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**Review**

# cGMP signalling in pre- and post-conditioning: the role of mitochondria

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Much of cell death from ischaemia/reperfusion in heart and other tissues is generally thought to arise from mitochondrial permeability transition (MPT) in the first minutes of reperfusion. In ischaemic pre-conditioning, agonist binding to $G_i$ protein-coupled receptors prior to ischaemia triggers a signalling cascade that protects the heart from MPT. We believe that the cytosolic component of this trigger pathway terminates in activation of guanylyl cyclase resulting in increased production of cGMP and subsequent activation of protein kinase G (PKG). PKG phosphorylates a protein on the mitochondrial outer membrane (MOM), which then causes the mitochondrial $K_{ATP}$ channel (mito$K_{ATP}$) on the mitochondrial inner membrane to open, leading to increased production of reactive oxygen species (ROS) by the mitochondria. This implies that the protective signal is somehow transmitted from the MOM to its inner membrane. This is accomplished by a series of intermembrane signalling steps that includes protein kinase C (PKC$\varepsilon$) activation. The resulting ROS then activate a second PKC pool which, through another signal transduction pathway termed the mediator pathway, causes inhibition of MPT and reduction in cell death.

## 1. Introduction

Cyclic nucleotides play key roles in regulating cardiac function. cAMP and cGMP act as second messengers for signalling by sympathetic and parasympathetic agonists, nitric oxide (NO), and natriuretic peptides. The effects of cAMP on heart physiology have been studied extensively, and signalling via the $\beta$-adrenergic receptor/protein kinase A pathway plays a key role in excitation–contraction coupling, gene transcription, and inotropy.[1–4] In ischaemic pre-conditioning (IPC), cGMP-mediated signalling protects the heart against ischaemia–reperfusion injury through mechanisms described below that involve mitochondria. cGMP may also protect through its effects on $Ca^{++}$ signalling[5] and hypercontracture.[6] In IPC brief cycles of ischaemia–reperfusion administered prior to a lethal ischaemic insult or even briefer cycles of reperfusion–ischaemia administered immediately after the ischaemic insult (post-conditioning) attenuate ischaemic cardiac damage. IPC exhibits two windows of cardioprotection: *acute*, lasting for 60–120 min, and *delayed*, observed 24–72 h after the pre-conditioning stimulus. All of these protective protocols involve cGMP-mediated signalling. The two targets for these signals are the mitochondrial ATP-sensitive $K^+$ channel (mito$K_{ATP}$) and the mitochondrial permeability transition (MPT). There are a host of opportunities for translating these signalling mechanisms into pharmacological post-conditioning. This new generation of agents capable of protecting the heart when administered at the time of reperfusion should provide powerful new tools that can reduce mortality and morbidity associated with acute myocardial infarction.

## 2. Mechanism of classical ischaemic pre-conditioning

Description and elucidation of the mechanism of the seemingly paradoxical IPC have provided the critical key to understanding cardioprotection. It had long been recognized that loss of viable myocardium was the primary problem in patients with acute myocardial infarction. The first breakthrough came when it was demonstrated that early reperfusion would limit infarction,[7] and that was quickly translated into clinical practice by the introduction of

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thrombolytic agents which effectively restored blood flow to the ischaemic myocardium. Because thrombolytic therapy did not completely eliminate infarction, scientists wondered whether the heart could be made resistant to ischaemia–reperfusion injury so infarct size could be further reduced for any given duration of ischaemia. Interventions such as anti-inflammatory agents and free radical scavengers were tested, but unfortunately with unequal success amongst the various investigators.[8] The impediment was lack of knowledge of what was actually killing ischaemic myocardium, and it was unknown if this goal of infarct reduction was even theoretically possible. All that changed when Murry *et al*.[9] described IPC. In that study, hearts were pre-conditioned with four series of 5-min coronary occlusions, each followed by 5 min of reperfusion, and these preceded a 40-min ischaemic insult. Infarct size was reduced to about a quarter of that in non-pre-conditioned hearts, despite the additional 20 min of ischaemia. This protection was unrelated to collateral flow or haemodynamics, and was truly a change in the vulnerability of the cardiomyocytes to ischaemia/reperfusion. Unlike previous interventions, IPC was reproducible by all who tried it, regardless of the species. Thus, it was indeed possible to make the heart resistant to infarction. The next challenge was to understand its mechanism. Today, more than 20 years after the initial breakthrough of Murry *et al*., we still do not fully understand IPC, but we have a grasp on many of the fundamental processes involved. The steps in the signalling pathway outlined below have mainly been documented in small animals: rabbit, rat, and mouse. Only key signalling elements like $K_{ATP}$ channels, protein kinase C (PKC) and $G_i$-protein-coupled receptors (GPCRs) have been confirmed in pigs and dogs. Even less is known about human hearts.

## 2.1 Ischaemic pre-conditioning's trigger pathway

IPC can be divided into two distinct phases: trigger and mediator. Events during the pre-conditioning occlusion/reperfusion cycles trigger onset of the protected phenotype which persists for several hours despite restoration of blood flow. IPC's protection against infarction is thought to be mediated early in the reperfusion period following the lethal ischaemic insult. The trigger phase is summarized in *Figure 1* (for a review of the trigger pathway see[10]). During the pre-conditioning ischaemia, the heart releases bradykinin and an endogenous opioid, and produces adenosine as a result of metabolic breakdown of ATP. These three ligands occupy their respective GPCRs which ultimately work in parallel to activate PKC. Any one of these agonists can fully pre-condition the heart. Blockade of any single receptor raises the threshold for protection but does not eliminate it if the other two remain active.

The pathway by which each of these ligands activates PKC is quite different, as shown in *Figure 1*. Bradykinin and opioid both activate phosphatidylinositol 3-kinase (PI3K), which phosphorylates membrane phospholipid's position 3 of the inositol moiety. Phosphatidylinositol trisphosphate then activates phospholipid-dependent kinases (PDK) which in turn cause phosphorylation and activation of Akt. PKG plays an important role in this pathway and will be discussed in detail later in this review, but a brief overview will be presented. Akt phosphorylates endothelial NO synthase (eNOS), causing it to generate NO. We had previously raised doubts about the importance of NO in IPC based on the failure of $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME) to block IPC in isolated hearts, a model dependent mainly on adenosine to trigger the signalling cascade. That is because NO is not in adenosine's trigger pathway (*Figure 1*).[11] However, in the *in situ* heart in which bradykinin and opioid receptors are also involved, NO is critical and L-NAME does block IPC.[11] NO activates soluble guanylyl cyclase (GC) causing it to make cGMP which then activates protein kinase G (PKG). PKG acts on mitochondria, causing opening of mito$K_{ATP}$ on the inner membrane. Opening of mito$K_{ATP}$ has two known actions: swelling of the matrix and generation of reactive oxygen species (ROS).[12,13] PKC has long been known to play a central role in pre-conditioning.[14,15] It is also known that ROS can directly activate PKC.[16] We believe that ROS produced by mitochondria act as second messengers to activate PKC through redox signalling.[17,18] ROS production is deficient in mice genetically engineered to lack connexin 43, a protein on the inner mitochondrial membrane that forms gap junctions, and this may account for the loss of IPC's protection in connexin 43 knockout mice.[19] Protection from a pulse of bradykinin or opioid can be blocked by co-administration of either wortmannin,[20,21] a PI3K inhibitor, L-NAME,[22] an eNOS inhibitor, ODQ,[22] a GC inhibitor, 5-hydroxydecanoate (5HD),[23] a mito$K_{ATP}$ blocker, or a ROS scavenger like N-acetyl cysteine (NAC) or N-(2-mercaptopropionyl) glycine (MPG).[23] The dependency on redox signalling explains why occupation of these receptors during ischaemia without IPC is not protective. Indeed, although the receptors are occupied during ischaemia, the brief reperfusion period is needed to supply oxygen for ROS generation that is required to complete the pathway.

Opioid receptors activate PI3K through transactivation of the epidermal growth factor receptor (EGFR).[24] The transactivation signalling sequence is initiated by the release of heparin-binding (HB) EGF through the action of a metalloproteinase cleaving a membrane-bound precursor.[25] The liberated HB-EGF then occupies the EGFR, causing it to dimerize, undergo transactivation through autophosphorylation of tyrosine residues, and form a signalling complex that includes activated PI3K. Bradykinin does not signal through the EGFR, although it does activate PI3K.[21] The mechanism by which it does so is unknown.

Although adenosine also activates PI3K,[26] it appears to have a second coupling to PKC that has not been fully elucidated. Both of adenosine's $G_i$-coupled receptors, $A_1$ and $A_3$ subtypes, can trigger PC. The $A_3$ receptor has been reported to activate PKC through its coupling to Rho.[27] As a result of these, more direct couplings to PKC, adenosine signalling largely bypasses the trigger cascade documented for opioid and bradykinin. Hence, activation of PI3K by adenosine is not the main event mediating this agonist's protection. Nevertheless, this observation is important because it could explain why blockade of the trigger pathway ending with mito$K_{ATP}$ opening and ROS generation will not abort adenosine-triggered protection.[23,28]

## 2.2 Ischaemic pre-conditioning's mediator pathway

Less is known about how IPC mediates its protection at reperfusion. For years, it was assumed that IPC protected by reducing injury during ischaemia. Then Hausenloy *et al*.[29]

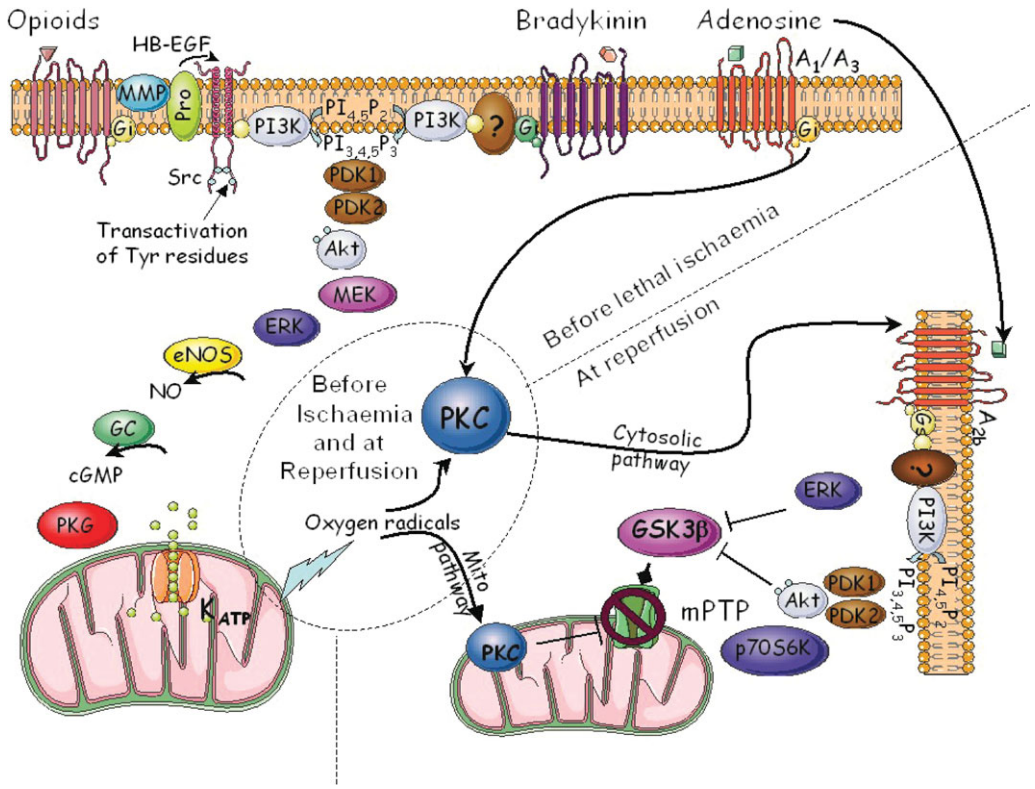


**Figure 1** Simplified signalling pathways of myocardial pre-conditioning. MMP; matrix metalloproteinases; HB-EGF, heparin-binding epidermal growth factor-like growth factor; Pro, pro-HB-EGF; PDK, phospholipid-dependent kinase; PI3K, phosphatidylinositol 3-kinase; $PI_{4,5}P_2$, phosphatidylinositol bisphosphate; $PI_{3,4,5}P_3$, phosphatidylinositol trisphosphate; MEK, mitogen activated protein kinase; ERK, extracellular-signal regulated kinase; NO, nitric oxide; NOS, NOS synthase; eNOS, endothelial NOS; GC, guanylyl cyclase; PKG, protein kinase G; PKC, protein kinase C; $K_{ATP}$, mitochondrial ATP-dependent potassium channel; p70S6K, p70S6 kinase; GSK-3β, glycogen synthase kinase-3β; MPT, mitochondrial permeability transition. (Modified from reference[94]).

discovered they could block IPC's protection with either PI3K or extracellular signal-regulated kinase (ERK) inhibitors given at reperfusion. Clearly, a protective event that was occurring early in the reperfusion period was controlled by these kinases. They referred to PI3K and ERK as reperfusion induced salvage kinases, or RISK. Hausenloy *et al*. also provided evidence of how these kinases might be protecting. MPT was first described by Hunter *et al*.,[30] and Halestrap and colleagues[31] proposed that under certain conditions this high-conductance pore or MPT can form in mitochondria, effectively connecting matrix with cytosol. When MPT forms, mitochondria are uncoupled and ATP production is blocked. The matrix swells which can cause the outer membrane to rupture. Drugs that block MPT like cyclosporin A mimic IPC, and those that promote MPT abort IPC's protection.[32] Interventions that trigger IPC also make mitochondria resistant to MPT,[33,34] and the RISK pathway inhibits MPT through GSK-3β.[35] MPT is promoted by ROS and elevated calcium and inhibited by low pH. MPT presumably is inhibited during ischaemia when pH is low and therefore occurs in the first minutes of reperfusion as pH normalizes (for a review of MPT see[36]). The MPT hypothesis of IPC implies that at the time of reperfusion the heart contains three populations of cells: those killed by ischaemia, those that will survive the insult, and a third population that is viable at reperfusion but will soon be killed by MPT. It is the latter population that IPC targets.

The signal transduction events that mediate IPC are beginning to be elucidated (*Figure 1*). Solenkova *et al*.[37] observed that adenosine receptors must be repopulated at reperfusion for IPC to protect the heart. Oddly a $G_s$-coupled $A_2$ adenosine receptor, presumably $A_{2b}$, was involved. Both Philipp *et al*.[38] and Kuno *et al*.[39] noted PKC had to be active at reperfusion and that directly activating PKC with phorbol ester at reperfusion would duplicate IPC's protection in a naïve heart. Furthermore, an $A_{2b}$-selective blocker aborted protection from the phorbol ester. Conversely, the $A_{2b}$-potent agonist NECA was protective at reperfusion, and chelerythrine, a PKC inhibitor, did not affect its protection.[38] Thus, PKC was upstream of the $A_{2b}$ receptor. Kuno *et al*.[39] noted PKC sensitizes signalling from the heart's $A_{2b}$ receptors. Because of their low affinity, these receptors are not populated by adenosine liberated by ischaemia. After PKC treatment, the threshold for activation is dramatically lowered and then endogenous adenosine can be protective during reperfusion. It is not known whether PKC targets the receptor itself or one of the coupling proteins downstream of the receptor. Protection from NECA can be aborted by either wortmannin, L-NAME, or by an ERK inhibitor (PD98059 or U0126)[40] showing that NOS and the RISK pathway reside downstream of the $A_{2b}$ receptor.

The exact function of PKG in the mediator phase is not clear. Because L-NAME blocks protection from the adenosine agonist NECA given at reperfusion,[40] a NOS step must reside downstream of the $A_{2b}$ receptor which in turn is downstream of PKC. Administering the PKG activator 8-(4-chlorophenylthio)-guanosine 3′, 5′-cyclic monophosphate to a heart at reperfusion is also protective.[41] Presumably PKG is downstream of NOS in this pathway, but this needs to be explicitly tested. Our unpublished studies suggest protection

from a direct PKG activator is dependent on activation of ERK and PI3K, indicating that the PKG activator protects through pre-conditioning's mediator pathway. Inhibition of phosphodiesterase-5 with sildenafil at reperfusion which would be expected to augment cGMP is also cardioprotective.[42] Furthermore, raising cGMP in the heart by activating particulate GC with natriuretic peptides at reperfusion reduces infarct size in rabbits.[41] In the large-scale J-WIND clinical trial recombinant atrial natriuretic peptide limited infarct size in patients with acute myocardial infarction undergoing coronary angioplasty.[43]

Solenkova *et al*.[37] observed that continuous Akt activity is required during the first hour of reperfusion in the IPC heart. Transient blockade of Akt activation any time in the first hour of reperfusion aborted protection, whereas Akt blockade thereafter had no effect on protection. Likewise occupancy of adenosine receptors and ERK activation must persist into reperfusion if cardioprotection is to be realized. It appears the RISK pathway protects the heart by inhibiting MPT for the first hour of reperfusion, during which injury incurred by mitochondria during ischaemia is repaired. Once the repair is completed, the heart is able to function without support of RISK. Finally, it should be pointed out ERK and Akt phosphorylate GSK-3β which then inhibits MPT.[35] Unlike most kinases, GSK-3β is constitutively active and is inhibited by phosphorylation. As a result, GSK-3β inhibitors mimic IPC.[20,44]

## 3. cGMP signalling in delayed pre-conditioning

When the heart is pre-conditioned with brief ischaemia or a receptor agonist, protection lasts for 1–2 h and then wanes. Some of that protection reappears 24 h later and may persist for up to 4 days. This second wave of protection is termed 'late' or 'second window' protection. It results from expression of protective proteins in the heart that provide long-term adaptation against ischaemia. Bolli's group has demonstrated that NO is an obligatory messenger in delayed pre-conditioning. Thus, L-nitro-arginine, a non-selective NOS inhibitor, blocks delayed PC,[45,46] and, conversely, exposure to exogenous NO in lieu of ischaemia results in late protection.[47] Some of the downstream signalling steps have been identified. Following NO production PKCε translocates to the membrane fraction followed by activation of the Raf1-MEK1/2-p44/p42 MAPK signalling cascade, phosphorylation of STAT 1/3, and upregulation of STAT-dependent genes including COX-2.[48] Recent investigations in $eNOS^{-/-}$ mice have confirmed and extended these pharmacologic studies.[49] Delayed cardioprotection cannot be induced in these mice with a standard IPC protocol, and there is no evidence of PKC translocation or activation of downstream signalling events. Thus, NO is a trigger in this pathway. This was confirmed when it was noted that exogenous NO (DETA/NO) induced rapid PKCε translocation, a robust increase in MEK1/2 and p44/p42 MAPK phosphorylation, and an increased expression of COX2 protein 24 h later.[49]

NO plays a dual role in delayed pre-conditioning: it is both a trigger during the initial ischaemic stress of the first day[50] and a mediator of cardioprotection 24 h later.[51] Kodani *et al*.[52] observed that the GC inhibitor ODQ administered before six cycles of brief ischaemia/reperfusion failed to prevent the cardioprotective effect of delayed pre-conditioning against either myocardial stunning or infarction 24 h later. On the other hand, ODQ infused on the second day completely aborted the late pre-conditioning effect against both stunning and infarction. Additionally, this mediator phase of delayed pre-conditioning is dependent on opening of $mitoK_{ATP}$,[53,54] and the delayed cardioprotective effect of the $mitoK_{ATP}$ opener diazoxide was blocked by L-NAME administered a few minutes prior to the index ischaemia on day 2.[54] Hence, NO must participate in delayed pre-conditioning in two distinct fashions. It triggers cardioprotection on the first day by a cGMP-independent mechanism, while it mediates protection on the second day through a cGMP-dependent pathway.

## 4. cGMP signalling in ischaemic post-conditioning

Protecting the myocardium with short cycles of reperfusion–ischaemia following a prolonged ischaemic period (ischaemic post-conditioning) has been demonstrated in dogs, rats, mice, pigs, rabbits, and humans[55–59] (reviewed by Gross and Gross[60]). It appears that most, if not all, of the signalling pathways in acute pre-conditioning are also involved in protection by post-conditioning. Most investigators find that combining the two interventions does not provide additional protection.[56] PI3K inhibition by LY294002 or wortmannin completely eliminates the infarct-size-limiting effects of post-conditioning.[56,61,62] Post-conditioning augments phospho-Akt levels similar to pre-conditioning and inhibition of PI3K blocks that increase,[5,61,62] which presumably results in decreased eNOS activity. Yang *et al*.[56] demonstrated that either the NOS inhibitor L-NAME or ODQ blocked the infarct-sparing effect of post-conditioning, and others have confirmed this observation.[63] Closure of $mitoK_{ATP}$ also blocked post-conditioning's protection.[56,64] Additionally, scavenging ROS at the time of reperfusion aborts post-conditioning's protection[64] as well as that from IPC.[65] Thus, many similarities are apparent between IPC and post-conditioning and signalling occurring during early reperfusion in the post-conditioned heart recapitulates that observed during the trigger phase of IPC. IPC is triggered in part by opioid receptors and Vinten-Johansen's group showed that ischaemic post-conditioning can be abolished by administration of the opioid receptor antagonist naloxone.[66] Furthermore, specific inhibition of κ or δ opioid receptor subtypes reversed the beneficial effects of post-conditioning on infarct size.[66] As in IPC, mitochondria removed from post-conditioned hearts are very resistant to induction of MPT[67,68] suggesting that inhibition of MPT may be the final step in cardioprotection's signalling pathway.

Finally, like IPC, post-conditioning is dependent on both PKC[38,69] and adenosine receptor activation.[38,70] The precise adenosine receptor subtype responsible for post-conditioning is controversial. Vinten-Johansen and co-workers believe it is the $A_{2a}$ and/or $A_3$ receptors which are involved.[70] However, we favour the $A2_b$ receptor.[38] We were unable to block post-conditioning with highly selective $A_1$ or $A_{2a}$ antagonists in the rabbit, but could with the $A_{2b}$ antagonist MRS 1754. We believe that the doses of putatively specific receptor subtype antagonists used in Kin's study[70]

were sufficiently high to make them non-selective thus rendering conclusions about specific receptor subtypes problematic.

### 4.1 The pH theory of post-conditioning

Why does the staccato reperfusion of post-conditioning protect? Cohen *et al.*[71] adapted the seminal observations of Lemasters and colleagues[72,73] and showed that persistent acidosis during the initial 2 min of reperfusion in rabbits could mimic the protection of post-conditioning, whereas a perfusate that promoted alkalosis in cardiomyocytes during post-conditioning cycles abolished its protection. Acidosis is known to prevent MPT, whereas alkalosis facilitates it. They hypothesized that during ischaemia injury to the mitochondria primes them for MPT, but acidosis prevents it. Restoration of flow quickly restores myocardial pH, thus prompting formation of MPT which halts ATP production and kills cells. During post-conditioning, however, normalization of pH is prevented by the cyclic coronary artery re-occlusions and MPT is inhibited. During the reperfusion phases of the reperfusion/ischaemia cycles, re-introduction of oxygen leads to generation of ROS that triggers IPC's signalling cascade. Once the IPC mechanism is in place, the survival kinases continue to inhibit MPT after the post-conditioning cycles have been suspended.[71]

## 5. Intramitochondrial signalling pathways

Mito$K_{ATP}$ appear to be involved in many modes of cardioprotection. Mito$K_{ATP}$ are normally closed *in vivo* because they are exposed to inhibitory concentrations of ATP and ADP. ATP inhibition of $K^+$ flux is pharmacologically reversed by $K_{ATP}$ channel openers such as diazoxide or cromakalim[12] and is physiologically reversed by phosphorylation.[74] As discussed in section 2, Oldenburg *et al.*[22] demonstrated that bradykinin triggers the protected phenotype by activating GC to produce cGMP, which then activates PKG. PKG was proposed to be the last step in the signalling pathway before involvement of the mitochondrion. This hypothesis was confirmed when it was shown exogenous PKG plus cGMP added to isolated mitochondria increased matrix volume and respiration to the same extent as cromakalim and diazoxide.[75] This effect was blocked by the specific PKG inhibitor KT5823, by mito$K_{ATP}$ blockers such as 5HD, glibenclamide, and $TPP^+$, by the PKC inhibitor chelerythrine, and by the PKCε isoform-specific inhibitor peptide, $\varepsilon V_{1-2}$.[75] PKG+cGMP triggered identical effects in mitochondria isolated from rat heart, liver, and brain, indicating that this is a general pathway. Thus, it seems clear that PKG is the terminal cytosolic kinase of the cardioprotective signalling pathway that originates with the bradykinin receptor.

PKG, which cannot cross the mitochondrial outer membrane (MOM), must phosphorylate a serine or threonine of an unknown MOM protein, labelled R1 in *Figure 2*. Little is known about PKG phosphorylation or binding proteins, and we presently do not have a candidate for R1. PKG-dependent mito$K_{ATP}$ opening seems to require an intact MOM and is reversed by the Ser/Thr phosphatase PP2A (Costa and Garlid, unpublished data). Phosphorylation of the MOM protein causes the signal to be transmitted to PKCε bound to the mitochondrial inner membrane (PKCε1 in *Figure 2*), which in turn phosphorylates mito$K_{ATP}$ and causes it to open.[75–77] Using phorbol 12-myristate-13-acetate (PMA), Korge *et al.*[33] showed PKC opened mito$K_{ATP}$. PKCε was shown to be specifically involved since mito$K_{ATP}$ opening was triggered by the specific activator ψεRACK and mito$K_{ATP}$ opening by all PKCε activators, including PMA, $H_2O_2$, and ψεRACK, was blocked by the specific inhibitor peptide, $\varepsilon V_{1-2}$.[76,77] Detergent extraction and chromatographic separation of mitochondrial membrane proteins, long used for reconstitution studies of mito$K_{ATP}$,[78] lead to co-reconstitution of PKCε with mito$K_{ATP}$.[76] This indicates that PKCε1 is actually bound to the inner mitochondrial membrane and associated in a complex with mito$K_{ATP}$. The association is fully functional. PKCε activators open mito$K_{ATP}$ and induce $K^+$ flux in vesicles. When mito$K_{ATP}$ are opened by a PKCε activator in liposomes, flux can be blocked by PP2A showing the importance of phosphorylation in this interaction.

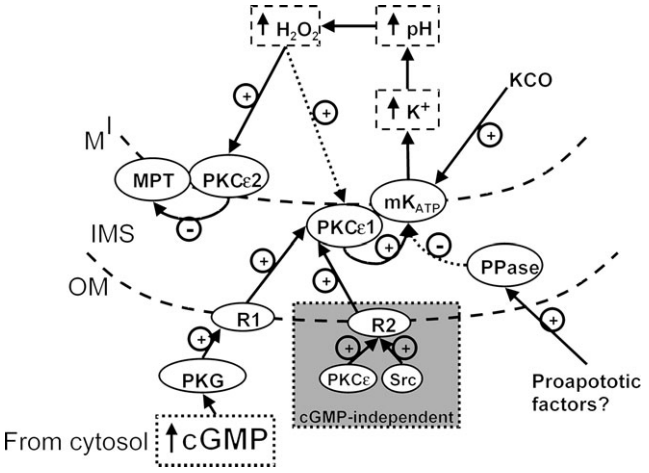


**Figure 2** cGMP-induced opening of mito$K_{ATP}$ triggers the intramitochondrial signalling pathway. Signals arising from $G_i$-coupled receptors are delivered to mitochondria via the terminal kinase PKG. PKG phosphorylates an unknown MOM receptor ('R1') which transmits the signal, by an unknown mechanism, to PKCε1 located at the inner membrane. The activated PKCε1 phosphorylates and opens mito$K_{ATP}$. PKCε1 activity is likely to be counteracted physiologically by Ser/Thr protein phosphatases (PPase) such as PP2A. Mito$K_{ATP}$ opening via PKCε1 or by $K_{ATP}$ channel openers such as diazoxide causes $K^+$ uptake, increased matrix pH, and increased $H_2O_2$ production from Complex I. $H_2O_2$ produced by mito$K_{ATP}$ activity now diffuses and activates both PKCε1 and PKCε2. PKCε2 inhibits MPT, thus reducing cell necrosis and infarct size. cGMP-independent protective mechanisms operate via a second unknown MOM receptor ('R2'), which also transmits the signal to mito$K_{ATP}$ via PKCε1.

As shown in *Figure 2*, the increase in $K^+$ influx that results from opening mito$K_{ATP}$ causes alkalinization of the matrix as $K^+$ replaces $H^+$ ejected by the respiratory chain.[12] Matrix alkalinization, in turn, retards the reduction of Q to $QH_2$ at Complex I of the respiratory chain, thus prolonging the lifetime of $Q^{\cdot-}$ anions and increasing the likelihood of single electron reduction of molecular oxygen to produce superoxide anion.[13,79,80] Mito$K_{ATP}$-dependent increases in pH and $H_2O_2$ production by rat heart mitochondria can be induced by a $K_{ATP}$ channel opener or by activation of the mito$K_{ATP}$-associated PKCε1. The increased pH and $H_2O_2$ are due specifically to an increase in $K^+$ influx, because the results are faithfully mimicked by administration of the potassium ionophore valinomycin at a concentration that catalyzes the same $K^+$ flux as opening of mito$K_{ATP}$.[12,13]

The intramitochondrial target for the mito$K_{ATP}$-dependent increase in ROS is a second pool of intramitochondrial PKCε,

termed 'PKCε2' in *Figure 2*, which can inhibit $Ca^{2+}$- and ROS-induced MPT. All activators of mitoK$_{ATP}$ including diazoxide, cromakalim, and nicorandil caused inhibition of MPT in isolated mitochondria. Importantly, the effect of each of these agents was replicated by valinomycin, showing that the key event is an increase in $K^+$ influx into the mitochondrial matrix. MPT was found to be inhibited by the direct PKC activator PMA[32] or by cGMP plus a cytosolic extract containing PKG.[81] MPT inhibition by PKG+cGMP could be prevented by a ROS scavenger or a mitoK$_{ATP}$ blocker whereas that by PMA could not. This suggested that there are actually two PKC pools in the mitochondria: one that acts to inhibit MPT downstream of ROS generation and a second that couples PKG's target to mitoK$_{ATP}$ opening that would be upstream of ROS production.[75]

Costa *et al*.[77] established that PKCε1, mitoK$_{ATP}$ and ROS are obligatory intermediates in MPT inhibition by signals coming from the cytosol. Thus, PKG-dependent inhibition of MPT is abolished by inhibitors of PKCε and mitoK$_{ATP}$ and by ROS scavengers. It was necessary to invoke a second pool of inner membrane PKCε for two reasons. First, PMA or $H_2O_2$ inhibited MPT even when mitoK$_{ATP}$ were blocked. Second, PMA inhibition of MPT was unaffected by ROS scavengers, again indicating a bypass of mitoK$_{ATP}$ and PKCε1. This association of PKCε2 with MPT is consistent with the finding of Baines *et al*.[82] that mice expressing PKCε targeted to cardiac muscle exhibited less $Ca^{2+}$-induced mitochondrial swelling and enhanced formation of a signalling complex between PKCε and proteins believed to be MPT components.

### 5.1 Involvement of GSK-3β

GSK-3β inhibition is cardioprotective[44] and Juhaszova *et al*.[35] showed that protection was related to inhibition of MPT. GSK-3β has been called a master switch immediately proximal to MPT and the end-effector of cardioprotection.[35] It should be pointed out, however, that most protective modalities involve inhibition of MPT. Whereas mitoK$_{ATP}$ opening has been shown to directly inhibit MPT in isolated mitochondria,[77] GSK-3β inhibition has no effect on isolated mitochondria, indicating that the kinase is cytosolic. Inhibiting GSK-3β, either pharmacologically or by phosphorylation, appears to be permissive, allowing the signal to travel from plasma membrane receptors to mitochondria.

### 5.2 Multiplicity of PKCε in cardioprotective signalling

The specific role of a given PKCε is determined entirely by its localization within the pathway. Multiple PKCε moieties participate in the various cardioprotective signalling pathways. Thus, there is at least one cytosolic isoform, as shown in *Figure 1*, and two mitochondrial isoforms, as shown in *Figure 2*. During ouabain signalling, there appear to be four PKCεs with distinct roles. In addition to the two mitochondrial enzymes, PKCε acts proximally in conjunction with EGFR transactivation,[83] and a second cytosolic PKCε phosphorylates receptor 'R2' on the MOM (*Figure 2*). Under physiological conditions, each PKCε is activated independently and phosphorylates its unique substrate. Since the kinases are biochemically identical, addition of general PKCε activators such as PMA should activate all of them, making it difficult to pinpoint their location within the signalling sequence.

### 5.3 Which reactive oxygen species does the signalling?

As stated earlier, ROS produced by mitochondria act as second messengers to activate cytosolic and mitochondrial PKC through redox signalling. ROS are also known to cause activation of other kinases, such as Akt[84] and p38 MAP kinase,[85] and these actions may also be mediated by ROS of mitochondrial origin. Superoxide is formed by single-electron reduction of oxygen in response, for example, to mitoK$_{ATP}$ opening.[13] $H_2O_2$ is formed by matrix superoxide dismutase, and hydroxyl radical is formed by the reaction of $H_2O_2$ with a Fenton reagent, a metal catalyst. We find that $H_2O_2$ and hydroxyl radical, but not superoxide, can activate mitochondrial PKCε. The question is whether $H_2O_2$ or hydroxyl radical does the signalling. $H_2O_2$ is a good candidate, because it is relatively stable and can diffuse to target kinases. Hydroxyl radical can be excluded as a second messenger *per se*, because it reacts with any reduced group it meets, its lifetime is short, and its diffusion distance is small. However, hydroxyl radical could act via a proxy, such as a lipid peroxidation product, which could move to kinases to oxidize their thiol groups. For example, 4-hydroxynonenal, the end product of lipid peroxidation, is known to be a signalling molecule.[86] We do find that hydroxyl radical is the oxidant that causes MPT inhibition through activation of PKCε2 (Costa and Garlid, unpublished). However, this is a special case, because these radicals are produced in the matrix by Complex I in response to mitoK$_{ATP}$ opening,[13] and there are indications that PKCε2 is localized on the matrix side of the inner membrane.[77] Thus, hydroxyl radical in this case is acting locally.

The scavengers MPG and NAC have been used to demonstrate the need for redox signalling in IPC[87] and diazoxide protection.[88] In the test tube, MPG is a selective scavenger that strongly quenches hydroxyl radical and peroxynitrite and only weakly scavenges superoxide or hydrogen peroxide.[89] NAC has similar properties. It is not clear, however, that these test tube results can be applied generally to the intact heart or to mitochondria. MPG and NAC maintain a reducing environment in cells, buffer cellular antioxidant capacity and maintain glutathione levels, and these actions may reduce $H_2O_2$ levels below their signalling threshold. In summary, it is accurate to say that ROS act as second messengers, but it is not yet known whether signalling occurs via $H_2O_2$ or a diffusible product of hydroxyl radical oxidation.

### 5.4 Can the intramitochondrial mechanism of MPT inhibition explain it all?

ROS production in the first minutes of reperfusion is required for IPC's protection,[65,90] indicating that ROS are part of the mediator pathway at reperfusion. Activating PKC in the first moments of reperfusion with PMA mimics IPC's anti-infarct effect and this protection can be blocked by an adenosine $A_{2b}$ receptor blocker.[38,39] These data place an adenosine receptor between the cytosolic PKC and inhibition of MPT. In this model a ROS scavenger does not block PMA's protection, because ROS and PMA are independent activators of PKC. Protection from PMA is also blocked by wortmannin[38] putting PI3K downstream of PKC. An $A_{2b}$ agonist given at reperfusion is also protective[39] and its protection can be blocked by either an ERK or a PI3K inhibitor[40] suggesting

that those kinases reside downstream of the $A_{2b}$ receptor. Taken together, these data suggest that at reperfusion ROS activates PKC in the cytosol which, through the $A_{2b}$ receptor, inhibits MPT in an ERK- and PI3K-dependent pathway. This cytosolic pathway presumably terminates at the MOM where it triggers the intramitochondrial signalling pathway described above. One important finding appears to conflict with this view: the $A_{2b}$-potent agonist NECA at reperfusion was protective, but chelerythrine, a PKC inhibitor, did not affect its protection.[38] Although all protective signalling must target mitochondria in order to inhibit MPT, this finding indicates that the mechanism is not fully resolved.

## 6. Opening mitoK$_{ATP}$ by non-cGMP pathways

cGMP is not the only signal that can open mitoK$_{ATP}$. The inotropic glycoside ouabain triggers cardioprotective signalling by a cGMP-independent pathway.[91] Ouabain binding to the $Na^+/K^+$-ATPase triggers a signalling cascade[92] that resembles the signalling triggered by IPC.[74] Ouabain signalling opens mitoK$_{ATP}$ and induces increased generation of mitoK$_{ATP}$-dependent ROS in cardiomyocyte mitochondria.[93] At sub-inotropic doses, ouabain protects rat hearts against infarction, promotes post-ischaemic functional recovery, and preserves adenine nucleotide compartmentalization in mitochondria.[83,91] A comparison of cardioprotective signalling by ouabain and bradykinin revealed that both are dependent on Src kinase, mitoK$_{ATP}$ activity, and increased mitochondrial ROS production. However, bradykinin protection was blocked by GC or PKG inhibitors, whereas ouabain protection was not.[91] A detailed examination of ouabain signalling suggests that the terminal cytosolic kinases of this pathway are Src and PKC$\varepsilon$ (Pasdois *et al.*[91] and unpublished data) and that they act in tandem to phosphorylate a p38 MAP kinase residing in the MOM (Costa and Garlid, unpublished data). Thus, there is more than one signalling pathway capable of inducing mitoK$_{ATP}$ opening and cardioprotection.

## 7. Summary

cGMP-mediated signalling is intimately involved in the pathways that trigger protection against ischaemia–reperfusion injury. These signalling pathways begin at the plasma membrane and carry messages to intracellular structures, including mitochondria. Emerging research indicates that the cytosolic signal is generally delivered to the MOM in the form of a phosphorylation event by PKG. The signal is transmitted across the intermembrane space to a PKC$\varepsilon$ that is associated with mitoK$_{ATP}$ causing the latter to open with subsequent triggering of increased production of $H_2O_2$ and activation of a second pool of PKC$\varepsilon$ that stimulates a pathway that ultimately terminates in inhibition of MPT. Although our knowledge of this scheme is still incomplete, we have a framework of the sequence of molecular events that can account for the reduction in cell death observed in cardioprotection.

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