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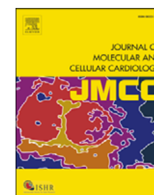

Review article

Cardioprotective signaling to mitochondria

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A B S T R A C T

Mitochondria are central players in the pathophysiology of ischemia–reperfusion. Activation of plasma membrane G-coupled receptors or the Na,K-ATPase triggers cytosolic signaling pathways that result in cardioprotection. Our working hypothesis is that the occupied receptors migrate to caveolae, where signaling enzymes are scaffolded into signalosomes that bud off the plasma membrane and migrate to mitochondria. The signalosome–mitochondria interaction then initiates intramitochondrial signaling by opening the mitochondrial ATP-sensitive K^+ channel (mitoK_{ATP}). MitoK_{ATP} opening causes an increase in ROS production, which activates mitochondrial protein kinase C epsilon (PKCε), which inhibits the mitochondrial permeability transition (MPT), thus decreasing cell death. We review the experimental findings that bear on these hypotheses and other modes of protection involving mitochondria.

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Contents

1. Mitochondria: the target for ischemia–reperfusion injury and cardioprotection . . . 859
2. Receptor-mediated signaling to open mitoK_{ATP} . . . 859
   2.1. Gi-protein coupled receptor (GPCR) pathways . . . 859
   2.2. Non-GPCR pathways of protection — digitalis . . . 859
3. From receptor to mitochondria by signalosomes . . . 859
   3.1. Rationale . . . 860
   3.2. The signalosome hypothesis . . . 860
   3.3. Experimental evidence for signalosome-mediated mitoK_{ATP} opening . . . 860
   3.4. Signalosomes phosphorylate MOM receptors . . . 860
   3.5. Signal transmission from MOM to PKCε1 . . . 861
4. Intramitochondrial signaling. . . . 861
   4.1. Step one — opening mitoKATP by activation of PKCε1 . . . 861
   4.2. Step two — mitochondrial K^+ uptake and its consequences. . . . 862
   4.3. Step three — activation of PKCε by endogenous ROS . . . 862
      4.3.1. ROS activation of PKCε2 and inhibition of MPT . . . 862
      4.3.2. ROS activation of PKCε1 and feedback mitoK_{ATP} opening . . . 862
   4.4. Intramitochondrial signaling and the literature . . . 862
5. Other mitochondrial mechanisms of cardioprotection . . . 862
   5.1. K_{ATP} channel openers (KCO) . . . 862
   5.2. Glycogen synthase kinase-3β (GSK-3β) . . . 863
   5.3. Amobarbital . . . 863
   5.4. Bromoenol lactone (BEL) . . . 863
   5.5. Hydrogen sulfide . . . 863
   5.6. Mitochondrial aldehyde dehydrogenase (ALDH2) . . . 863
   5.7. Ca^{2+}-activated mitochondrial K^+ channel (mitoK_{Ca}) . . . 863
6. Summary . . . 863
References . . . 863

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1. Mitochondria: the target for ischemia–reperfusion injury and cardioprotection

Mitochondria are effectors of both ischemia–reperfusion injury (IRI) and cardioprotection. As pointed out 30 years ago by Jennings and Ganote [1], the heart is strictly aerobic and consequently extremely vulnerable to a decrease in oxygen supply. Thus, ischemia causes profound and immediate mitochondrial derangements. These include cessation of ATP synthesis, inhibition of respiration, and a drop in $\Delta\Psi$. This is accompanied during ischemia by cellular changes, especially an increase in Ca^{2+} and phosphate, and, during reperfusion, by large increases in reactive oxygen species (ROS) originating from the respiratory chain [2,3]. Together, these factors promote opening of the mitochondrial permeability transition (MPT), a high-conductance pore in the inner mitochondrial membrane, which is the main cause of necrotic cell death in IRI [4–8]. Consequently, as pointed out by Weiss et al. [4], cardioprotection by preconditioning or postconditioning must ultimately involve the prevention of MPT.

In addition to their role as mediators of cell death, mitochondria have been shown to be major effectors of diverse self-defense mechanisms, including ischemic pre- and post-conditioning [7,9–11]. These and other conditioning protocols have been shown to require opening of the mitochondrial ATP-sensitive K^+ channel (mitoK_{ATP}) and inhibition of MPT opening. Since cardioprotection involves both mitoK_{ATP} opening and a decrease in MPT opening, it is reasonable to hypothesize that these two phenomena are part of the same signaling pathway. Indeed, this connection has been demonstrated [12], and will be discussed in this review.

This review describes our current understanding of the signaling mechanisms that originate at plasma membrane receptors, go to mitochondria, and terminate with MPT inhibition. For space reasons, we have not discussed mechanisms for the prevention of apoptosis. For different perspectives, readers are referred to excellent reviews by other authors [13–18].

2. Receptor-mediated signaling to open mitoK_{ATP}

2.1. Gi-protein coupled receptor (GPCR) pathways

Ischemic preconditioning (IPC) and ischemic postconditioning are receptor-mediated processes that are triggered by GPCR agonists released by the ischemic heart, primarily bradykinin, opioid peptides, and adenosine [19]. Other GPCR ligands, including acetylcholine, catecholemines, endothelin, and angiotensin II, are also cardioprotective [20–24], but they were found not to be physiological triggers of IPC [14]. A composite diagram of the GPCR signaling pathways is given in Fig. 1. GPCR signaling has been extensively studied by Downey and Cohen and their coworkers, and is the subject of an excellent review by these authors [14]. It should be emphasized that each G_i-coupled receptor ligand triggers its own unique signaling cascade. Opioids and acetylcholine instigate transactivation of the epidermal growth factor receptor (EGFR), leading to downstream activation of phosphatidylinostitol 3-kinase (PI3-K) and Akt. Bradykinin also induces activation of PI3-K and Akt, but without transactivation of EGFR. These two pathways then converge and ultimately lead to mitoK_{ATP} opening and production of ROS. The adenosine signaling pathway has not yet been fully characterized. MitoK_{ATP} opening is not involved during the *trigger* phase of adenosine preconditioning (i.e., when 5-HD administration brackets adenosine perfusion) [25], but mitoK_{ATP} opening is required during the *mediator* phase (i.e., when 5-HD administration precedes ischemia) [26–29].

Protection afforded by all of the trigger substances is blocked by PKC inhibitors, and PKC, probably PKCε, is thought to be a common target of cardioprotective signaling [14]. It has been difficult to localize the critical PKCε, because multiple PKCε isoforms participate in cardioprotection [30]. In ouabain signaling, PKCε acts proximally in conjunction with EGFR transactivation [31]. The adenosine A1 receptor is believed to directly stimulate PLC and PLD to activate PKC [21]. These PKCs are cytosolic. As discussed below, two PKCε isoforms regulate mitoK_{ATP} and MPT at the level of the inner mitochondrial membrane [32]. Thus, the physiological effect of PKCε activation depends entirely on its location and not on its biochemistry, which appears to be invariant.

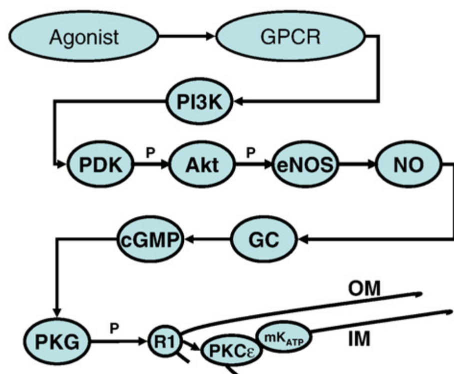

Fig. 1. GPCR-mediated signaling to mitochondria. Occupation of the GPCR leads to activation of PI3-kinase, phosphorylation of phosphatidylinositol bisphosphate, and activation of the phosphatidylinositol-dependent kinases (PDKs) [128]. PDKs then phosphorylate Akt, which initiates the remainder of the cytosolic signaling pathway: endothelial nitric oxide synthase (eNOS) is phosphorylated, leading to production of NO. NO stimulates guanylyl cyclase, and the cGMP produced activates protein kinase G (PKG) [129], which causes mitoK_{ATP} opening [62,130–132].

2.2. Non-GPCR pathways of protection — digitalis

Cardiac glycosides are classic inhibitors of the plasma membrane Na^+/K^+-ATPase, but this enzyme also has important non-canonical functions that are triggered by digitalis. Thus, ouabain interaction with the Na,K-ATPase activates src kinase, causing formation of a “binary receptor” that phosphorylates and assembles other proteins into signaling modules that transmit signals to intracellular compartments [33,34]. Ouabain signaling has been shown to depend on mitoK_{ATP} opening and mitochondrial ROS production [35]. Ouabain is cardioprotective in rat heart [31,36,37], and this cardioprotection is blocked by the mitoK_{ATP} blocker 5-hydroxydecanoate (5-HD), the ROS scavenger N-2-mercaptopropionylglycine (MPG), and the src kinase inhibitor PP2 [36]. It is interesting to note that, whereas inhibition of the pump and consequent increase in intracellular Na^+ and Ca^{2+} is required for positive inotropy, ouabain cardioprotection occurs at doses (about 10 μM in rat) that do not produce significant enzyme inhibition [37] or increased contractility [31,36,37]. These distinctions further emphasize the dissociation of the pumping and signaling functions of Na,K-ATPase. Ouabain cardioprotection does not depend on guanylyl cyclase or PKG activities, showing that this signaling pathway differs from that triggered by GPCR agonists [36]. Ouabain-induced inotropy also requires mitoK_{ATP} opening and ROS production [36,38].

The rat heart Na,K-ATPase exhibits a low sensitivity to cardiac glycosides; however, we have observed qualitatively similar phenomena in the ouabain-sensitive rabbit heart. Thus, cardioprotection occurs at lower ouabain doses than those required for inotropy, and both cardioprotection and inotropy require mitoK_{ATP} opening (S. Pierre, unpublished data).

3. From receptor to mitochondria by signalosomes

We propose that cardioprotective signals are transmitted to mitochondria by signalosomes, which are vesicular, multimolecular signaling complexes that are assembled in caveolae and deliver signals

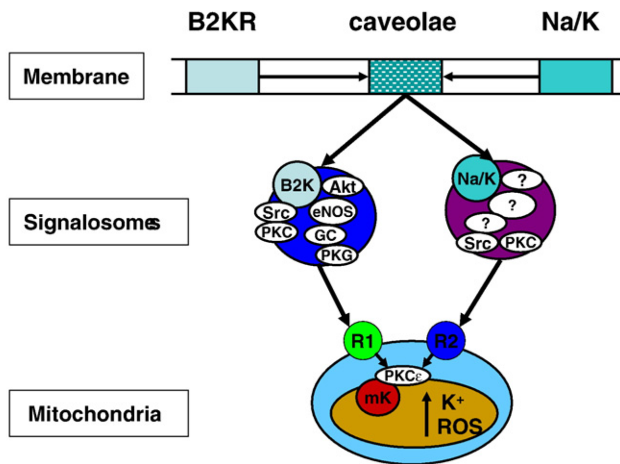

Fig. 2. Signal transmission by signalosome. It is proposed that interaction of bradykinin (and other GPCR agonists) or ouabain with their plasma membrane receptors induces formation of a vesicular caveolar signaling platform (signalosome) that phosphorylates rreceptors (R1 and R2) on the mitochondrial outer membrane (MOM). The terminal kinase of the bradykinin signalosome is PKG. The terminal kinases of the ouabain signalosome are PKCε and Src kinase. Following phosphorylation of the MOM receptor, the signal is transmitted across the intermembrane space to activate PKCε on the mitochondrial inner membrane. PKCε, in turn opens mitoK_{ATP} by a phosphorylation reaction [32].

to the mitochondrial outer membrane (MOM) [39]. A diagram of the signalosome hypothesis is presented in Fig. 2.

3.1. Rationale

Signaling cascades such as the one portrayed in Fig. 1 must occur rapidly and precisely. We consider it unlikely that these spatio-temporal requirements can be met by random diffusional collisions. Cytosolic proteins are extensively hydrated, and the organization of this water causes a phase separation from bulk cytosolic water. Minimization of the phase boundary, in turn, causes proteins to coalesce within their common hydration phase [40]. If the proteins of the cardioprotective signaling pathway were randomly distributed in the cytosol, they too would coalesce within the hydration phase, with losses of directionality and specificity. Accordingly, we suggest that the signaling cascade is compartmentalized to promote metabolic channeling and that the entire reaction sequence moves through the cytosol as a unit. This hypothesis agrees with and extends the proposal by Ping and coworkers [41,42] that intracellular signaling involves assembly and regulation of multiprotein complexes.

3.2. The signalosome hypothesis

Upon activation, GPCR migrate to caveolae, where caveolins organize and compartmentalize receptors and signaling molecules [43–49]. Caveolae are 50–100 nm membrane invaginations that are rich in cholesterol, sphingolipids, and caveolin proteins [50]. EGFR, the Src family of tyrosine kinases, G-protein α-subunits, PKC isoforms, and transporters such as the Na/K-ATPase have been found to associate with caveolins, which also regulate the activity of many of these proteins [50–56]. Caveolar assembly of the signaling platform is followed by budding off and internalization [57]. Receptor endocytosis has been observed for both GPCRs [45,47,58–60] and the α1-subunit of Na,K-ATPase [61]. We propose, therefore, that the receptor-specific signaling platform is assembled in caveolae, then separates and internalizes as a signalosome. The signalosome migrates via the cytoskeleton to mitochondria, where it binds to receptors on the MOM, designated in Fig. 2 as R1 (for GPCR-induced signalosomes) and R2 (for ouabain-induced signalosomes). The terminal kinase of the signalosome phosphorylates its specific receptor, which causes the signal to be transmitted across the MOM and intermembrane space to PKCε1 on the mitochondrial inner membrane. This is followed by the intramitochondrial signaling pathway described in Section 4 and Fig. 4.

3.3. Experimental evidence for signalosome-mediated mitoK_{ATP} opening

We developed protocols for purifying signalosomes from hearts subjected to various preconditioning or postconditioning protocols and then assayed their functional activity [39]. When the signalosome fractions were added to mitochondria from untreated hearts, they caused mitoK_{ATP} opening, as shown in Fig. 3. Based on the finding that functionally active signalosomes were obtained from hearts exposed to bradykinin, ouabain, ischemic preconditioning, and ischemic postconditioning (Fig. 3), we conclude that this is a general mechanism of signal transmission. Signalosome preparations also inhibited MPT when added to mitochondria from untreated hearts. The signalosomes were dissolved by the cholesterol binding agent methyl-β-cyclodextrin and were resistant to Triton X-100. These properties support their origin in caveolae. Electron microscopy reveals that the signalosomes are 100–140 nm in diameter and can be decorated with immunogold labeled caveolin 3 antibodies [39]. The signalosome induced by bradykinin stimulation contains eNOS, guanylyl cyclase, and cGMP-dependent protein kinase (PKG), and we were able to demonstrate the participation of each of these enzymes in the mitoK_{ATP} assay when proper substrates were supplied (Quinlan and Garlid, unpublished).

3.4. Signalosomes phosphorylate MOM receptors

As shown in Fig. 3, addition of signalosomes from preconditioned hearts to mitochondria from non- preconditioned hearts results in activation of the mitoK_{ATP}. Activity of signalosomes induced by GPCR-mediated protection (bradykinin, IPC, and postconditioning) is inhibited by KT5823, and we conclude that GPCR signalosomes use PKG as the terminal kinase that interacts with mitochondria. In contrast, the activity of signalosomes induced by ouabain is not inhibited by KT5823, in agreement with the finding that KT5823 does not block protection by ouabain [36]. Activity of the ouabain signalosome is inhibited by preincubation with $εV_{1-2}$ and PP2 (both

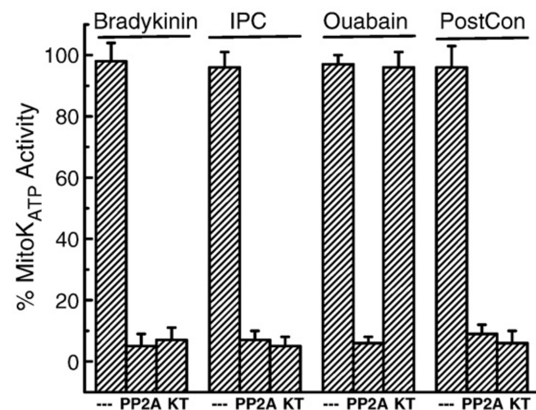

Fig. 3. Signalosomes from treated hearts induce mitoK_{ATP} opening in mitochondria from untreated hearts. Shown are changes in "MitoK_{ATP} activity (%)" when signalosome fractions from rat hearts treated with bradykinin, ouabain, ischemic preconditioning, or ischemic postconditioning were added to assay mitochondria from untreated rat hearts. Ischemic preconditioning consisted of two 5 min ischemia–reperfusion cycles, and ischemic postconditioning consisted of six 10 s cycles of ischemia–reperfusion. Also shown are the effects of PP2A (11 ng/mL) and KT5823 ("KT", 0.5 μM) added to the assay. Note that PP2A cannot cross the MOM of intact mitochondria, so its effect is on the MOM. Data are averages ± SD of 3 to 4 independent experiments. The figure is from Quinlan et al. [39].

are required), indicating that the terminal kinases are PKCε and Src kinase. We have found that recombinant PKG [32,62] or recombinant PKCε plus Src (Quinlan and Garlid, unpublished) can also induce mitoK$_{ATP}$ opening in isolated mitochondria.

That the signalosomes interact with the MOM was demonstrated by the finding that neither the signalosomes nor the recombinant terminal kinases induce mitoK$_{ATP}$ opening in mitoplasts lacking the MOM [32,39]. That signalosomes interact by phosphorylation was demonstrated by the finding that activity was blocked in the presence of the Ser/Thr phosphatase PP2A (Fig. 3). The ouabain signalosome was also blocked by tyrosine phosphatase, confirming the action of its Src kinase.

We have not yet determined the molecular identity of R1. R2 is an endogenous MOM MAP kinase, as revealed by Western blot showing increased phosphorylation of p38 MAPK (Thr 180/Tyr 182) after the heart was treated with ouabain and functional studies showing that the MAP kinase inhibitor SB203580 blocked mitoK$_{ATP}$ opening by the ouabain signalosome (Quinlan and Garlid, unpublished studies).

3.5. Signal transmission from MOM to PKCε1

Signalosome-dependent mitoK$_{ATP}$ opening is also blocked by the PKCε inhibitors chelerythrine and $εV_{1-2}$ [62], confirming a role for PKCε ("PKCε1" in Fig. 2), which is discussed in the next section. Signaling from R1 or R2 to PKCε1 is not prevented by MPG, and therefore this step does not involve ROS. This is all we know at this stage about the nature of the link between the MOM receptors and PKCε1.

4. Intramitochondrial signaling

The diagram in Fig. 4 summarizes several years of studies on intramitochondrial signaling [12,32,39,62–66]. The primary function of this pathway is to inhibit MPT opening, which is widely considered to be the cause of cell death after ischemia–reperfusion [5,6,11].

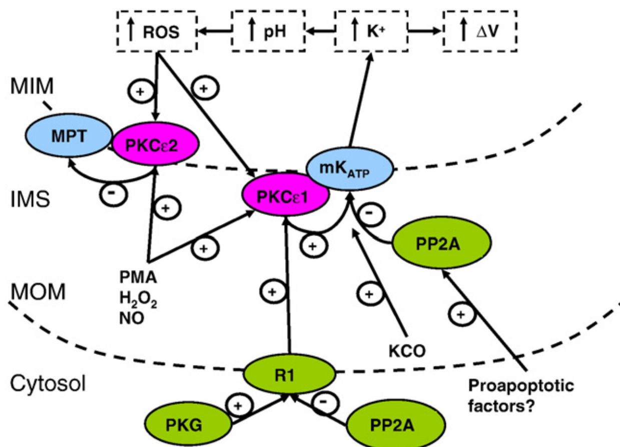

Fig. 4. The intramitochondrial signaling pathways. The pathways leading to mitoK$_{ATP}$ opening, ROS production, and MPT inhibition are shown. Signals arising from Gi-coupled receptors are delivered to mitochondria via the terminal kinase PKG. Signals arising from ouabain action upon the Na-K ATPase are delivered to mitochondria via two terminal kinases, PKCε and Src. The terminal kinases are localized in the signalosomes. PKG phosphorylates an unknown MOM receptor, "R1", whereas PKCε and Src act in conjunction to activate a distinct MOM receptor, "R2". These MOM receptors transmit the signal by an unknown mechanism to PKCε1 located at the inner membrane. The activated PKCε1 phosphorylates and opens mitoK$_{ATP}$. PKCε1 activity is likely to be counteracted physiologically by Ser/Thr protein phosphatases (PPase) such as PP2A. MitoK$_{ATP}$ opening via PKCε1 or by K$_{ATP}$ channel openers such as diazoxide causes K^+ uptake, increased matrix pH, and increased ROS production from ComplexI. ROS produced by mitoK$_{ATP}$ activity now diffuses and activates both PKCε1 and PKCε2. PKCε2 inhibits MPT, thus reducing cell necrosis and infarct size. The figure is from Costa and Garlid [32].

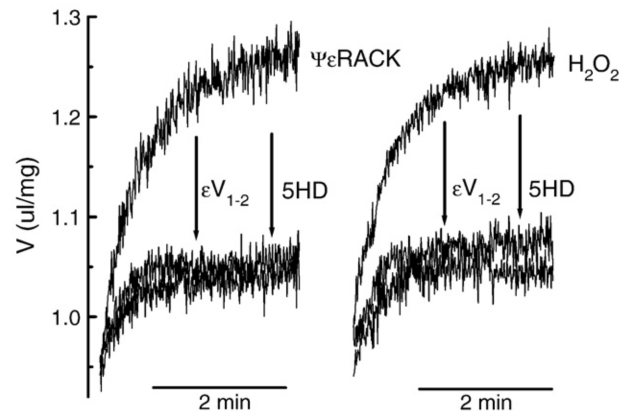

Fig. 5. PKCε-mediated mitoK$_{ATP}$ opening. Changes in mitochondrial matrix volume (V) are plotted versus time. Rat heart mitochondria (0.1 mg/ml) were suspended in standard assay medium [32]. H_2O_2 (2 μM) or ΨεRACK (0.5 μM) were added to medium in the presence of ATP (0.2 mM) approximately 1 s after the mitochondria. Other additions to the assay medium were 5-HD (0.3 mM) and $εV_{1-2}$ (0.5 μM). Traces are representative of at least 5 independent experiments. The figure is from Costa and Garlid [32].

4.1. Step one — opening mitoKATP by activation of PKCε1

Ischemic preconditioning, ischemic postconditioning, and pharmacological preconditioning by plasma membrane receptor agonists cause mitoK$_{ATP}$ opening by activating a PKCε that is constitutively expressed in mitochondria and associated with the mitochondrial inner membrane [64]. The PKCε-specific peptide agonist ΨεRACK and the PKCε agonists H_2O_2, NO, and phorbol 12-myristate 13-acetate (PMA) each open mitoK$_{ATP}$ (see Fig. 5) [32]. That these agents were acting via PKCε ("PKCε1" in Fig. 4) was verified by showing that the PKCε-specific binding antagonist $εV_{1-2}$ blocked all four modes of PKCε activation of mitoK$_{ATP}$ but did not block mitoK$_{ATP}$ opening by diazoxide [32,64]. Neither the PKCδ-specific peptide antagonist, $δV_{1-1}$ nor a scrambled analog of $εV_{1-2}$ had any effect on H_2O_2-dependent mitoK$_{ATP}$ opening. Moreover, the PKC inhibitor Gö6983, which inhibits PKCα, PKCβ, PKCγ, and PKCζ, did not block PKCε-dependent mitoK$_{ATP}$ opening, excluding a role for these isoforms [62].

Jaburek et al. [64] showed that ΨεRACK and $εV_{1-2}$ open mitoK$_{ATP}$ in liposomes reconstituted with partially purified mitoK$_{ATP}$. Thus, mitoK$_{ATP}$ and PKCε copurify and remain associated during multiple purification steps carried out in the presence of Triton X-100. This, together with the finding that PKCε remains associated with mitochondria in mitoplasts [12], suggests that PKCε and mitoK$_{ATP}$ are part of a functional complex. When given access in mitoplasts to the mitochondrial inner membrane ("MIM"), PP2A prevented mitoK$_{ATP}$-dependent swelling induced by PKCε agonists [32], and we conclude that PKCε-dependent mitoK$_{ATP}$ opening requires phosphorylation, perhaps of mitoK$_{ATP}$ itself.

PKCε requires anionic phospholipids for activity, a requirement met in this case by cardiolipin, which is abundant in mitochondria. PKCε is activated physiologically by diacylglycerol (or phorbol ester) or by a sulfydryl oxidizing agent, such as H_2O_2 [67] or NO [32]. PMA or H_2O_2 open up one of the two zinc fingers in PKCε [68, 69]. ΨεRACK, PMA, H_2O_2, or NO cause conformational changes that expose the substrate domain on PKCε and cause its binding to its RACK (receptor for activated C kinase [70]). ΨεRACK is a PKCε-specific peptide agonist that acts by regulating intramolecular PKCε binding, and $εV_{1-2}$ is a PKCε-specific peptide antagonist that acts by preventing protein–protein interactions between PKCε and its binding protein [70–72]. Murriel and Mochly-Rosen [73] found that ΨεRACK protected cardiac cells from ischemic damage, whereas $εV_{1-2}$ caused a loss of protection.

4.2. Step two — mitochondrial K^+ uptake and its consequences

Once mitoK_{ATP} is opened, the increase in K^+ uptake leads to several changes in the matrix. Electrophoretic K^+ influx is balanced by electrogenic H^+ efflux driven by the respiratory chain. Uncompensated, this would cause an increase of matrix pH of about 2 pH units. Partial compensation is provided by electroneutral uptake of substrate anions, such as phosphate. The compensation is partial because the concentration of phosphate in the cytosol is much lower than that of K^+, and this imbalance leads to matrix alkalinization [65,66,74].

Matrix alkalinization now releases the K^+/H^+ antiporter from inhibition by matrix protons [75], causing K^+ efflux to increase in response to increased K^+ uptake until a new K^+ steady state is achieved. The uncoupling caused by increased futile cycling of K^+ induces is about 3% of the maximum activity of the electron transport chain. This low level of uncoupling reflects the low activity of mitoK_{ATP}, a property that is essential for mitochondrial survival. Thus, if we add sufficient valinomycin to double the mitoK_{ATP}-mediated K^+ influx, the MOM ruptures with loss of cytochrome *c* [65].

Uptake of K^+ salts and osmotically obligated water leads to increased matrix volume ("ΔV" in Fig. 4), which is the basis of the light scattering (LS) assay for mitoK_{ATP} activity [65]. LS is the only practical method to study this process in isolated mitochondria, because mitoK_{ATP}-dependent K^+ flux is rapid ($t_{1/2}\sim30$ s) and small in magnitude. This technique has been successfully employed by several laboratories to measure K^+ flux in mitochondria [76–80].

MitoK_{ATP}-dependent matrix alkalinization plays an essential role in intramitochondrial signaling. It causes Complex I to produce increased amounts of superoxide and its products, H_2O_2 and hydroxyl anion radical [66]. As seen below, the ROS produced by this mechanism play two important roles in cardioprotection, through their ability to activate PKCε.

We note that each of the consequences of mitoK_{ATP} opening is due specifically to the increased K^+ influx. Thus, valinomycin (approximately 1 pmol/mg mitochondria) duplicates the effects of KCO on K^+ uptake, respiration, matrix alkalinization, volume increase, ROS production, and MPT inhibition [12,65,66].

4.3. Step three — activation of PKCε by endogenous ROS

4.3.1. ROS activation of PKCε2 and inhibition of MPT

The increased ROS activates a second mitochondrial PKCε, "PKCε2" in Fig. 4, which inhibits the mitochondrial permeability transition ("MPT") in a phosphorylation-dependent reaction [12]. H_2O_2 and NO, but not superoxide, also activate PKCε2 and inhibit MPT [12,32]. The dichotomy between protective and damaging ROS was strikingly demonstrated in an experiment in which 100 μM H_2O_2 plus 2 μM free Ca^{2+} were used to induce MPT opening in heart mitochondria. This ROS-induced MPT opening was inhibited when the mitochondria were first conditioned with 2 μM H_2O_2 [12]. Thus, cardioprotective mitoK_{ATP} opening leads to inhibition of MPT, and, therefore, to reduction of cell death after ischemia–reperfusion injury [5,6,11].

The evidence for two distinct mitochondrial PKCε is that the specific agonist ΨεRACK can activate PKCε1and open mitoK_{ATP}, but it cannot activate the PKCε that regulates MPT [12]. This establishes a clear difference between the two PKCεs. Our tentative explanation for the observation is that PKCε2 faces the matrix side of the inner membrane. ΨεRACK is an anionic peptide that cannot enter the matrix, whereas εV_{1-2}, which inhibits both PKCε1 and PKCε2, can readily enter the matrix.

4.3.2. ROS activation of PKCε1 and feedback mitoK_{ATP} opening

The mitoK_{ATP}-dependent increase in ROS plays an additional role in cardioprotection. Note in Fig. 4 that PKCε1 is bypassed when KCOs are administered to the heart; however, we have found that PKCε1 is soon activated by mitoK_{ATP}-dependent ROS, leading to a persistent phosphorylation-dependent open state of mitoK_{ATP} [32]. These data define a new, positive feedback loop for mitoK_{ATP} opening, whose existence, which has been suggested by a number of authors [81–83], means that mitoK_{ATP} may be either upstream or downstream of PKCε, depending on the triggering stimulus. We suggest that feedback phosphorylation of mitoK_{ATP} is the mechanism of memory, which is seen with all PC triggers [84,85]. Thus, cardioprotective stimuli can be washed away from the system and the perfused heart remains protected from a major ischemic assault, thanks to phosphorylation of mitoK_{ATP}. We infer, but have not demonstrated, that mitoK_{ATP} opening is eventually reversed by an endogenous phosphatase ("PP2A" in Fig. 4) within the intermembrane space. For example, PP2A has been found in mitochondria where it is activated by proapoptotic factors [86].

4.4. Intramitochondrial signaling and the literature

The model in Fig. 4 helps to support and extend results of previous studies. Jiang et al. [87] observed PKC and 5-HD regulation of the human cardiac mitoK_{ATP} in lipid bilayers. Garg and Hu [88] showed that PKCε modulates mitoK_{ATP} activity in cardiomyocytes and COS-7 cells. Penna et al. [89] demonstrated that protection by postconditioning involves a redox mechanism and persistent activation of mitoK_{ATP} and PKC. Facundo et al. [80] showed that H_2O_2 induces mitoK_{ATP} activity in isolated mitochondria, but did not identify participation of PKCε. Zhang et al. [90] found that superoxide anion activated mitoK_{ATP} in planar bilayers, and we showed that this effect is mediated, not by superoxide, but by H_2O_2 acting indirectly via PKCε1 [32]. Sasaki et al. [91] suggested that NO may open mitoK_{ATP} directly; however mitoK_{ATP} opening by NO is blocked by εV_{1-2} [32], showing that NO opens mitoK_{ATP} indirectly through PKCε1. Several authors have shown that exogenous and endogenous NO are cardioprotective and have attributed this effect to MPT inhibition [92–95]. Brookes et al. [92] showed that NO inhibited MPT and cytochrome *c* release in isolated liver mitochondria. We showed that this effect occurs via activation of PKCε2 [32]. Korge et al. [96] found that diazoxide prevented MPT opening and cytochrome *c* loss, and that both effects were mimicked by the PKC activator PMA and blocked by 5-HD. Kim et al. [97] found that a cytosolic extract, together with cGMP and ATP, blocked MPT in isolated mitochondria, an effect that was blocked by PKG inhibition. Forbes et al. [98] and Pain et al. [85] found that N-acetylcysteine or MPG reversed the protective effect of diazoxide in perfused hearts. Our data suggests that blockade of protection occurred because mitoK_{ATP}-dependent ROS was scavenged and unavailable to activate PKCε2 and inhibit MPT. Lebuffe et al. [81] found that PMA-induced protection was blocked by 5-HD and that this block was reversed by coadministration of H_2O_2 and NO. This is also consistent with the model of Fig. 4 in that H_2O_2 and NO can bypass the blocked mitoK_{ATP} and act directly on PKCε2, thereby inhibiting MPT and protecting the heart.

5. Other mitochondrial mechanisms of cardioprotection

5.1. K_{ATP} channel openers (KCO)

KCOs have been shown to be cardioprotective in all species examined [99,100]. The ability of KCOs to open mitoK_{ATP} in their therapeutic dose range was described in 1996 [101]. Diazoxide was 1000 times more potent in opening mitoK_{ATP} than in opening sarcK_{ATP}, making diazoxide a valuable tool to determine whether cardioprotection was mediated by the sarcolemmal or the mitochondrial K_{ATP} channel. It was found that diazoxide was as effective as cromakalim in protecting the heart. Moreover, diazoxide protection, unlike that mediated by cromakalim, was not accompanied by APD shortening, thus demonstrating that cardioprotection was not due to sarcK_{ATP} opening. These findings led to the hypothesis that mitoK_{ATP} is the

receptor that mediates the cardioprotective effects of KCOs [101,102]. KCOs act on the regulatory sulfonylurea receptors (SUR) of K_{ATP} channels. Pinacidil, cromakalim, and nicorandil are effective openers of cardiac K_{ATP} through their action on SUR2A, but ineffective on pancreatic beta cell K_{ATP}, which uses SUR1. Conversely, diazoxide is an effective opener of beta cell K_{ATP}, but ineffective on the cardiac channel [101,103]. All KCOs we have examined open mitoK_{ATP} and protect the heart [65,101,102,104–108].

5.2. Glycogen synthase kinase-3β (GSK-3β)

The GSK-3β inhibitors lithium and SB 216763 are cardioprotective. IPC and diazoxide cause phosphorylation and inactivation of GSK-3β [83,109], suggesting that GSK-3β may be downstream of mitoK_{ATP}. Inhibition of GSK-3β has no effect on MPT opening in isolated mitochondria [32], suggesting that the GSK isoform that interferes with cardioprotection resides outside of mitochondria. Importantly, cardioprotection by GSK-3β inhibition is blocked by 5-HD [110], indicating that the ultimate deleterious effect of GSK-3β activity may be to cause mitoK_{ATP} inhibition.

5.3. Amobarbital

Amobarbital, administered 1 min before 25 min global ischemia, is cardioprotective in rat, causing marked improvement of contractile function and reduction of infarct size [111]. Amobarbital is a short-acting barbiturate that is a classic, reversible inhibitor of Complex I at the rotenone site. Amobarbital treatment was associated with preservation of cytochrome *c* [111], which is otherwise released after ischemia–reperfusion, due in part to oxidative degradation of cardiolipin [112]. Cardioprotection by amobarbital is consistent with the authors' overall hypothesis that ROS arising from Complex III during ischemia causes mitochondrial damage that contributes to myocardial injury during reperfusion [113].

5.4. Bromoenol lactone (BEL)

BEL, administered before global ischemia, is cardioprotective in rat [114] and rabbit [115], causing marked reduction of infarct size. BEL is a specific inhibitor of calcium-independent phospholipase A(2) (iPLA2), which is the major phospholipase A(2) in myocardium and is present in heart mitochondria [115]. Ischemia causes fatty acid accumulation in the heart, caused by phospholipase-mediated degradation of membrane phospholipids [112,116,117]. Protection by BEL was reversed, in both rat and rabbit hearts, by the simultaneous perfusion of 5-HD, implying participation of mitoK_{ATP}.

5.5. Hydrogen sulfide

H_2S, administered before global ischemia, is cardioprotective in rat [118–120]. H_2S is synthesized in the heart and other tissues by cystathionine λ-lyase. Cytoplasmic [H_2S] is determined by the balance between its constitutive production and its oxidation by mitochondria. When tissue oxygen levels fall, H_2S oxidation decreases, and [H_2S] increases, and Olson et al. [121] consider H_2S to be the oxygen sensor of cells. Protection by H_2S was abolished by chelerythrine, implicating participation of PKC [119]. Infarct size reduction by H_2S was also abolished by glibenclamide and 5-HD, implicating participation of mitoK_{ATP} in H_2S protection [120]. H_2S increased the open probability of sarcolemmal K_{ATP} in cardiomyocytes [122] and may have a similar effect on mitoK_{ATP}.

5.6. Mitochondrial aldehyde dehydrogenase (ALDH2)

An activator of ALDH2, administered before global ischemia, is cardioprotective in rat, causing marked reduction of infarct size [123]. This recent discovery was the result of a directed proteomic search. The authors speculate that protection by ALDH2 activation is due to its metabolism of cytotoxic aldehydes, such as 4-hydroxynonenol.

5.7. Ca^{2+}-activated mitochondrial K^+ channel (mitoK_{Ca})

NS1619, an activator of the large conductance mitoK_{Ca} is cardioprotective in guinea pig, and protection is blocked by the inhibitor paxilline [124–126]. Sato et al. [127] found that there was no cross-talk between mitoK_{ATP} and mitoK_{Ca} — that is, paxilline blocked effects of NS1619 but not diazoxide, and 5-HD blocked effects of diazoxide but not NS1619. Cao et al. [125] observed similar absence of cross-talk in cardioprotection experiments. The latter findings suggest distinct channels with distinct pharmacology and suggest that these two channels constitute alternative mechanisms for raising matrix K^+ and generating ROS.

6. Summary

Recent years have brought robust advances in our understanding of cardiac signal transduction during cardioprotection against ischemia–reperfusion and the pivotal role of mitochondria in these processes. This review exposes our current understanding of the mechanistic link between plasma membrane receptors and MPT, the ultimate mitochondrial target of cardioprotection. We suggest that interaction of the cardioprotective ligand with its receptor induces the formation of a signaling platform that is scaffolded by caveolins, that contains the activated enzymes of the pathway, and that is delivered to mitochondria as a signalosome. We believe that the signalosome mechanism [39] provides a means to resolve the mystery of receptor-specificity described by Downey et al. [14]:

> "It is still a mystery how identical Gi proteins, when activated by binding of the different agonists to their individual receptors, can initiate such distinct signaling pathways."

This review also points to several areas that require further investigation as to how signalosomes are directed to mitochondria, how cytoskeleton is involved, and what receptors are involved at the mitochondrial level. Even in light of these questions, the elucidation of the interactions among signaling components and mitochondria is a valuable tool for understanding the molecular controls in the decision between cell survival and cell death.

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