100 μmol/L), indicating that surfaceK_{ATP} channels were present and operable despite the inability of diazoxide to open them. As summarized in Figure 4B, diazoxide and PMA did not affect I_{K,ATP} measured at 0 mV, although DNP significantly increased the membrane currents measured at 0 mV to 3.09 ± 0.86 nA (n=6). These results provide further evidence that the oxidative effect of diazoxide and its regulation by PKC reflect the selective activation of mitoK_{ATP} channels.

Discussion

We found that exposure to the PKC-activating phorbol ester PMA, but not to the inactive 4α-phorbol, accelerates and augments the mitochondrial oxidation induced by diazoxide. Several aspects of the results are notable. From a technical perspective, the results in Figures 2 and 3 are from cells resting in the experimental chamber without invasion by patch pipettes or their contents. The fact that these results agree qualitatively and quantitatively with those of Liu et al, and with those in Figure 4, provides reassurance that the findings are not restricted to the specialized conditions of whole-cell electrophysiological recordings, in which the intracellular milieu is purposely controlled and the cells undergo repetitive electrical stimulation.

Conceptually, the results have obvious and direct implications for the mechanism of IPC. A variety of G protein–coupled agonists (adenosine, bradykinin, and opioids) are believed to confer ischemic tolerance by activating PKC and a variety of downstream kinases (for review see Reference 20). Our findings suggest the following hypothesis for IPC: conditioning ischemia activates PKC, which primes the mitoK_{ATP} channel to open earlier and more intensely during the lethal ischemia. By mechanisms that remain obscure, this functional alteration of the channels (presumably due to phosphorylation, although this has not yet been demonstrated) confers tolerance to ischemia,
delaying the process of necrosis. Several observations in simulated or genuine ischemia support this hypothesis: diazoxide protects ventricular cells in a perfusing model of ischemia\(^1\) (a model in which PKC activation has been shown to confer cardioprotection\(^2\)), improves functional recovery after ischemia in Langendorff-perfused hearts,\(^3,4\) and reduces infarct size in vivo in rabbits (J.M. Downey, unpublished data, 1998). The mitoK\(_{ATP}\) blocker 5HD, which we confirmed to be selective in our present experiments (Figure 1), prevents the cardioprotective effects of diazoxide\(^6,11\) and blocks genuine IPC.\(^12-14\)

It would be logical and desirable to extend the present functional studies to the structural level and to determine whether the mitoK\(_{ATP}\) channels are phosphorylated and, if so, how this alters their function. Such studies are not yet possible because the molecular identity of the channel is unknown. Much more is known about surface K\(_{ATP}\) channels, which are octamers of 4 sulfonylurea receptors and 4 pore-forming subunits of the Kir6 family.\(^22-25\) The initial patch-clamp study of Inoue et al\(^26\) in mitoplasts demonstrated that mitoK\(_{ATP}\) channels are activated by ATP depletion, that they have a smaller single-channel conductance than the surface K\(_{ATP}\) channels, and that they are susceptible to various K\(_{ATP}\) agonists and blockers. Subsequent work by other investigators has extended the pharmacological profile, and although less direct techniques were used, a distinctive set of drug sensitivities has emerged.\(^27,28\) Little is known at the structural level. Szewczyk et al\(^29\) have described a small glibenclamide-binding protein in crude mitochondrial extracts that may have a smaller single-channel conductance than the surface clamp study of Inoue et al\(^26\) in mitoplasts demonstrated that mitoK\(_{ATP}\) channels are activated by ATP depletion, that they have a smaller single-channel conductance than the surface K\(_{ATP}\) channels, and that they are susceptible to various K\(_{ATP}\) agonists and blockers. Subsequent work by other investigators has extended the pharmacological profile, and although less direct techniques were used, a distinctive set of drug sensitivities has emerged.\(^27,28\) Little is known at the structural level. Szewczyk et al\(^29\) have described a small glibenclamide-binding protein in crude mitochondrial extracts that may represent all or part of a mitochondrial sulfonylurea receptor. Suzuki et al\(^26\) have found that an antibody to a C-terminal epitope of Kir6.1 labels the inner membrane of mitochondria in skeletal muscle by immunogold histochemistry. The function of Kir6.1 itself is not entirely clear, although it has been argued to form a small-conductance ATP-activated channel in the surface of smooth muscle cells.\(^31\) Taken together, these observations hint that mitoK\(_{ATP}\) channels consist of a complex of an unknown sulfonylurea receptor and a pore-forming subunit with partial homology to Kir6.1.

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References


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