**Early Opening Of Sarcolemmal ATP-Sensitive Potassium Channels Is Not A Key Step
In PKC-Mediated Cardioprotection**

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Abstract

ATP-sensitive potassium (K_{ATP}) channels are abundantly expressed in the myocardium. Although a definitive role for the channel remains elusive they have been implicated in the phenomenon of cardioprotection, but the precise mechanism is unclear. We set out to test the hypothesis that the channel protects by opening early during ischemia to shorten action potential duration and reduce electrical excitability thus sparing intracellular ATP. This could reduce reperfusion injury by improving calcium homeostasis.

Using a combination of contractile function analysis, calcium fluorescence imaging and patch clamp electrophysiology in cardiomyocytes isolated from adult male Wistar rats, we demonstrated that the opening of sarcolemmal K_{ATP} channels was markedly *delayed* after cardioprotective treatments; ischemic preconditioning, adenosine and PMA. This was due to the preservation of intracellular ATP for longer during simulated ischemia therefore maintaining sarcolemmal K_{ATP} channels in the closed state for longer. As the simulated ischemia progressed, K_{ATP} channels opened to cause contractile, calcium transient and action potential failure, however there was no indication of any channel activity early during simulated ischemia to impart an energy sparing hyperpolarization or action potential shortening.

We present compelling evidence to demonstrate that early opening of sarcolemmal K_{ATP} channels during simulated ischemia is not part of the protective mechanism imparted by ischemic preconditioning or other PKC-dependent cardioprotective stimuli. On the contrary, channel opening was actually delayed. We conclude that sarcolemmal K_{ATP} channel opening is a consequence of ATP depletion, not a primary mechanism of ATP preservation in these cells.

Highlights:

- Opening of the SarcoK_{ATP} channel was proposed to be cardioprotective
- Channel opening was *delayed* after cardioprotective stimuli
- Ca²⁺ & ATP levels were maintained during ischemia independent of SarcoK_{ATP} opening
- Mitochondrial function preserved during ischemia, independent of SarcoK_{ATP} opening
- Early opening of SarcoK_{ATP} is not involved in PKC-dependent cardioprotection

Keywords:

ATP-sensitive potassium (K_{ATP}) channels, Protein Kinase C (PKC), cardioprotection, Ischemic Preconditioning

1. Introduction:

ATP-sensitive potassium (K_{ATP}) channels are abundantly expressed throughout the myocardium, however a definitive role in myocardial function has yet to be fully elucidated [1]. K_{ATP} channels are ubiquitously expressed protein complexes hypothesized to provide a link between metabolic state and electrical excitability by virtue of their inhibition by ATP at normal physiological concentrations following direct binding of ATP to the Kir6 pore-forming subunit. In normal physiological conditions the channel is predominantly inhibited, whilst a fall in the ATP:ADP ratio causes activation.

The K_{ATP} channel is expressed as a hetero-octameric structure comprising four Kir6 (Kir6.1 or Kir6.2) and four sulphonylurea (SUR) subunits (SUR1 or SUR2). SUR2 has two predominant splice variants SUR2A and 2B. Two K_{ATP} channel subtypes have been proposed in the heart; the sarcolemmal (sarco K_{ATP}) and mitochondrial (mito K_{ATP}). The cardiac ventricular sarco K_{ATP} channel has been identified as comprising Kir6.2 and SUR2A as the predominant subunit combination [2-4]. Other K_{ATP} subunits are expressed in cardiomyocytes and may form components of the postulated mito K_{ATP} [4].

An early hypothesis used to define the role of K_{ATP} channels suggested that during an ischemic insult, activation of the sarco K_{ATP} channel by a depletion of ATP and concurrent elevation in ADP, would cause a shortening of the cardiac action potential duration (APD) [5] and might lead to complete action potential failure, as the weakly rectifying K_{ATP} channel activity short-circuited the triggered depolarization. These actions could limit the rise in intracellular calcium and preserve cellular ATP. Several studies attempted to address the relationship between APD and cardioprotection at the whole organ level (for example Yao 1994[6], Grover 1995[7]) but these relied on responses to pharmacological agents to infer K_{ATP} activity and provided little information about what was happening to APD, the calcium or membrane potential of individual cardiomyocytes.

Following Murray's description of ischemic preconditioning (IPC), as the intrinsic ability of the myocardium to be protected from a prolonged ischemic insult by a series of short ischemic episodes prior to the main insult[8], pharmacological agents have been used to both implicate and exclude K_{ATP} channel involvement in the protection afforded by ischemic preconditioning and other cardioprotective stimuli [9-12]. The definitive mechanism behind this remarkable intrinsic protection still remains elusive with an increasingly complex, and often contradictory, literature. Recently, protein kinases forming part of the RISK pathway, specifically PKC ϵ , have been implicated in the IPC phenomenon[13]. Experimentally, in cardioprotective protocols, PKC ϵ is translocated to the mitochondria [13-15]. It has been demonstrated in differing tissue types that K_{ATP} channels can be regulated by a number of different cell signaling pathways; in particular by PKA and PKC [16]. However, the mechanism by which either of the cardiac K_{ATP} channel subtypes may be directly involved in the protection afforded by IPC, or other cardioprotective stimuli, is still unclear [17].

Evidence for a key role for the cardiac K_{ATP} channel comes from the observation that pharmacological blockade [12, 18], disruption of channel expression with interacting fragments of SUR[19], shRNA knockdown[3] and knockout of Kir6.2 subunits[20, 21] all have deleterious effects on the ability of IPC and other cardioprotective stimuli to impart protection.

The importance of the sarco K_{ATP} in IPC has been the subject of much debate since the existence of a mitochondrial K_{ATP} (mito K_{ATP}) channel was first hypothesized[22]. The case for mito K_{ATP} has, until recently, relied mainly on pharmacological agents, most notably diazoxide and 5-hydroxydecanoate (5-HD); however these agents have been suggested to have effects on metabolism[23] and so the use of these compounds is perhaps flawed as direct evidence for the existence of a mito K_{ATP} channel. More recently, Kir1 (ROMK) subunits, not part of the classically accepted K_{ATP} channel subunit family, have been demonstrated in mitochondria and proposed as a molecular component of the mito K_{ATP}

channel[24]. Where a mitoK_{ATP} channel would act and what the consequences of such activation would be are still unclear.

In this current study we present strong evidence to demonstrate that an early activation of the sarcoK_{ATP} channel, activation of a channel that may have a role in cardioprotection, is *not* involved in the protection afforded by common cardioprotective stimuli (IPC, direct activation of PKC or pre-treatment with adenosine or diazoxide). Furthermore, we demonstrate that, after cardioprotection, rather than opening early during metabolic inhibition, sarcoK_{ATP} opening actually occurs *later*. These data demonstrate that the protection afforded by cardioprotective stimuli does *not* involve an early activation of sarcoK_{ATP} to reduce electrical excitability; however this does not exclude a role for mitoK_{ATP} in cardioprotection. Our findings suggest that cardioprotective stimuli prolong normal mitochondrial function during ischemia and that the delay in opening of sarcoK_{ATP} channels is a consequence of continuation of ATP production. Similar to the original hypothesis for the role of the sarcoK_{ATP} channel [5], we suggest that sarcoK_{ATP} channel opening is the last defense of the cardiomyocyte to preserve ATP and limit Ca²⁺ overload during ischemia.

2. Methods:

2.1. Solutions:

Cardiomyocytes were maintained at room temperature until use in 2 mmol/L Ca^{2+} Tyrode's solution (2CaT). 2CaT contained, in mmol/L, 135 NaCl, 5 KCl, 0.33 NaH_2PO_4 , 10 HEPES, 5 Glucose, 5 Mannitol, 5 Na Pyruvate, 1 MgCl_2 and 2 CaCl_2 , pH to 7.4 with NaOH. Nominally Ca^{2+} -free Tyrode's (0CaT) solution was as above with no added CaCl_2 . Substrate-free metabolic inhibition Tyrode's (SFT-MI) contained, in mmol/L, 140 NaCl, 5 KCl, 0.33 NaH_2PO_4 , 10 HEPES, 10 Sucrose, 1 MgCl_2 , 2 CaCl_2 , 2 cyanide and 1 iodoacetic acid, pH to 7.4 with NaOH. The 'Ischaemic buffer' contained, in mmol/L, 108 NaCl, 16 KCl, 0.33 NaH_2PO_4 , 10 PIPES, 20 deoxyglucose, 20 Na-Lactate, 1 MgCl_2 and 2 CaCl_2 , 0.2 2,4-dinitrophenol (DNP), pH 6.8 with NaOH. Pipette solution was as described previously[19, 25].

2.2. Isolation of cardiomyocytes:

Adult male Wistar rats (300–400 g) were killed by concussion followed by cervical dislocation. The care and sacrifice of the animals conformed to the requirements of Directive 2010/63/EU of the European Parliament. The protocol for isolation of cardiomyocytes was as described previously[25, 26]. Ischemic preconditioning was imparted to cardiomyocytes using a protocol adapted from Rodrigo and Samani[27]. Briefly, prior to enzymatic digestion, the heart was perfused with a 2CaT solution for 5 minutes. Perfusion was then halted for 5 minutes to simulate ischaemia, followed by 5 minutes of reperfusion. This cycle was repeated 3 times. The solution was then exchanged for 0CaT and enzymatic digestion proceeded as described previously[25, 26].

2.3. Metabolic inhibition and reperfusion (MI/R) model and contractile function measurements:

Metabolic inhibition and simulated reperfusion (MI/R) were used as described previously[19, 26, 28, 29]. For MI/R experiments, cardiomyocytes were perfused with 2CaT solution for 3

minutes, SFT-MI for 7 minutes, followed by 10 minutes of washout (simulated reperfusion) with 2CaT. Time to contractile failure, percentage contractile recovery and hypercontracture (confirmed by Trypan blue uptake into hypercontracted cells) were recorded. Use of the 'ischaemic buffer' solution necessitated an adapted protocol where the duration of the ischaemic buffer perfusion was 10 minutes, rather than 7 minutes for SFT-MI.

2.4. Patch-clamp electrophysiology

Action potential and cell-attached patch recordings were made as previously described[19].

2.5. Fura-2 measurement of $[Ca^{2+}]_i$

Cardiomyocytes were loaded with 5 μ mol/L Fura-2-AM for 20 minutes at room temperature, and allowed to settle for 5 minutes in a heated perfusion chamber. During the recording, cardiomyocytes were perfused at $32\pm 2^\circ\text{C}$ with 2CaT and stimulated to contract via 1Hz external field stimulation. Cardiomyocytes were illuminated with 340 and 380 nm wavelengths alternately from a monochromator (PTI, Birmingham, NJ, USA) and fluorescence emissions captured above 520 nm using a Cascade 512B CCD camera (Photometrics, Tuscan, AZ, USA). Data was acquired using EasyRatioPro software (PTI). Images were acquired throughout the recording at a rate of one ratio image every five seconds to minimize photo bleaching. At the end of 3 minutes of 2CaT perfusion and 10 minutes of reperfusion images were acquired for 30 seconds at a rate of 32 frames per second (16 ratio images per second) to measure single Ca^{2+} transients. Data are expressed as the ratio of the 340:380 nm signals.

2.6. Magnesium Green measurement of free intracellular Mg^{2+}

Magnesium Green, a single wavelength dye, was used as a surrogate for measuring intracellular [ATP][30]. Cardiomyocytes were loaded at room temperature with 5 μ mol/L Magnesium Green-AM for 20 minutes, and allowed to settle for 5 minutes as described above. Quiescent cardiomyocytes were investigated to avoid changes in fluorescence

caused by movement artefacts. Data are expressed as a ratio of the fluorescence/mean basal fluorescence (F/F_0) over the last 30 seconds of 2CaT perfusion.

2.7. Mitochondrial depolarisation measured using TMRM fluorescence:

Tetramethylrhodamine methyl ester (TMRM) fluorescence was used to measure mitochondrial membrane potential. Isolated cardiomyocytes were loaded with 5 $\mu\text{mol/L}$ TMRM for 10 minutes in Tyrode's solution containing 5 mM glucose where TMRM accumulated in the mitochondria and its fluorescence was autoquenched. Cardiomyocytes were illuminated with 550 nm light for 200 ms at 0.1 Hz.

3. Results

3.1. Cardioprotective pre-treatment protocols delayed the time to contractile failure and increased the contractile recovery in isolated cardiomyocytes

Pharmacological pre-treatment has been demonstrated to impart a cardioprotected phenotype to isolated cardiomyocytes *in vitro*. Rodrigo and Samani demonstrated that cells displaying a cardioprotected phenotype may be prepared using a “stop-start” perfusion protocol in a whole heart mounted on a Langendorff perfusion system prior to enzymatic isolation of cardiomyocytes[27]. Using the metabolic inhibition and reperfusion (MI/R) protocol with the isolated cardiomyocytes, the degree of cardioprotection can be assessed by measuring the proportion of cells recovering contractile function at the end of a period of simulated reperfusion and also by measuring the proportion of hypercontracted cardiomyocytes, as a surrogate measure of reperfusion injury. This protocol gives levels of hypercontracture in isolated cardiomyocytes similar to infarct size in the whole heart reported in the literature (up to 40% of the area at risk in control, and as low as 10% after cardioprotective stimuli)[9, 31]. Furthermore, using this protocol, the contractile function of the cardiomyocytes can be measured throughout the periods of metabolic inhibition and reperfusion. This gives additional functional data, which cannot be recorded using other protocols, such as ischemic pelleting, whilst reproducing the survival data obtained using such techniques.

Cardiomyocytes were pre-treated with cardioprotective agents by perfusion with Tyrode's solution (2CaT) containing either 100 $\mu\text{mol/L}$ diazoxide, 50 $\mu\text{mol/L}$ pinacidil or 100 $\mu\text{mol/L}$ adenosine for 5 minutes followed by wash out with 2CaT in the first 3 minutes of the MI/R protocol prior to substrate-free metabolic inhibition Tyrode's (SFT-MI) perfusion. For direct PKC activation, 5 $\mu\text{mol/L}$ phorbol 12-myristate 13-acetate (PMA) was added to the perfusion chamber and cardiomyocytes incubated for 5 minutes prior to starting the 2CaT perfusion for 5 minutes leading into the MI/R protocol. Figure 1A(i) shows the outline control and pre-treatment protocols and some example images of cardiomyocytes taken at the time points

indicated in the control protocol (Figure 1A(ii)). Figure 1B(i) shows the mean time course for the proportion of contractile cells for control cardiomyocytes, those treated with the known cardioprotective stimulus adenosine and cardiomyocytes after IPC. For each treatment, including direct PKC activation with PMA, the contractile recovery was greater than control.

Pre-treatment with 5-HD (500 $\mu\text{mol/L}$), a postulated $\text{mitoK}_{\text{ATP}}$ channel inhibitor[32], had no effect on the time to contractile failure or on the contractile recovery on reperfusion compared to control. Figure 1B(ii-iii) show the mean times to contractile failure and percentage contractile recovery, respectively. These data confirm that in all cases, with the exception of diazoxide, the time to contractile failure was increased by cardioprotective treatments (Figure 1B(ii)) and that all improved the contractile recovery at the end of simulated reperfusion (Figure 1B(iii)) with the exception of 4 α -PMA, an inactive analogue of PMA which neither increased the time to contractile failure (Figure 1B(ii)) or improved the contractile recovery (Figure 1B(iii)) compared to control cardiomyocytes. Additionally, Figure 1B(iv) shows that the cardioprotective stimuli reduced the proportion of hypercontracture after reperfusion, whereas a 'cardiotoxic' agent, 5-HD, increased hypercontracture.

Compared to control cardiomyocytes, IPC, or adenosine and pinacidil pre-treatment, significantly delayed the time to contractile failure. With a fixed period of metabolic inhibition and simulated reperfusion, this meant that control cardiomyocytes spent a longer period of time in a state of contractile failure than those pre-treated with adenosine or pinacidil. To determine whether there was still a cardioprotection if this time in contractile failure was normalized, a longer SFT-MI period was used after pre-treatment with these agents. Significant cardioprotection, as assessed by an increased percentage contractile recovery and reduced hypercontracture, was still observed using a '9 minute SFT-MI' protocol in pinacidil and adenosine pre-treated cardiomyocytes, but not in those pre-treated with diazoxide (Figure 2A and B) when compared to the '7 minute SFT-MI' control group.

Although the CN/IAA SFT-MI model employed in this study is widely used, we tested our findings using an 'ischaemic buffer'. This buffer was designed to mimic conditions seen in ischaemia more than just a simple substrate removal/metabolic poison. This model used a high K^+ , low pH, non-metabolized deoxyglucose, high lactate solution with the addition of the mitochondrial uncoupler (DNP). Our findings of a delayed time to contractile failure in adenosine, pinacidil and PMA were replicated in this model (Figure 2C). The time to contractile failure was not significantly different after diazoxide pre-treatment, as in the CN/IAA model (Figure 2C). The contractile recovery was increased (Figure 2D) and the hypercontracture, as confirmed by Trypan blue uptake (Figure 2E), was decreased consistent with the findings reported in the CN/IAA model in figure 1.

3.2. The time to action potential failure during metabolic inhibition was associated with the activity of PKC ϵ .

Figure 1B, Figure 2C-E, and our previous findings[26], showed that direct activation of PKC by PMA imparts a cardioprotected-like phenotype to cardiomyocytes. Tat-peptide linked PKC isoform-specific inhibitor peptides were used to dissect the contribution of specific PKC isoforms to the delay in action potential failure. Isolated cardiomyocytes were incubated with Tat-PKC inhibitors (100 nmol/L) for 10 minutes, followed by a further 5 minutes with 5 μ mol/L PMA prior to experimentation. Cardiomyocytes were then perfused for 10 minutes with 2CaT prior to metabolic inhibition with SFT-MI solution. Action potentials were stimulated at 1 Hz via the patch pipette and the APD₉₀ and time to action potential failure recorded. In cardiomyocytes pipette-loaded with the scrambled peptide, the time to action potential failure after PMA treatment was significantly increased compared to PKC ϵ inhibitor peptide loaded via the patch-pipette and control cardiomyocytes (Figure 3A and B). Figure 3C shows the mean time to action potential failure on selective inhibition of PKC isoforms after PMA treatment. PMA treatment delayed action potential failure in isolated cardiomyocytes, except for cells treated with PKC ϵ or non-specific (20-28) peptide inhibitors. Additionally, if PKC ϵ was activated directly using a Tat- ψ RACK ϵ activating peptide[13, 33] the time to action

potential failure was prolonged, in the absence of PMA. Mean time to action potential failure, measured electrophysiologically, and contractile failure, as measured in figure 1, was not significantly different suggesting little detrimental effect of using the ruptured patch recording in these experiments.

3.3. The delay in action potential failure in IPC and adenosine treated, but not in diazoxide or pinacidil treated, cardiomyocytes is attenuated by PKC ϵ inhibition.

The role of PKC ϵ in the time to action potential failure was investigated; inhibition of PKC ϵ attenuated the delay seen in response to IPC and adenosine stimuli but not diazoxide and pinacidil (Figure 4 B(i and ii)). Additionally, the hyperpolarization associated with action potential failure was also markedly delayed after cardioprotective stimuli (Figure 4C(i and ii)). Mirroring the attenuation of the increase in time to action potential failure, the increase in time to hyperpolarization was also attenuated by inhibition of PKC ϵ using Tat-PKC ϵ inhibitor peptide (Tat-PKC ϵ -IP). In the absence of metabolic inhibition, only pinacidil affected the action potential duration or the membrane potential of cardiomyocytes acutely or after a pre-treatment (Figure 4C).

3.4. Cardioprotection by IPC, adenosine and diazoxide, but not pinacidil, is attenuated by PKC ϵ inhibition.

Having shown that the delay in action potential failure was PKC ϵ -dependent, we investigated the effect of inhibiting PKC ϵ on cardioprotection using contractile function measurements. As expected Tat-PKC ϵ -IP attenuated the delay in contractile failure seen in IPC, PMA-treated and adenosine treated cardiomyocytes (Figure 5A). Inhibition of PKC ϵ markedly reduced the contractile recovery and attenuated the reduction in hypercontracture of IPC, PMA-treated cardiomyocytes and those pre-treated with adenosine (Figure 5B and C). In contrast to IPC and adenosine treatment, diazoxide did not significantly delay contractile failure. However, the increased contractile recovery and reduced hypercontracture associated with diazoxide pre-treatment were both attenuated by Tat-PKC ϵ -IP.

Cardioprotection by direct activation of K_{ATP} channels using a 5 minute pre-treatment of 50 μ mol/L pinacidil was not significantly affected by PKC ϵ inhibition.

3.5. SarcoK_{ATP} channel opening is delayed after cardioprotective stimuli.

It has been hypothesized that sarcoK_{ATP} channels open during ischemia, or metabolic inhibition, when intracellular [ATP] falls with a concomitant rise in intracellular [ADP], so activating the hyperpolarizing IK_{ATP} current. To investigate this directly, activation of sarcoK_{ATP} by metabolic inhibition was investigated in cell-attached patch recording. Cell-attached, rather than conventional whole cell recording, was used to allow recording of the time to channel opening without interfering with the intracellular ionic buffering and so that opening of sarcoK_{ATP} could be identified using the conductance of the channel.

Figure 6A shows exemplar cell-attached patch recordings of sarcoK_{ATP} channel opening in SFT-MI. Expanded example traces of channel openings at 150 s (approximately the time at which contractile failure occurred in control cardiomyocytes) are shown in figure 6B. With each of the cardioprotective stimuli tested the time to sarcoK_{ATP} channel opening was significantly delayed compared to control (Figure 6C). Furthermore, the delay in channel opening time was attenuated by 15 minutes of pre-treatment with Tat-PKC ϵ -IP (Figure 6C). These findings confirmed our hypothesis that early opening of sarcoK_{ATP} channels during ischemia is not part of the mechanism of PKC-dependent cardioprotection.

3.6. The protection afforded by cardioprotective stimuli preserves mitochondrial function during SFT-MI.

Activation of PKC ϵ by IPC has been demonstrated to cause a translocation of this isoform to mitochondria[14]. To investigate whether the cardioprotective stimuli used in this study preserved mitochondrial function during metabolic inhibition, $[Ca^{2+}]_i$ (Fura-2-AM), $[Mg^{2+}]$ (a surrogate for ATP concentration (MgGreen-AM)) and Tetramethylrhodamine methyl ester (TMRM) fluorescence (to investigate mitochondrial depolarization) were measured.

Figure 7A shows an example recording of Fura-2 fluorescence from 5 control cardiomyocytes exposed to the MI/R protocol. These cardiomyocytes show a typical profile of an increased $[Ca^{2+}]_i$, shown as an increase in Fura-2 ratio during SFT-MI, followed by a further rise in Ca^{2+} on reperfusion. Only 1 cell recovered a contractile phenotype. Figure 7B shows similar example data for 5 IPC cardiomyocytes, however in this recording 4 out of 5 cardiomyocytes recovered a contractile phenotype. Similar to the contractile recovery in figure 1, the percentage of cardiomyocytes recovering Ca^{2+} -transients was markedly increased after cardioprotective stimuli (Figure 7C). The mean time to Ca^{2+} -transient failure, together with contractile and action potential failure times, are plotted in Figure 6D demonstrating that, as would be expected, all of these events occurred concurrently, irrespective of the measurement of contractile function employed.

Additionally, the depletion of intracellular ATP (Figure 8A and B), as measured using an increase in free intracellular Mg^{2+} as a surrogate marker for ATP depletion, was less pronounced after cardioprotective stimuli. Finally, the time to mitochondrial depolarization during metabolic inhibition, as measured by the time at which basal fluorescence was increased to 10% of the maximum, was markedly delayed after cardioprotective stimuli (Figure 8C to E).

Together, these data show that mitochondrial function is preserved for longer during the simulated ischemia, so preserving intracellular ATP, thus delaying the opening of $sarcoK_{ATP}$ during metabolic inhibition. These findings are consistent with the hypothesis that opening of the $sarcoK_{ATP}$ channel does not play a significant role in cardioprotection.

4. Discussion

The literature suggests that sarcoK_{ATP} channels are of fundamental importance in the myocardium with evidence suggesting that Kir6.2 plays a key role in IPC[20, 21]. It is, therefore, somewhat of a paradox that the data presented here suggest that the opening of cardiac sarcoK_{ATP} channels during ischemia is not a key step in the protection afforded by IPC or other cardioprotective stimuli. Contractile function experiments demonstrated that the time to contractile failure, and by inference sarcoK_{ATP} channel opening, is markedly delayed in response to all cardioprotective stimuli tested (with the exception of diazoxide). Furthermore, by use of specific PKC isoform inhibitors, we demonstrated that indicators of IPC, adenosine, diazoxide and PMA cardioprotection (namely, improved contractile recovery and reduced hypercontracture, confirmed by Trypan blue uptake) are dependent on PKC ϵ . The delay in contractile failure was also shown to be PKC ϵ -dependent.

Further evidence supporting a delay in sarcoK_{ATP} activation was provided by action potential measurements, where the time to failure was increased following cardioprotection. Consistent with these data was the hyperpolarization of the membrane potential seen prior to contractile or action potential failure, again suggesting a contribution of a K⁺ current to contractile failure; which we hypothesized was carried by sarcoK_{ATP} channels. Finally, direct measurement of the time to sarcoK_{ATP} channel activation in cell-attached patch recording confirmed that the sarcoK_{ATP} channel does indeed open later after cardioprotection.

These data, along with previous findings by others [34, 35], demonstrate that an early opening of sarcoK_{ATP} is not part of the PKC ϵ -dependent cardioprotective processes. Here we demonstrate that sarcoK_{ATP} opening is actually *delayed* suggesting that the channel is responding to, rather than causative, of the ATP preservation after cardioprotection.

4.1. Cardioprotection by pinacidil.

It is important to note that our data suggests that pinacidil pre-treatment imparts cardioprotection via a different mechanism to that involving PKC activation. Inhibition of the

known cardioprotective PKC isoform (PKC ϵ) did not markedly affect any measure of cardioprotection when cardiomyocytes were pre-treated with pinacidil. The action potential and membrane potential data presented suggest that pinacidil caused a marked shortening of APD₉₀ independent of PKC activation and a significant hyperpolarization of the sarcolemmal membrane potential, that was presumably limiting the cyclic increases in [Ca²⁺]_i normally seen for each cardiac cycle. A reduced [Ca²⁺]_i and shortened APD would use less ATP and so would, as originally hypothesized for sarcoK_{ATP} channel activation[8], preserve ATP for longer during metabolic inhibition. Example and mean data for cell-attached patch recordings after pinacidil pre-treatment are not shown, as the channel was already active and so the time to specific MI-induced activation was difficult to determine accurately. Ca²⁺ fluorescence, Mg²⁺ and TMRM fluorescence recordings all supported this hypothesis with intracellular [Ca²⁺]_i, ATP and mitochondrial membrane potential being maintained for longer during MI. We hypothesize that, although the pre-treatment stimulus with pinacidil was washed off, there was still either a residual amount of pinacidil within the membrane or bound to channels so contributing a larger than control K_{ATP} channel current as evident with a shorted APD and more hyperpolarized membrane potential (Figure 4).

4.2. Cardioprotection by diazoxide does not delay the time to sarcoK_{ATP} channel activation.

Diazoxide-induced cardioprotection bore all of the hallmarks of a classic protected phenotype, including sensitivity to PKC ϵ inhibition; however the time to sarcoK_{ATP} channel activation (and hence contractile failure) was not significantly increased compared to control. This initially puzzling finding may be explained by a clearer understanding of diazoxide pharmacology. Diazoxide has been widely reported as a mitoK_{ATP} channel specific opener, however it has also been reported that the sarcoK_{ATP} channel may be activated by diazoxide under conditions of reduced ATP, or elevated ADP, such as occur in ischemia[36]. It was hypothesized that diazoxide may reduce the ATP, or increase the ADP, sensitivity of sarcoK_{ATP} channels directly. Indeed it has been reported that if diazoxide is perfused as a 5

minute pretreatment, then the sarcoK_{ATP} channel actually opens *earlier* than in control cardiomyocytes [28]. If this hypothesis were correct, under normal physiological conditions the channel would remain closed in the presence of diazoxide, but during ischemia sarcoK_{ATP} channels would require a smaller depletion of ATP to become activated.

As diazoxide has been shown to protect via a PKC ϵ -dependent mitochondrial mechanism [37], the time to ATP depletion would be delayed, making interpretation difficult. In our findings presented in figure 1, there was a trend towards a delay in contractile failure after diazoxide pre-treatment, however this was not significant compared to control cells (148.3 $\pm$ 4.7 vs 173.4 $\pm$ 4.6 s, n=6 ($\geq$ 93 cells), p=0.176). This non-significant delay of contractile failure may represent opening of sarcoK_{ATP} channels at a higher ATP concentration than would be seen in control cardiomyocytes despite the *rate* at which ATP depletion occurs being slowed after diazoxide treatment [28].

4.3. Evidence for a role of sarcoK_{ATP} channel subunits in cardioprotection.

Whilst our data suggests that the opening of K_{ATP} at the sarcolemmal membrane may not be a key determinant of the protection afforded by the cardioprotective stimuli tested here, there is evidence that sarcoK_{ATP} channels are fundamentally important to cardiomyocyte survival. Evidence from knockout animal models for Kir6.2 has demonstrated that ischemic preconditioning does not occur in these animals[20, 21]. Rather than contributing effect at the sarcolemma, there is a suggestion that Kir6.2 may be trafficked to the mitochondria on activation by cardioprotective stimuli[38]. These findings suggest that intracellular effects of Kir6.2 redistribution, rather than merely opening as part of the sarcoK_{ATP} channel complex at the cell surface, may be of importance. Indeed, our data in Figure 7 demonstrated that the measured 'mitochondrial parameters' of cardioprotection, ATP level and mitochondrial membrane potential, were preserved for longer after cardioprotection and we cannot exclude trafficking of Kir6.2 to the mitochondria as part of the protective mechanism. The mitoK_{ATP} channel, hypothesized to open in conditions of metabolic stress to preserve the mitochondrial function, may be a key determinant in cardioprotection[39]. Pharmacological

evidence, using the classical mitoK_{ATP} channel agents diazoxide and 5-HD, would suggest that mitoK_{ATP} channels do indeed play a role; however the specificity of these agents has been called into question[23]. The recent identification of ROMK as a putative mitoK_{ATP} channel subunit[24] provides a potential target for further investigation. Additionally, the role of sarcoK_{ATP} channels in cardioprotection may be as part of a larger signalosome-type complex[40]. In such a complex, sarcoK_{ATP} channels may act as a scaffold for signaling proteins, to facilitate a cellular response perhaps intimately linked with mitochondrial function.

A potential limitation of any study of ionic currents within cardiomyocytes is the relationship between what occurs at the single cell level and what is seen in a whole heart. Our data is consistent with that of Suzuki and colleagues who found that although IPC was blocked in Kir6.2 KO mice there was no difference in infarct size between KO and control[20]. Other groups however, have shown that the hearts of KO animals show decreased stress tolerance[41, 42], and the importance of sarco or mitoK_{ATP} in whole heart responses to ischemia remains an area of controversy in the literature. Transgenic approaches have their limitations; genetic background-dependent variations in phenotype are well recognised in other fields and developmental compensation, which may obscure or distort 'true' phenotypes, has been demonstrated in mice expressing an ATP-insensitive mutant of Kir6.2[43]. Pharmacological approaches are equally at risk of confounding; we have previously demonstrated that HMR 1098 inhibition of Kir6.2/SUR2A, like that of glibenclamide[44] and glimepiride[45], is markedly attenuated by elevated intracellular [ADP][25] as would be found in the sub-sarcolemmal environment during ischemia.

SarcoK_{ATP} is an abundantly expressed myocardial ion channel, but our data suggests that an early opening of the channel does not contribute to the protection afforded by a number of PKC-dependent cardioprotective stimuli, as had been previously hypothesized, although it is clear that the opening of the sarcoK_{ATP} channel does occur when ATP concentrations have fallen far enough. We believe, as was originally suggested for these channels, that

sarcoK_{ATP} channel opening is the last opportunity the cardiomyocyte has to reduce ATP consumption and limit the potential damage from [Ca²⁺]_i overload. These data suggest that this property is distinct from the earlier acting mechanisms of cardioprotection, which appear to preserve mitochondrial function for longer during an ischemic insult.

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6. Conflicts of Interest: none declared

7. References

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8. Figures

Figure 1

Cardioprotective stimuli delay contractile failure, increase contractile recovery and reduce hypercontracture after a metabolic inhibition and reperfusion protocol.

A, (i) Representation of the MI/R protocol used throughout the manuscript. (ii) Example images of cardiomyocytes taken at the time points (a – c) as indicated in (i). B(i) Time course showing the percentage of contractile cardiomyocytes throughout the MI/R protocol in control, IPC cardiomyocytes, or in cells pre-treated with 100 $\mu\text{mol/L}$ adenosine prior to MI/R. Histograms showing mean time to contractile failure (ii), mean percentage contractile recovery (iii) and hypercontracture (iv) after MI/R with cardioprotective or cardiotoxic stimuli. IPC or pre-treatment of cardiomyocytes with PMA, adenosine and pinacidil caused a significant delay in contractile failure, increase in contractile recovery and reduction in hypercontracture. 4 α -PMA, an inactive analogue of PMA, had no significant effect on any of the measured parameters compared to control. Diazoxide significantly increased contractile recovery and reduced hypercontracture, but did not significantly delay contractile failure. 5-HD had no significant effect on contractile failure or recovery, however it significantly worsened hypercontracture. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, $n = 6$ (≥ 86 cardiomyocytes) from ≥ 4 animals for each data set, ANOVA with Bonferroni's Post-hoc test).

Figure 2

A cardioprotective phenotype is still maintained after a prolonged period of metabolic inhibition or use of an alternative 'ischaemic buffer' protocol.

A, histogram showing the mean percentage contractile recovery and (B) mean percentage hypercontracture with cardiomyocytes perfused with either 7 minutes SFT-MI (standard protocol) or 9 minutes SFT-MI. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, $n = 6$ (≥ 156 cardiomyocytes)

from ≥ 4 animals for each data set, ANOVA with Bonferroni's Post-hoc test). Histogram showing mean time to contractile failure (C) mean percentage contractile recovery (D) and mean percentage hypercontracture (E) using an alternative method of metabolic inhibition with an 'ischaemic buffer'. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, $n = 4$ (≥ 119 cardiomyocytes) from ≥ 3 animals for each data set, ANOVA with Bonferroni's Post-hoc test).

Figure 3

Direct activation of PKC with PMA delays action potential failure in a PKC ϵ -dependent manner.

Cardiomyocytes were treated with 100 nmol/L Tat-Peptide linked PKC isoform specific inhibitor peptide for 10 minutes, followed by 5 μ mol/L PMA for 5 minutes prior to perfusion with 2CaT solution. Action potential recordings were made in current clamp mode after allowing the cardiomyocytes to equilibrate for 10 minutes after achieving the whole-cell configuration. For cell-impermeant inhibitor peptides loaded via the patch electrode, cardiomyocytes were allowed to equilibrate for 15 minutes prior to application of SFT-MI. A, Example action potential recordings from cardiomyocytes pipette loaded with PKC ϵ inhibitor peptide (i) or with scrambled control peptide (ii). B, Mean time course plot showing control, PMA-treated, PMA-treated with PKC ϵ inhibitor peptide in the patch pipette or PMA-treated with scrambled PKC ϵ peptide in the patch pipette. PMA significantly delayed action potential failure (white circles), whilst the PKC ϵ inhibitor reversed this delay back to control (light grey circles). PKC ϵ -scrambled peptide treated cells still showed a delay in action potential failure after PMA pre-treatment (dark grey circles). C, Histogram showing mean time to action potential failure in control cardiomyocytes, after direct PKC activation with Tat- ψ RACK ϵ activating peptide or after PMA pre-treatment for a number of PKC inhibitor peptides. Direct activation of PKC ϵ , in the absence of PMA treatment significantly delayed action potential failure (** $P < 0.01$, ANOVA with Bonferroni's post-hoc test, $n = 8$ from 4 animals for each

group). Inhibition of all PKC isoforms (Tat-PKC20-28) or inhibition of PKC ϵ reversed the PMA-induced delay in action potential failure. (**P<0.001, ANOVA with Bonferroni's post-hoc test, n $\geq$ 7 cardiomyocytes from $\geq$ 3 animals for each group).

Figure 4.

Delay in action potential failure and concomitant delay in membrane potential hyperpolarization following cardioprotective stimuli, other than diazoxide or pinacidil, is attenuated after PKC ϵ inhibition.

A(i) Example action potential recordings from single cardiomyocytes during MI/R in IPC control (i) and IPC cardiomyocytes pre-treated with Tat-PKC ϵ -IP at the time points indicated. B(i), Time course showing APD₉₀ in control, IPC and IPC cardiomyocytes pre-treated with Tat-PKC ϵ -IP. Action potential failure was delayed in IPC cardiomyocytes compared to control, however this delay was reversed by pre-treatment with Tat-PKC ϵ -IP. (ii) Histogram of mean time to action potential failure showing that the delay seen in IPC, PMA and adenosine treated cardiomyocytes was reversed by Tat-PKC ϵ -IP pre-treatment. Diazoxide pre-treatment showed no significant delay in action potential failure time. Pinacidil-induced delay in action potential failure time was unaffected by PKC ϵ inhibition. C(i), Measurement of membrane potential showed a significant hyperpolarization at the time of contractile failure, which was delayed after cardioprotective pre-treatments. This was quantified by measuring the mean V_m prior to and after the hyperpolarization and measurement of the time taken to elicit half of that change (Δ V_{m50%}). (ii) Histogram showing the mean time to Δ V_{m50%} in cardiomyocytes with and without Tat-PKC ϵ inhibition. The time to Δ V_{m50%} was increased with IPC, PMA and adenosine pre-treatment, and reversed with Tat-PKC ϵ inhibitor, however it was not increased with diazoxide pre-treatment and was unaffected by Tat-PKC ϵ -IP after pinacidil pre-treatment. (**P<0.01, ***P<0.001, ANOVA with Bonferroni's Post-Hoc Test, n $\geq$ 6 cardiomyocytes from $\geq$ 3 animals for each data set). C. Cardiomyocytes

were perfused with 2CaT solution for 5 minutes, followed by 10 minutes of perfusion of 2CaT containing pinacidil, diazoxide or adenosine to investigate the direct effects of these on APD₉₀ and membrane potential. Pinacidil caused a significant shortening of APD₉₀ and hyperpolarisation, however diazoxide and adenosine had no significant effect on either parameter (***P<0.001, n=6 from 3 animals for each data set, Paired T-test). B, The mean APD₉₀ (i) and mean Vm (ii) for control, IPC cardiomyocytes, and cardiomyocytes pre-treated with PMA, adenosine, diazoxide or pinacidil for 5 minutes, followed by 5 minutes of washout, prior to MI/R. Only pinacidil significantly shortened mean APD₉₀ and none significantly affected membrane potential. (*P<0.05, n≥6 cardiomyocytes from ≥3 animals for each data set, ANOVA with Bonferroni's Post-hoc test).

Figure 5.

Inhibition of the known cardioprotective PKC isoform ϵ attenuates the changes in contractile failure, contractile recovery and hypercontracture after MI/R.

A, Histogram showing the mean contractile failure times during SFT-MI perfusion in control, cardiomyocytes from IPC-hearts or cardiomyocytes pre-treated for 5 minutes with 5 μ mol/L PMA, 100 μ mol/L adenosine, 100 μ mol/L diazoxide or 50 μ mol/L pinacidil in control conditions, or after 15 minutes pre-incubation with Tat-PKC ϵ inhibitor peptide prior to MI/R. PKC ϵ had no significant effect on the time to contractile failure in control and diazoxide pre-treated cardiomyocytes, but attenuated the delay in contractile failure in IPC, PMA-treated cardiomyocytes and after adenosine pre-treatment. There was no significant effect on the delay in contractile failure seen after pinacidil pre-treatment in the presence of PKC ϵ inhibitor. B, Histogram showing mean contractile recovery at the end of the MI/R protocol in cardiomyocytes as described in A. PKC ϵ inhibition attenuated the improvements in contractile recovery seen with IPC, PMA, adenosine or diazoxide but did not affect the improvements seen with pinacidil. C, Histogram showing the hypercontracture at the end of MI/R in cardiomyocytes described in A. Inhibition of PKC ϵ attenuated the reduction in

hypercontracture after IPC, PMA, adenosine or diazoxide treatment but had no effect on the pinacidil-induced reduction in hypercontracture. (**P<0.01, ***P<0.001, n=6 (≥97 cardiomyocytes) from ≥4 animals for each data set, ANOVA with Bonferroni's Post-hoc test).

Figure 6.

Time to sarcoK_{ATP} channel opening in cell-attached patches is markedly increased by IPC, PMA and adenosine pre-treatment.

A, Example traces of cell-attached patch recording from control, IPC, PMA or adenosine pre-treated cardiomyocytes. Arrows indicate time at which contractile failure occurred in control cardiomyocytes and was the time point at which the expanded recordings (B) were taken. C, Time to sarcoK_{ATP} channel opening was defined by the first burst of channel openings (sarcoK_{ATP} channel calculated to pass 10 pA per channel opening using these recording conditions) longer than 500 ms in duration. Mean time to sarcoK_{ATP} channel opening was markedly increased by IPC, PMA or adenosine pre-treatment. This delay was reversed in cardiomyocytes also pre-treated with Tat-PKCε-IP (D). (**P<0.01, ***P<0.001, n=≥6 from ≥3 animals per data set, ANOVA with Bonferroni's post-hoc test,).

Figure 7

Cardioprotective stimuli delay Ca²⁺-transient failure and increase the proportion of cardiomyocytes regaining Ca²⁺-transients after MI/R.

Example traces from Fura-2 fluorescence recording in isolated control (A) and IPC (B) cardiomyocytes. (ii) Expanded traces show the calcium transients over a 7 second period at time points #1 and #2 as indicated in (i). Ratio images were acquired at 0.2Hz except for the indicated time points where fast data acquisition was used to record calcium transients (16

ratios per second, 32 images). During the SFT-MI perfusion the acquisition rate was increased to 2 Hz until the Ca^{2+} transients failed when the rate was returned to 0.2Hz. Cardiomyocytes failing to recover their Ca^{2+} -transients at the end of MI/R are shown in grey, while those that did are shown in black. C, significantly more cardiomyocytes recovered their Ca^{2+} -transients after IPC, adenosine, diazoxide or pinacidil pre-treatment compared to control. (** $P < 0.001$, $n = 8$ experiments (≥ 48 cardiomyocytes) from ≥ 3 animals per data set, ANOVA with Bonferroni's post-hoc test). D, Mean time to Ca^{2+} -transient failure plotted with times to contractile failure and action potential failure. Cardioprotective stimuli other than diazoxide delayed the time to Ca^{2+} -transient failure. Times to action potential, Ca^{2+} -transient and contractile failure followed the same trend of being increased by cardioprotective protocols, other than diazoxide treatment. (** $P < 0.001$, (Ca^{2+} -transient failure data) $n = 8$ (≥ 48 cardiomyocytes) from ≥ 3 animals per data set, ANOVA with Bonferroni's Post-hoc test).

Figure 8

Mitochondrial function is maintained for longer during SFT-MI perfusion after application of cardioprotective stimuli.

A, Example traces of MgGreen fluorescence recorded from (i) control and (ii) adenosine (iii) diazoxide or (iv) pinacidil pre-treated cardiomyocytes in SFT-MI solution. B, Histogram showing mean change in MgGreen fluorescence (F/F_0) at the end of SFT-MI perfusion in control and cardioprotected myocytes. Cardioprotective stimuli reduced the elevation in intracellular Mg^{2+} (depletion in ATP) in each group tested (* $P < 0.05$, ** $P < 0.001$, $n = 9$ (≥ 137 cardiomyocytes) from ≥ 5 animals per group, ANOVA with Bonferroni's post-hoc test). C, Example traces of TMRM fluorescence recorded from (i) control and (ii) adenosine (iii) diazoxide or (iv) pinacidil pre-treated cardiomyocytes in SFT-MI solution. D, Histogram showing the mean time to a increase of fluorescence that was 10% of the maximal fluorescence at the end of SFT-MI. E, histogram showing the mean change in TMRM

fluorescence at the end of SFT-MI solution as a measure of mitochondrial membrane potential depolarisation in control and cardioprotected myocytes. Cardioprotective stimuli reduced the increase in fluorescence at the end of SFT-MI perfusion, and also delayed the time to mitochondrial depolarisation. (* $P < 0.05$, *** $P < 0.001$, $n = 9$ (≥ 168 cardiomyocytes) from ≥ 5 animals per group, ANOVA with Bonferroni's post-hoc test).

Figure 1

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