UNIVERSITY OF CALGARY

Differential Roles for sarcK_{ATP} and mitoK_{ATP} Channels in Hypoxia and Reoxygenation Injury:

A Study of Cell Viability and Ion Channel Pharmacology

by

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Abstract

Ischemic preconditioning is a novel cardioprotective mechanism in the heart. ATP-sensitive K (K_{ATP}) channels have been suggested as key elements of this pathway; however, their mechanisms of activation and involvement remain unclear. To determine the ability of the plasma membrane K_{ATP} ($sarcK_{ATP}$) and mitochondrial K_{ATP} ($mitoK_{ATP}$) channels to mitigate injury, calcium overload and trypan blue viability assays were conducted. In addition, characterization of a new $sarcK_{ATP}$ selective drug, HMR 1098, was performed by patch clamp experiments. Testing of HMR 1098 on a number of different K^+-channels showed a selective, dose-dependent block of the cardiac $sarcK_{ATP}$ channel. Calcium loading experiments and viability assays demonstrated that both $sarcK_{ATP}$ and $mitoK_{ATP}$ channels afford some level of protection at different, well-defined stages of the response to metabolic stress. The data also suggest that PKC stimulation as well as adenosine-A_1 receptor activation can mediate this effect via activation of the sarc and mito K_{ATP} channels

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Introduction:

In 1986 Murry et al. discovered a novel phenomenon in the heart that has potential clinical applications in ischemic heart disease. The discovery illustrated a mechanism whereby the heart could be conditioned to protect itself from ischemic insult. The phenomenon was termed Ischemic Preconditioning (IPC), a situation whereby a brief period of ischemia and reperfusion before an extended period reduced the extent of myocardial injury (see figure 1, Murry et al., 1986). This "classical" form of IPC has since been reported in many animal species including humans (Tomai et al., 1999). Though IPC has been extensively described, the task of elucidating the mechanism, series of events and effector(s) in mediating this process has left investigators bewildered. Since the initial report of this phenomenon over 15 years ago, many different theories proposing mechanisms have been suggested, but to date there remains much uncertainty in the field. Though the exact pathway of protection remains unclear, one key element is the cardiac K_{ATP} channel (Auchampach et al., 1992; Koning et al., 1996).

There is a vast amount of data that supports the notion that activation of the adenosine 5'-triphosphate (ATP)-sensitive potassium channel is one of the most likely contributors to this phenomenon (Ashraf et al., 1994; Auchampach et al., 1992; Tomai et al., 1994). Therefore, the majority of present studies focus on understanding how this channel is activated, and more importantly, how opening of this channel might afford protection against ischemic injury. To add to the already perplexing mechanism, the discovery and involvement of the mitochondrial K_{ATP} isoform has created another element of obscurity. The recent emphasis and debate on the involvement of the sarcolemmal vs. the

mitochondrial channel in cardiac protection is forcing the traditional hypothesis of how these channels are involved to be remodeled. It was believed that the sarcK_{ATP} channel was the a major mediator of protection against ischemic injury by affecting action potential duration. This hypothesis, due to emerging data, is being questioned and a role for the mitoK_{ATP} channel isoform is becoming more prevalent (Gross and Fryer, 1999).

One feature that is unique to the K_{ATP} channel is its ability to couple cellular metabolism and excitability. The channel is predominantly modulated by the ATP/ADP concentrations within the cell. At millimolar concentrations of ATP the channel is closed and conversely when ATP is lowered and the subsequent ATP/ADP ratio is lowered the channel is more likely to be in its open state (Bernardi et al., 1992). An example of this property is best illustrated during insulin release in the pancreatic β-cell. In the β-cell, K_{ATP} channels are present in high density and their role is well characterized. During glucose metabolism, the K_{ATP} channel "senses" a rise in internal [ATP], which results in closure of the channel, which under normal functioning is open (Van Winkle et al., 1994a). Closure of the channels results in depolarization and thus opening of the voltage gated L-type calcium channel. An influx of calcium initiates insulin release by exocytosis. This mechanism is referred to as the "fuel hypothesis": the K_{ATP} channel is therefore pivotal in maintaining the proper function and release of insulin from the β-cell. Mutations in the channel subunits result in familial hyperinsulinism or persistent hyperinsulinemic hypoglycemia of infancy (PHHI, OMIM 256450) (Meissner et al., 1999; Sperling and Menon, 1999; Thomas et al., 1996).

In cardiac myocytes however, the question of whether K_{ATP} channels are active under normal physiological conditions remains controversial. Under normal cellular functioning, the K_{ATP} channel is reported to remain closed signifying no apparent role for this channel under this condition. To date, no conclusive evidence has been presented that defines a role for this channel in the heart, raising the question of why nature and evolution have equipped the heart with such an abundance of these channels without apparent purpose.

Ischemic Preconditioning:

Initial studies on anesthetized dogs have revolutionized our understanding of the heart and its ability to react to ischemic insult. Murry et al. subjected anesthetized dogs to four sequential 5-minute periods of regional ischemia each followed by reperfusion, prior to a sustained 4-minute global ischemic insult (Murry et al., 1986). Surprisingly, the results demonstrated that the short initial regional ischemic events, too brief to cause necrosis themselves, preconditioned the heart and protected it, reducing injury from the expected 30% of the affected region to only 7%. Although the cumulative ischemic time was increased, preconditioning had a remedial effect. This phenomenon has inspired the investigation by many with the hope of understanding the mechanisms involved.

Cardioprotection by virtue of ischemic preconditioning is a short-lived phenomenon. The protective window fades as a function of the time between the preconditioning event and the sustained infarct generating ischemia, when this interval is extended beyond 1 hour

(Van Winkle et al., 1994b). Another factor that affects the interval of protection is the duration of the preconditioning stimulus. In rats and rabbits a full 5 minutes of deep ischemia is required to put the heart into a preconditioned state. However, shorter periods of ischemia can also successfully precondition the heart if it is concomitantly metabolically challenged by diminishing glycogen stores (Van Winkle, 1996).

Recently, investigators have reported a second window of protection that appears 24 hours after the preconditioning event. This second window is thought to be mediated by the induction of a protective protein, though no conclusive evidence supports this claim at present (Kim et al., 1999). Not only does this "second window" of protection appear much later than classical conditioning, it has been reported to protect against injury for up to 3 days after the preconditioning event. This has led investigators to speculate that the protection may be mitigated by transcriptional factors in lieu of the local mediators such as channels that are thought to afford protection under classical preconditioning. There still remains much to be learned in the realm of IPC and the effectors and mechanisms that cause protection.

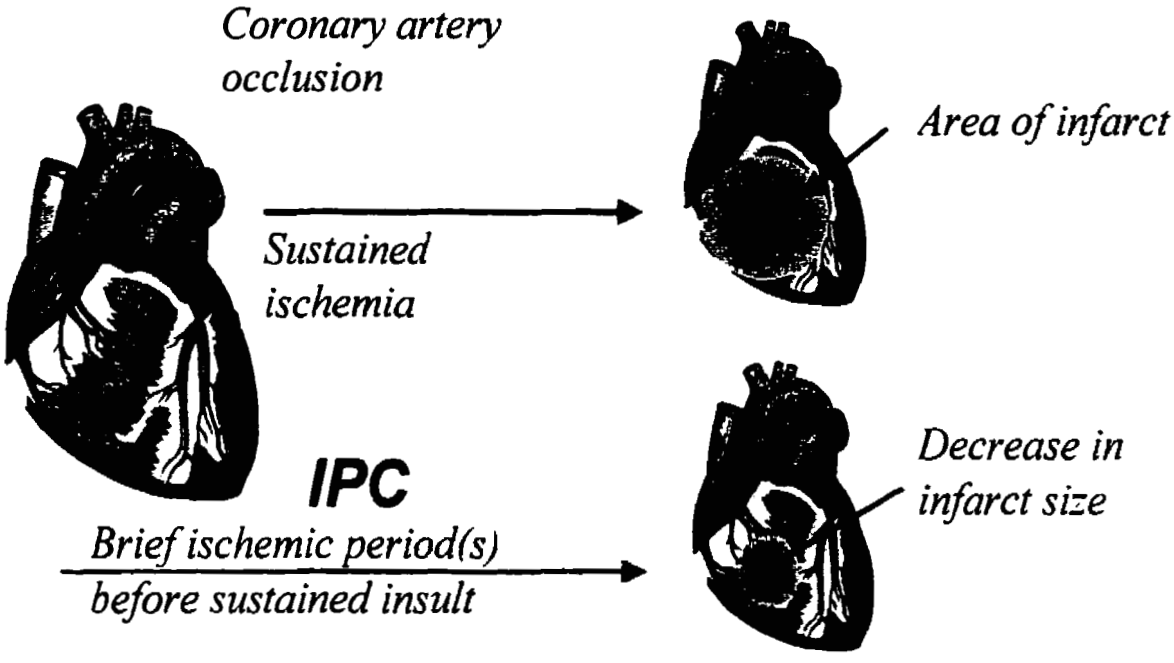


Figure 1. Ischemic Preconditioning

The diagram illustrates the phenomenon of Ischemic Preconditioning (IPC). Short bouts of ischemia before a sustained ischemic insult precondition and protect the heart against damage. The resulting infarct in the preconditioned heart is markedly reduced when compared to the unconditioned heart.

The K_{ATP} Channel

In addition to the pancreatic β-cells and cardiac myocytes, the K_{ATP} channel has been characterized in many tissues including neurons, vascular smooth muscle and skeletal muscle. Reconstitution and cloning of the K_{ATP} channel has revealed that it is an hetero-octamer composed of two types of subunits that associate with a 1:1 stoichiometry (see figure 2). The subunits comprise the pore forming unit which belongs to the inward rectifying K^+ channel family (see figure 3) and the sulfonylurea receptor (SUR), a member of the ATP binding cassette (ABC) superfamily which is the target for ATP and sulfonylurea compound binding (Aguilar-Bryan and Bryan, 1999; Baines et al., 1998). The mitochondrial channel remains somewhat of a mystery. The first and the only direct patch clamp experiment to date conducted by Inoue et al. describes the channel as having a smaller conductance and being pharmacologically distinct from the sarcolemmal channel (Inoue et al., 1991). This discrepancy in properties has provided the means to allow investigators to selectively target the sarcolemmal or mitochondrial channel. Since direct activity of mitoK_{ATP} channel has not been surveyed extensively, the selectivity of pharmacological agents for this channel is being challenged. Recent evidence proposes that certain agents that were reported to exclusively target the mitoK_{ATP} channel may in fact be acting on both the sarcK_{ATP} and mitoK_{ATP} channels. The details of this finding will be described more extensively in the Discussion.

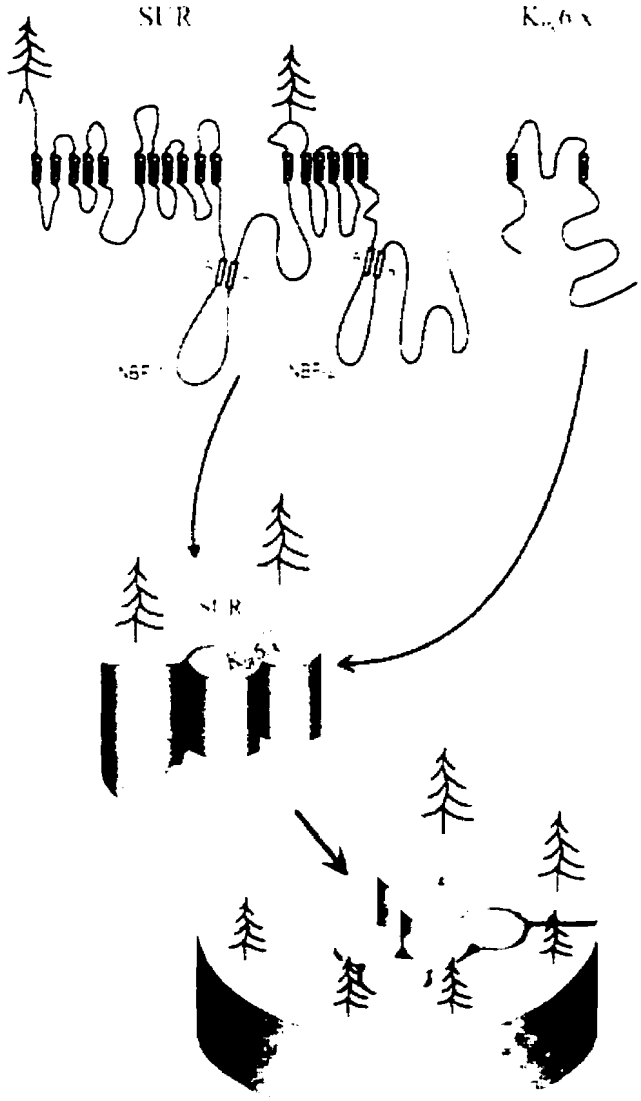


Figure 2. K_{ATP} Channel Subunit Composition

K_{ATP} channels are composed of the pore forming unit that belongs to the inward rectifying K^+ channel family and the sulfonylurea receptor (SUR), a member of the ATP binding cassette (ABC) family. These subunits associate with a 1:1 stoichiometry to form a hetero-octameric channel complex. (Adapted from A.P Babenko, L Aguilar-Bryan, J. Bryan. A View of SUR/K_{ir}6.X, K_{ATP} channels. Annu. Rev. Phyiol. 1998. 60:667-87.)

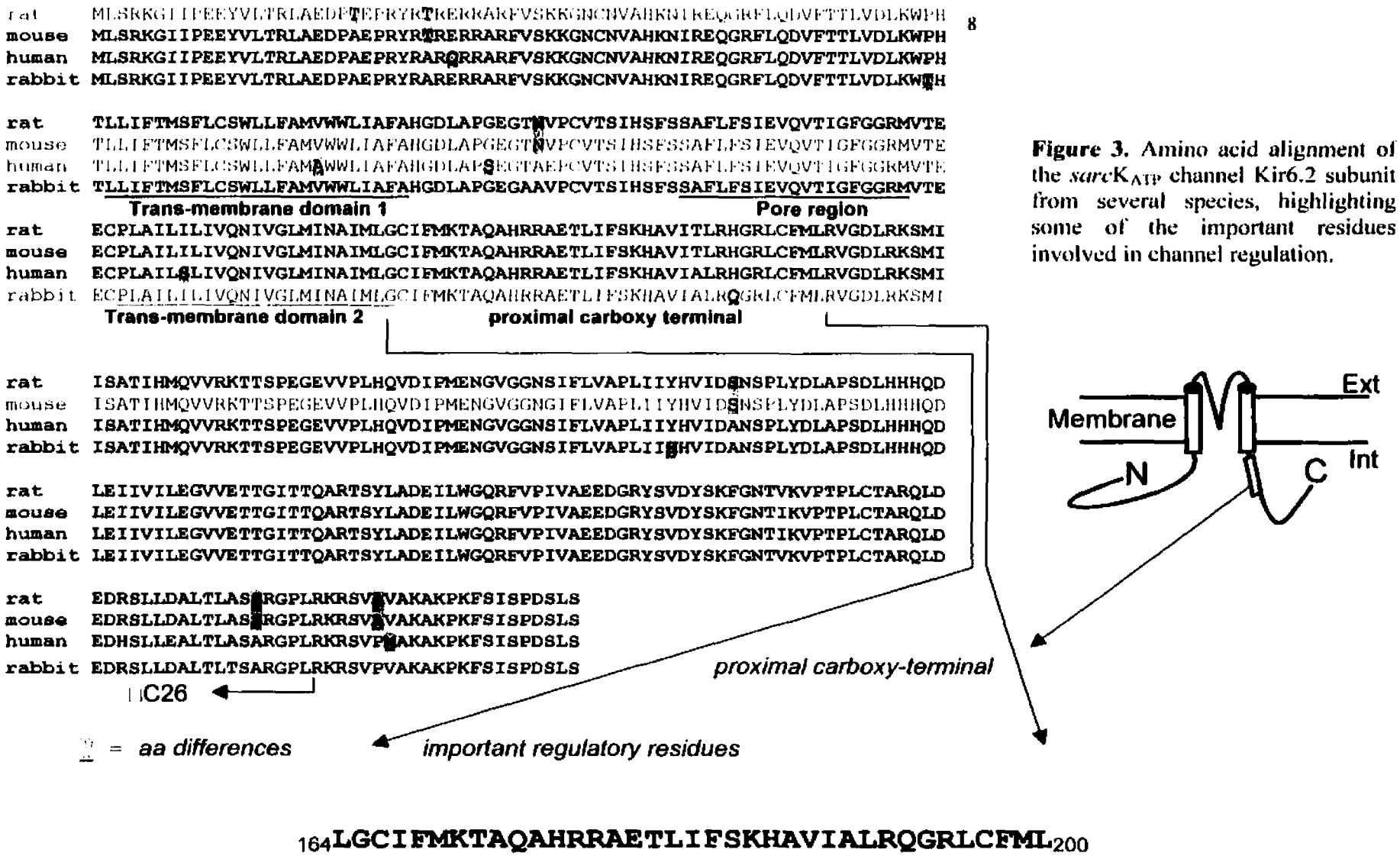


Figure 3. Amino acid alignment of the *sarc*K_{ATP} channel Kir6.2 subunit from several species, highlighting some of the important residues involved in channel regulation.

$_{164}$**LGCIFMKTAQAHRRAETLIFSKHAVIALRQGRLCFML**$_{200}$

K_{ATP} Channel in IPC

While unraveling the mechanisms underlying this protective phenomenon is a complex process, some of the important elements involved have been deciphered. There is ample evidence suggesting that the activation of the K_{ATP} channel is not only important but also an imperative trigger or effector of IPC (Kouchi et al., 1998; Parratt and Kane, 1994). Evidence for the involvement of this channel has come from studies that report that K_{ATP} channel openers such as cromakalim, pinacidil and diazoxide are capable of pharmacologically inducing IPC. Conversely, this effect can be abated by the concomitant addition of K_{ATP} channel blockers, namely glibenclamide and 5-hydroxydecanoate (Schulz et al., 1994). Though this finding appears to be straightforward, it is unquestionably the greatest source of confusion and debate. Although the K_{ATP} channel is involved, an intense debate continues on whether it is the sarcK_{ATP} or mitoK_{ATP} that is responsible for the protection. Recent pharmacological advances have made it possible for investigators to target selectively either the mitochondrial or the sarcolemmal channel. Presently, support appears to be shifting from the sarcolemmal to the mitochondrial channel as being responsible for the protection; however, studies that propose the involvement of both channels are uncommon. The data remain inconclusive and the role of each channel is still in question.

Although many investigators are concerned with determining which channel is involved, there is an increased focus on understanding how the channel affords protection. There is an even greater paucity in this field and answers have not been forthcoming.

Understanding how channels modulate injury or afford protection may provide equally important insight into possible clinical exploitation of this process.

sarcK_{ATP} Channel in IPC

Evidence for K_{ATP} channel involvement in acute IPC was first presented by Gross and Auchampach in their studies on canine heart (Gross and Auchampach. 1992a). Several studies confirmed both in animals and humans that K_{ATP} channels are likely the effectors mediating acute conditioning in cardiomyocytes (Carr and Yellon. 1997). When studies began on the K_{ATP} channel. the sarcK_{ATP} channel was the primary focus of investigation. Noma et al. first postulated that the opening of the sarcK_{ATP} channel by hypoxia. ischemia or pharmacological agents leads to a shortened action potential duration (APD). The shortened APD was postulated to reduce Ca^{2+} entry by inhibiting the L-type calcium channels (Noma. 1983: Schulz et al.. 1994). In addition. membrane hyperpolarization would also inhibit Ca^{2+} entry and slow or prevent the reversal of the sodium-calcium exchanger that normally extrudes calcium. The result of these processes would be reduced calcium loading. energy preservation by virtue of a lessened contractile force and thus greater cell viability. Cole et al.. using an isolated guinea pig right ventricular wall preparation. showed that glibenclamide inhibited the shortening of the action potential during ischemia and resulted in poor recovery of ventricular function (Cole et al.. 1991). Yao and Gross found similar results. as did many other investigators. linking action potential duration to the cell survival via K_{ATP} channels (Yao and Gross. 1994a). This

hypothesis was termed the "action potential hypothesis" and was thought to be the mechanism by which the K_{ATP} channel afforded protection to the cardiac myocyte.

Although the AP hypothesis appeared to rationalize the way in which IPC afforded protection. investigators remained unconvinced that this mechanism was responsible for mediating protection. Further experiments testing the theory yielded contradictory results. Schulz et al.. using anesthetized pigs as a model of IPC. discovered that the reduction of AP duration was less than 10% and concluded that this seemed unlikely to account for the extent of cardioprotection observed. In addition to this study. many investigators have since reported that cardioprotection is observed while pharmacologically activating the sarcK_{ATP} channel in the absence of reduction in action potential duration. This was achieved by pharmacological activation of the sarcK_{ATP} channel in a non-beating model (Jovanovic et al.. 1998a). These results have led to dismissal of the action potential hypothesis. leaving investigators to ponder once again how the sarcK_{ATP} channel affords protection.

In favor of the sarcolemmal channel as a mediator of protection. Haruna et al. reported that digoxin. an inhibitor of the Na-K ATPase. blocked the cardioprotective effect of IPC (Haruna et al.. 1998). This effect was assumed to occur as a function of sarcK_{ATP} since increased cytosolic ATP levels due to reduced utilization were thought to have an inhibitory effect on opening of the channel. Furthermore. under the same protocol. addition of diazoxide. a selective mitoK_{ATP} opener. at doses that have been reported to reduce infarct size. had no effect. The infarct size remained unaltered at doses up to 10-fold greater than those reported to reduce infarct size (Garlid. 2000). This finding. in

addition to many others, disputes the idea that the mitoK_{ATP} channel is the sole participant or effector in the conditioning pathway and is consistent with a role for the sarcolemmal channel as requisite contributor to the preconditioning pathway (Gross and Auchampach, 1992b).

Most convincing are the recent experiments performed by Jovanovic et al. (Jovanovic et al., 1999). COS-7 cells were transfected with the Kir6.2 and SUR2A subunits, the cardiac K_{ATP} channel isoform, in order to study the effects of sarcK_{ATP} channels on Ca^{2+} overload as a function of hypoxia and reoxygenation. K_{ATP} channel-deficient cells, exposed to three minutes of chemically induced hypoxia, by administration of dinitrophenol (DNP), and subsequent removal of inhibition and reoxygenation, were observed to "overload" with Ca^{2+}. An increase in the intracellular level of Ca^{2+} has been identified as one of the events that ultimately leads to cell death (Ashcroft and Gribble, 1998). The Ca^{2+} overload was markedly diminished by the transfection and pharmacological activation of both SUR2A and Kir6.2. Furthermore, it was noted that both subunits were required for protection to be significant. This protection was observed independently of APD shortening, but nonetheless strongly supports a protective role for the sarcolemmal channel.

The evidence suggests that the sarcK_{ATP} channel plays an integral role in mediating injury by preconditioning. Though the action potential hypothesis has been refuted, evidence persists for an important role for the sarcK_{ATP} channels. The recent studies by Jovanovic et al. provide the most compelling evidence for the involvement of these channels in IPC. The role of sarcK_{ATP} channel appears most significant during reperfusion/reoxygenation

injury, which suggests that its role may be important in ionic balance between intracellular and extracellular media. The ability of the K_{ATP} channel to be activated in this situation suggests that it may have an osmo-protective effect. This being said, there is still convincing evidence that suggests that the mitochondrial K_{ATP} isoform has an equally significant role in the IPC pathway.

mitoK_{ATP} Channel in IPC

Evidence has been shifting in favor of the mitoK_{ATP} channel as the effector of IPC. This notion has been primarily fueled by the discovery that the action potential effect of the K_{ATP} channel could be divorced from the protective conditioning pathway. The first study to propose that protection was independent of APD shortening, and therefore assumed to be independent of sarcK_{ATP} channels was published by Yao and Gross in 1994 (Yao and Gross, 1994b). In addition, various studies on isolated non-beating cardiac myocytes implicated the K_{ATP} channel in mediating the protective effects of K_{ATP} openers or IPC in the absence of ventricular action potentials (Armstrong et al., 1995). A detailed study of the mitochondrial population of channels has been enabled by the characterization of the drug, diazoxide. Garlid et al. demonstrated that diazoxide was 2000 times more potent for the opening of mitochondrial K_{ATP} channels than sarcolemmal channels in the heart (Garlid et al., 1997a). Selective activation of the mitoK_{ATP} channel has proved to be very advantageous in learning more about the specific role of the mitoK_{ATP} channels in affording protection. In addition, 5-HD (5-hydroxydecanoate) has been described as

selectively inhibiting mitoK_{ATP} channels without having any effect on sarcK_{ATP} channels (Sato et al., 1998a). These two agents used in concert have been important in understanding the role of the mitoK_{ATP} channel independent of the sarcK_{ATP} channel in IPC.

Inoue et al. first identified an ATP-sensitive K^+ channel in the inner mitochondrial membrane in rat liver by patch clamping giant mitoplasts prepared from rat liver mitochondria (Inoue et al., 1991). Evidence that the mitoK_{ATP} channel is involved in cardiac protection was first presented by Garlid et al. in 1997 (Garlid et al., 1997a). Marban's laboratory similarly demonstrated how selective closure of these channels via a pharmacological agent 5-HD inhibited protection during IPC. An analysis of flavoprotein fluorescence (due to oxidation from mitochondrial depolarization) and sarcK_{ATP} currents supported the conclusion that mitochondrial channels were the effectors of this observed protection (Liu et al., 1998). Sato et al. recently addressed the issue of mitochondrial function in the preconditioning pathway (Gross and Fryer, 1999; Sato and Marban, 2000). In their report they provided evidence that PKC has the ability to potentiate the effect of diazoxide in activating mitoK_{ATP} channels. In support of these findings, Gogelein et al. have recently characterized the pharmacology of a new sarcK_{ATP} channel antagonist, HMR 1883, which appears to be a cardioselective sarcK_{ATP} antagonist (Gogelein et al., 1998). The authors present data that demonstrate that this drug does not block infarct size reduction during IPC in rabbits but does affect the cardiac action potential. Recent observations suggest that this compound has no effect on mitoK_{ATP} channels as observed by flavoprotein fluorescence analysis in the presence of diazoxide (Sato et al., 1998a). In addition to the above, it was reported that, in isolated rat hearts

subjected to 25 min of global ischemia and 30 min reperfusion. diazoxide increased the time to onset of contracture and improved postischemic functional recovery in a manner similar to that of cromakalim (Garlid et al.. 1997b).

Experiments have implicated PKC as being involved in curtailing cellular injury by activating both K_{ATP} channels (Takashi et al.. 1999). This finding suggests that both sarcK_{ATP} and mitoK_{ATP} channels could be activated through similar pathways. It is postulated that opening of the mitoK_{ATP} channel results in dissipation of inner mitochondrial matrix potential that in effect results in a reduction of calcium influx and consequently a reduction of calcium overload. Furthermore. it has been observed that opening of these channels results in an enhancement of the inhibition of mitochondrial ATP-synthase by the regulatory protein IF_1 and subsequent conservation of ATP (Miyamae et al.. 1996). This finding was supported by Wang et al. who demonstrated that intracellular ATP was preserved in diazoxide-treated hearts. However. no significant differences existed in LDH release and tissue ATP levels between diazoxide and calcium preconditioned (CPC)-treated hearts after Ca^{2+} repletion (Wang and Ashraf. 1999).

Mitochondria are known to play at least two roles that are essential in cell survival: ATP synthesis and maintenance of Ca^{2+} homeostasis (Gross. 2000). It is possible that the enhancement of ATP production in the diazoxide-preconditioned heart maintains Ca^{2+} homeostasis. A Ca^{2+} increase within the physiological range stimulates the activity of 3 enzymes: pyruvate dehydrogenase. isocitrate dehydrogenase and α-ketogluterate dehydrogenase. Ca^{2+}-induced increase in NADH redox potential might increase the rate of ATP synthesis (Gunter and Pfeiffer. 1990; Haworth and Hunter. 1980). In addition.

the mitoK$_{ATP}$ channel is believed to be in part responsible for the maintenance of potassium homeostasis within the mitochondrion and the control of intramitochondrial osmotic pressure and mitochondrial volume, which are important in the modulation of metabolic processes (Halestrap, 1989). When mitoK$_{ATP}$ channels are opened the depolarization by the influx of K^+ has been reported to result in swelling of the mitochondria (Gross and Fryer, 2000). It has also been reported that ATP production is up-regulated secondary to this effect. Therefore it appears that the mitoK$_{ATP}$ channel is responsible for ATP conservation but also some form of up-regulation of ATP production. Understanding the role of the mitoK$_{ATP}$ channel is starting to help investigators realize the significance of this elusive channel in the mitochondrial membrane and how it may confer resistance to injury.

Mechanism of Action: PKC and Receptor-Coupled Initiation

IPC has been observed in both animal models and humans; evidence supports the K$_{ATP}$ channel as an effector of this phenomenon. Nevertheless, initiators and factors modulating this pathway are a source of debate. Recently, PKC has been reported to be a pivotal element in regulating the mechanistic pathway leading to IPC (Liu et al., 1991; Walsh et al., 1995; Ytrehus et al., 1994). Downey et al. have described a mechanism whereby the stimulation of G-protein coupled receptors activates PKC which is believed to translocate from the cytoplasm to the sarcolemma in order to phosphorylate an effector that is possibly the sarcK$_{ATP}$ channel. This phosphorylation is assumed to be the action

responsible for protection (Downey and Cohen, 1996). Light et al. have shown that phosphorylation by PKC increases P_o of the sarcK_{ATP} channel in the presence of physiological concentrations of ATP and have recently determined the specific residue responsible for activation (Light et al., 1996a; Light et al., 2000). It appears that pretreatment with PMA or other PKC activators results, in addition to a direct PKC effect on the channel, in depressed metabolic activity. This claim is supported by the observation that low levels of lactate are produced in cells activated by PMA under anoxic conditions (Ladilov et al., 1998). The reduced ATP depletion appears to be a result of a lowered consumption rather than accelerated anaerobic metabolism by pretreated cells. It appears therefore that the effect of PKC is two-fold, acting both directly on the channel and more generally on the metabolic state of the cell under insult.

Pathways exist that appear to activate IPC. These systems are redundant, suggesting a powerful selective pressure over the course of evolution to incorporate this regulatory machinery into the cardiovascular system. An analysis of the PKC-induced protection motivates the obvious question: what causes the activation of PKC and how is the activity modulated? Protection, working presumably through the PKC pathways, has been shown to be mediated by several classes of receptor-coupled pathways, namely adenosine (A_1/A_3), bradykinin B_2, and opioid receptors (Tsuchida et al., 1992; Wall et al., 1994). The exact nature of the signal pathways involved in preconditioning is a focus of current investigation. In a report by Mizumura, an open chest, anaesthetized dog model of IPC resulted in not only reduced infarct size, but also resulted in adenosine release (Mizumura et al., 1995). Furthermore, administration of the adenosine A_1 receptor agonist before coronary artery ligation produced a significant reduction in infarct size (Baxter and

Yellon, 1999). This phenomenon was investigated at the cellular level where administration of adenosine agonists was seen to be cardioprotective in instances of hypoxia and reoxygenation (Dougherty et al., 1998; Liang, 1998). 2-Chloro-N^6-cyclopentyladenosine (CCPA), an A_1 receptor agonist, in lieu of the 5 min pre-ischemia, mimicked ischemic preconditioning in the rabbit, whereas the A_2-selective agonist CGS-21680 had no protective effect (Thornton et al., 1992). In addition, the A_3 receptor was also deemed important in this cascade of events. The adenosine mechanism is made even more plausible considering that the myocardium is reported to release large amounts of adenosine soon after the onset of ischemia. In linking PKC to adenosine, the 5'-nucleotidase, an enzyme responsible for converting AMP to adenosine, is reported to be up-regulated as a result of PKC activation (Kitakaze et al., 1999). The exact mechanism has not yet been described; however, many studies collectively construct a model that is persuasive in involving receptor-coupled pathways, PKC and K_{ATP} as constructive elements (Miyamae et al., 1996). The detailed cellular mechanism of protection through such pathways has not been worked out in detail. However, current investigation is bringing us closer to identifying the series of events responsible. It is known that PKC is a potent activator of both the sarcolemmal and mitochondrial channels (Light et al., 2000; Liu et al., 1996; Sato et al., 1998a). Initiators of this and the function of the K_{ATP} channels are another topic of investigation (refer to figure 4).

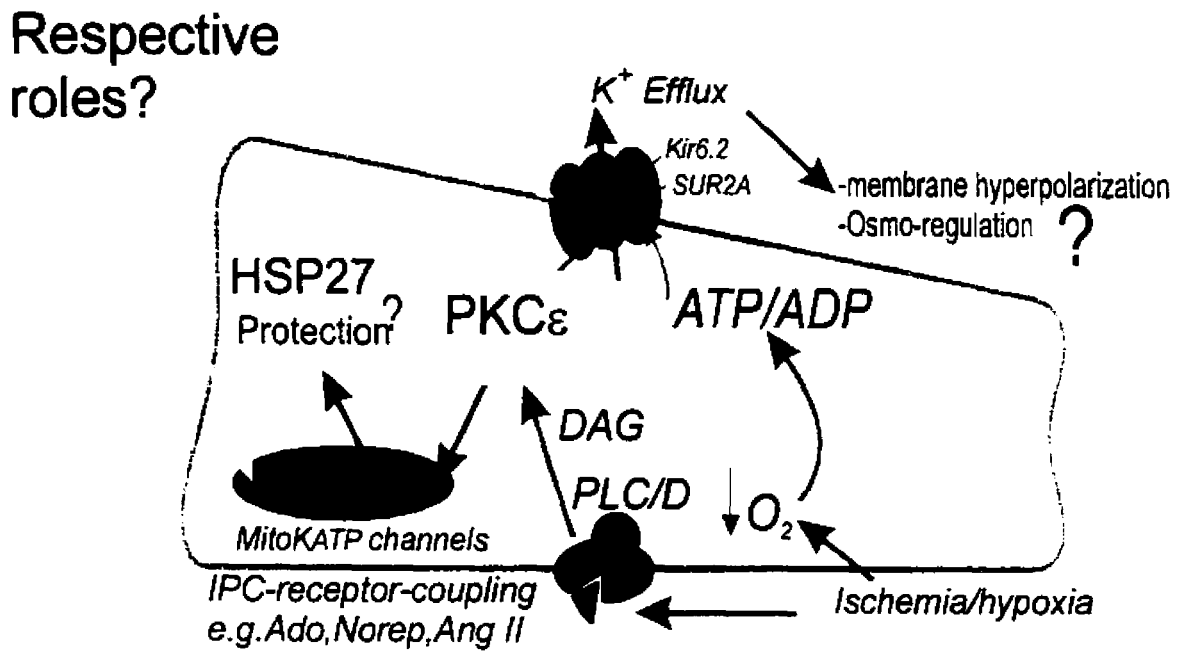


Figure 4. Current understanding of IPC pathways and the involvement of sarcK_{ATP} and mitoK_{ATP} channels

Opening of the sarcK_{ATP} channel is thought to be mediated by a change in the ATP/ADP ratio and by phosphorylation by PKC. The functional significance of this is still unknown. PKC is thought to also activate the opening of the mitoK_{ATP} channels resulting in the release and activation of mediators that are postulated to be involved in the second window of protection.

(HSP27: Heat Shock Protein 27, Ado: Adenosine, Norep: Norepinephrine, Ang II: Angiotensin II, PKC: Protein Kinase C, PLC/D: Phospholipase C/D, DAG: Diacylglycerol)

Ischemic Preconditioning – Human Model

Ischemic preconditioning has been defined as improved ischemic tolerance induced by prior exposure of the myocardium to a brief period of ischemia (Murry et al., 1986). It is important to understand and identify this mechanism in humans so that insights from animal models can be applied in a clinical setting. The phenomenon of IPC cannot be directly extrapolated to humans due to different intracellular mechanisms among the various animal models studied. Unfortunately due to ethical and logistical obstacles, direct human experiments are not a possibility. However, empirical studies in conjunction with surrogate end points have been used to assess this condition and have shown that the phenomenon is apparent (Claeys et al., 1996). Speechly-Dick et al. showed that functional recovery in human isolated atrial trabeculae following an extended period of hypoxia was greatly enhanced by a preconditioning event (Speechly-Dick et al., 1995). In addition, in vitro studies have shown that human cardiomyocytes can in fact be preconditioned (Carr et al., 1997; Morris and Yellon, 1997; Speechly-Dick et al., 1995). Interestingly, this effect can be chemically induced by the activation of PKC, or by adenosine receptor agonists, and is prevented by K_{ATP} channel blockers (Ladilov et al., 1999).

Cardiac Injury

Before one can delve into the details of cardiac protection, understanding mechanisms of injury is imperative. Cellular damage is a function of several participating elements including ionic imbalance, osmotic injury, and Ca^{2+} overload. In addition, the question of

whether cell death occurs via necrosis or apoptosis remains unanswered. Among the several factors proposed to initiate ischemic myocardial injury, excess influx of Ca^{2+} is one of the most potent, resulting in sarcolemmal destruction, necrosis of the cell, depletion of high energy phosphates, and loss of intracellular contents (Alto and Dhalla, 1979; Ashraf et al., 1994; Tanno et al., 1992). It was therefore originally postulated that K_{ATP} channels modulate the influx of calcium by shortening of the action potential duration (APD).

Another proposed form of damage is reperfusion injury, defined as injury caused by the restoration of blood flow after ischemic insult of those cells that were reversibly damaged during the ischemic transient. During ischemia, metabolites accumulate in cardiomyocytes increasing the intracellular osmolarity. The discrepancy of osmolarity between the internal and external solutions upon restoration of flow results in cell swelling during reperfusion (Jennings et al., 1975a). It is known that cell swelling and the loss of cell volume regulation play pivotal roles in ischemic injury in the myocardium (Jennings et al., 1975b; Jennings and Reimer, 1991). Cell swelling can activate a variety of transport pathways that can cause a net efflux of K^+, Cl^-, organic anions and organic osmolites that are thought to be important in regulating ischemic injury (Vandenberg et al., 1996).

Hypercontracture of the myofibrils has also been postulated as one of the major causes of cellular injury and death. This injury may be secondary to the energization during reperfusion that enables the cell to restore contractile forces (Piper et al., 1998a). It has been shown in earlier studies that combination of cytosolic Ca^{2+} overload with the re-

supply of energy during the reperfusion or reoxygenation is associated with hyperactivation of myofibrils and hypercontracture of cardiomyocytes (Piper et al., 1992; Vander Heide et al., 1986). This hypercontracture causes tissue disruption and cell death due to excessive cell-to-cell force transduction (Ganote, 1983).

Many reasonable hypotheses have been proposed regarding mechanisms leading to injury. Understanding these factors will help identify effectors in IPC and yield some insight as to how the K_{ATP} channel functions and participates to enhance cellular viability.

Methods and Materials

Overview of Methods

The objective of this research was to enhance our understanding of K_{ATP} mediated protection against hypoxia and reoxygenation injury. In order to further the understanding of the involvement of these channels, interest was focused on the specific involvement of the mito and sarcK_{ATP} isoforms, and the pathways that lead to activation of these channels was of particular interest.

In studying the implications of these channels during hypoxia we had to fulfill three requirements. First we required a model of hypoxia and reoxygenation injury. Second we needed an assay to measure viability and cellular injury during this state of hypoxia. Lastly, we faced the obstacle of studying the individual contribution of each of mito and sarcK_{ATP} channels in isolation. In a recent study by Jovanovic et al Ca^{+2} entry into COS-7 cells was measured by fluorescence of FLUO-2-AM dye as an index of cellular injury. Using this protocol for $[Ca^{2+}]_i$ measurement and inducing hypoxia by administration of cynaide and 2-deoxy-glucose afforded us a model whereby we could measure real-time $[Ca^{2+}]$ changes as an effect of sarc and mitoK_{ATP} channel activation and inhibition during hypoxia. By using an array of pharmacological agents we were able to selectively activate and inhibit the individual channel isoforms. The non selective K_{ATP} channel opener levcromakalim and the sarcK_{ATP} inhibitor HMR 1098 in concert with mitoK_{ATP} channel opener diazoxide and inhibitor 5-hydroxydecanoate were the pharmacological agents with which we were able to target the sarcolemmal and mitochondrial channels specifically (refer to table 1).

	SUR1/Kir6.2 β-cell	**SUR2A/Kir6.2** Heart. Brain. Skeletal Muscle	**SUR1/Kir6.1** Mitochondrial?
Diazoxide	Sensitive EC_{50} ~100 nM	Very weak EC_{50} ~1 mM	Sensitive EC_{50} ~100nM
Cromakalim	insensitive	Sensitive EC_{50} ~1-10 μM	Sensitive EC_{50} ~1-10 μM
HMR1098 (sulphonylurea)	insensitive	Sensitive	Insensitive
Glibenclamide (sulphonylurea)	very sensitive IC_{50} ~1-100 nM	Sensitive IC_{50} ~1-10 μM	Sensitive IC_{50} ~100-500 nM
5-hydroxydecanoate (5-HD)	sensitive	Insensitive (even to 500 μM)	Sensitive

Table 1. Pharmacology of K_{ATP} channels

The K_{ATP} channel has a rich and varied pharmacology. The distinct pharmacology of the different isoforms. namely the cardiac. β-cell and mitochondrial channels provides a way to selectively target the channel of interest.

HMR1098 is a relatively new drug and though it has been assumed to be selective for the sarcK_{ATP} channel there is a paucity of data that conclusively supports this. It was therefore in our interest to assure the selectivity of HMR 1098 for the sarcK_{ATP} channel and to determine a suitable isoform-selective concentration. This was conducted by testing several concentrations of the drug on sarcK_{ATP} channels from native myocytes and looking at the effect of the drug on recombinant K_{ATP} isoforms of this channel. We transiently transfected the channel of interest in tsA201 cells. These channels were: Kir6.2/SUR1, the β-cell isoform and pharmacology thought to resemble the mitoK_{ATP} channel: the Kir6.2/SUR2A, the sarcolemmal cardiac isoform: Kir6.2(ΔC26), an ATP sensitive channel construct able to function without the SUR subunit due to a C-terminal truncation on the Kir subunit: and Kir2.1, a strong inward rectifier also able to function in the absence of the SUR subunit. We determined the blocking potency of this drug for these different recombinant channels.

Another area of interest was to learn what possible upstream activators of the K_{ATP} channels could mitigate ischemic injury, since many investigators have suggested that PKC is involved in phosphorylation of the channel, subsequent activation, and thus protection (Light et al., 1996a). Studies dealing with the role of PKC as a mediator of cellular injury through the K_{ATP} channel were conducted. A set of experiments, in which the channels were activated via PMA activation of PKC during hypoxia, allowed us to observe the involvement of these channels via a downstream PKC activator. This also allowed us to observe if the channels maintain their respective roles under regulation by this pathway as well as after direct pharmacological activation. The Ca^{2+} overload protocol was used under these conditions as another way to measure the effect of the

PKC pathway. Furthermore PMA in conjunction with pharmacological agents that inhibit a specific population of channels allowed us to characterize the selectivity of channel activation by PKC. In addition, we used a molecular approach to test the role of the sarcolemmal channel during hypoxia and reoxygenation. Earlier studies by Light et al. determined the phosphorylation site on the sarcK_{ATP} channel responsible for activation of this channel by PKC (Light et al., 2000). By mutating this site we created a mutant (KirT180A) that was incapable of being phosphorylated and thus was insensitive to PMA. Using this mutant in Ca^{2+} overload experiments enabled us to prevent sarcK_{ATP} channel activation by PMA, and to compare the Ca^{2+} fluxes with those cells expressing the wild-type channel. This method of selectively targeting the sarcK_{ATP} channel during hypoxia and reoxygenation provided evidence for the involvement of this channel in mediating injury.

Since Ca^{2+} imaging and overload were used to indicate cell viability, further studies to confirm the correlation of Ca^{2+} overload and cell death were performed. Trypan blue viability assays of cells subjected to the same hypoxic insults and pharmacological agents as for the Ca^{2+} imaging protocol served to correlate Ca^{2+} overload with a decrease in cell viability (refer to figure 5 and 6).

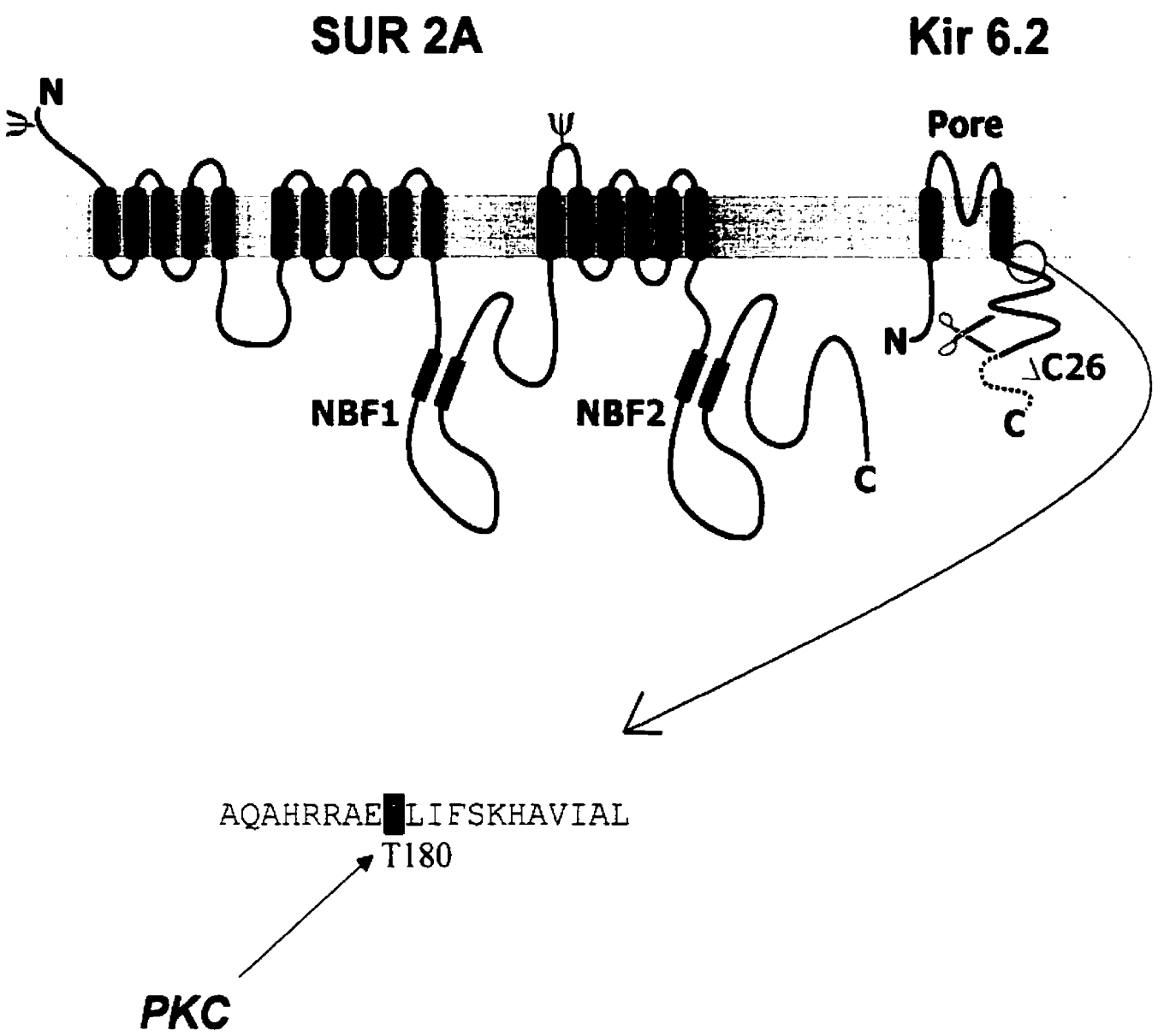


Figure 5. Membrane Topology of Kir 6.2/SUR2A and PKC Phosphorylation Site

K_{ATP} channels are activated by phosphorylation by PKC at physiological [ATP]. The mechanism is identical to that observed in native K_{ATP} channels. PKC acts to phosphorylate the Kir 6.2 subunit at the T180 residue. Mutating this site prevents phosphorylation and subsequent activation of the channel by PKC. Truncation of the last 26 residues of the Kir subunit results in the ΔC26 isoform of the channel .which is able to form a functional channel without associating with the SUR subunit.

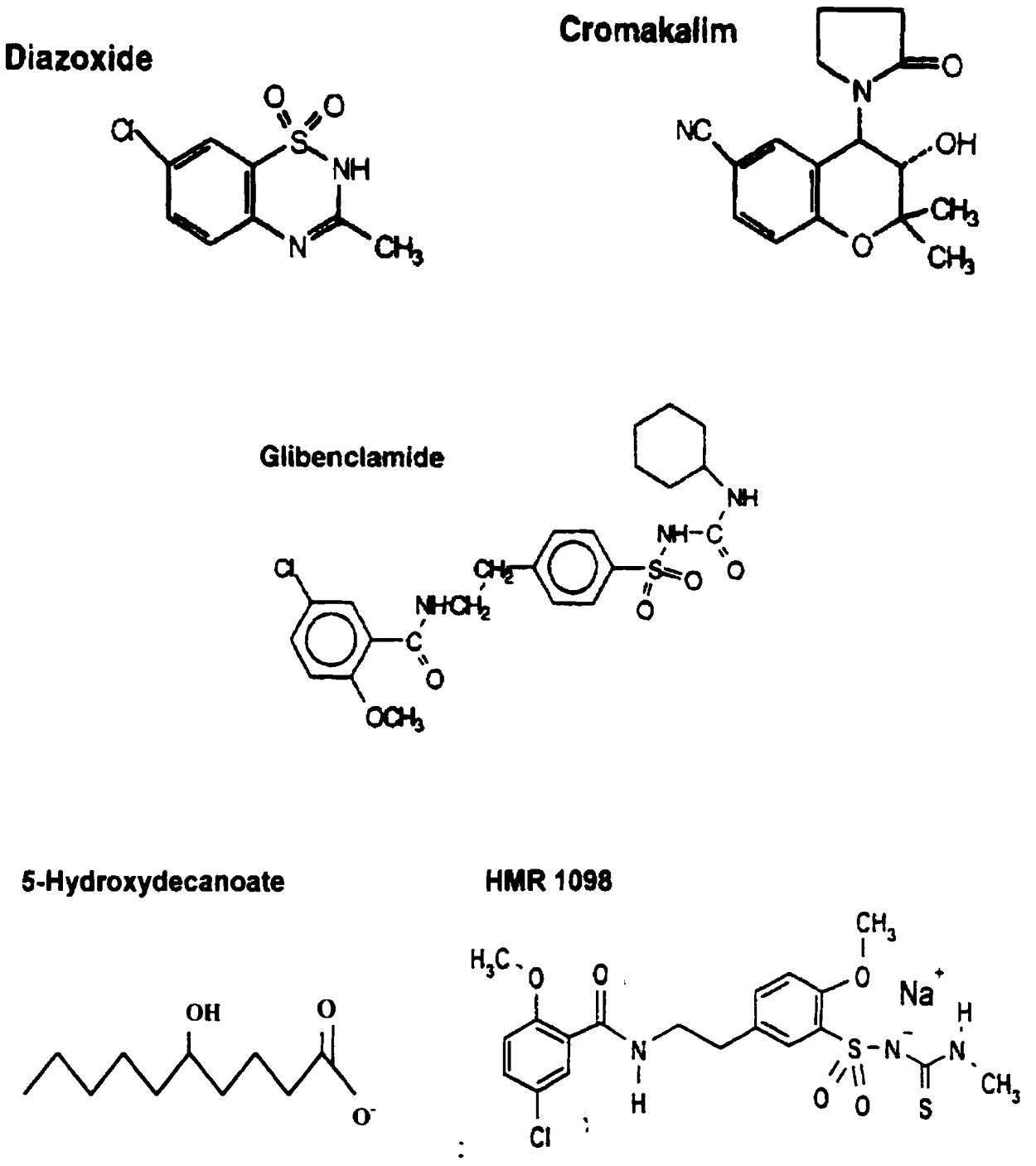


Figure 6. Structural formulae of K_{ATP} channel activators and inhibitors

Illustrated are the structural formulae of the pharmacological agents used to target the selective opening and closure of mitochondrial or sarcolemmal K_{ATP} isoforms in our experiments.

Ezymatic Isolation of Right Ventricular Myocytes:

Prior to the surgical extraction of the heart. all solutions were prepared and bubbled for 20 min with 95-5%O_2:CO_2 preceding the perfusion. Sprague-Dawley rats weighing 250-350 g were used. The rats were anesthetized with metofane followed by an intraperitonial injection of 1.0 ml heparin (1000 IU/ml). The rat was left in this state for 5 minutes. then was sacrificed by cervical dislocation. The heart was then immediately excised and immersed in ~ 50 ml of chilled isolation buffer (bicarbonate buffer). After a brief chilling. the heart was mounted on a modified non-circulated Langendorff apparatus. and immediately perfused with bicarbonate buffered solution containing 1.8 mM Ca^{2+} at 37 °C and constant flow for ~5 min. The bicarbonate buffer solution contained (in mmol/L): NaCl 123.0. KCl 5.0. NaAc 2.8. $MgCl_2$ 2.13. Na_2HPO_4 1.0. $NaHCO_3$ 20.8. and glucose 10. The heart was perfused with the bicabonate solution (calcium free) for 3 min. This was then followed by a perfusion with the bicarbonate solution containing 0.1 mM Ca^{2+} and collagenase type II (12 mg / 100mL . Worthington cat# 4176) for 10-12 min or until the outer myocardium appeared digested. Once completed. the right ventricular wall was removed. cut into small pieces and placed in the digestion solution (bicarbonate buffer containing 8 mg collagenase type 2 and 2 mg Protease type XIV. Sigma cat# P5147). Myocytes were left in this solution at 37 °C to slough off. Digestion was halted once sufficient individual intact myocytes are observed under microscopic (20X magnification) view. The myocytes were then stored in a bicarbonate solution containing taurine (0.75 % w/v Sigma cat# T0625) and BSA (3 % w/v. Sigma cat# A3350). Myocytes were used within 12 hours of extraction.

Cell Culture

To obtain expression of the different K_{ATP} channel isoforms of interest, a human embryonic kidney (HEK) cell expression system was used. HEK cell lines have traditionally served as efficient test systems for functional and biochemical studies of ion channel proteins and K_{ATP} channels in particular. It is a suitable system in that it is of mammalian origin and does not contain endogenous K_{ATP} current. TsA201 cells (an SV40 transformed variant of the HEK293 human embryonic kidney cell line) were maintained in Dulbecco's modified Eagle's medium supplemented with 10 mmol/L glucose, 2 mmol/L L-glutamine, 10% fetal calf serum and 0.1% penicillin/streptomycin at 37 °C (10% CO_2). Cells were plated at 30-40% confluency on 35 mm culture dishes 4 hours prior to transfection. The K_{ATP} channel subunit clones were generously provided by Dr. S. Seino (mouse Kir6.2 and rat SUR2A GenBank accession number AF087468, Inagaki et al., 1995; Inagaki et al., 1996) and Drs. Aguilar Bryan & J. Bryan (rat SUR1 GenBank D83598, Aguilar-Bryan et al., 1995a; Aguilar-Bryan et al., 1995b). Clones were inserted into mammalian expression vectors (pCDNA3 or pCMV6) and were transfected into tsA201 cells using the calcium phosphate precipitation technique. Transfected cells were identified using fluorescence optics in combination with co-expression of the K_{ATP} channel subunit clones with the green fluorescent protein (Clontech). Recordings were made from cells 48-72 hours after transfection. Expression of the mammalian expression vector pCDNA3 without any channel constructs inserted did not produce any measurable current under whole-cell conditions (Light et al., 2000).

Single-channel Recording

Standard patch-clamp techniques were used to record single-channel currents in the inside-out patch configuration from native myocytes. This allowed us access to native cardiac sarcK_{ATP} channels through tight control of the intracellular environment. By testing the effect of different concentrations of HMR 1098, we were able to determine the potency of this drug. The internal faces of the patches were directly exposed to test solutions via a multi-input perfusion pipette (time to change solution at the tip of the recording pipette was less than 2 s). Single channel currents were recorded at a holding potential, amplified (Axopatch 200, Axon Instruments), digitized (Neuro-corder DR-384, Neuro Data Instruments Corp.) and then stored on videotape. Data were sampled at 500 Hz and filtered at 200 Hz. The pipette solution used for all patch recordings contained the following (in mmol/L): KCl 140, HEPES 10, $MgCl_2$ 1.4, EGTA 1, glucose 10. The pH of the solution was adjusted to 7.4 with KOH. This solution was also used in the recording chamber to superfuse the cells/patches for experiments using symmetrical [K^+]. Several concentrations of HMR 1098 were tested. Voltage was stepped from resting 0 mV to +50 mV and current was recorded in the inward direction. To assure identity of the K_{ATP} channel in the patch an ATP sensitivity test was performed. The addition of 1 mM ATP resulted in closure of K_{ATP} channels and subsequent current confirming the identity of the sarcK_{ATP} channels being recorded.

Whole-Cell Recording

Standard patch-clamp techniques were used to record currents in the whole cell patch configuration from tsA201 cells transfected with Kir6.2/SUR2A, Kir6.2/SUR1,

Kir6.2(ΔC26) and Kir2.1. Current was amplified (Axopatch 200B. Axon Instruments). digitized (Digidata 1320A. Axon Instruments) and stored digitally. Data were filtered at 200 Hz and sampled at 500 Hz and analyzed using pclamp 8 and clampfit 8.0 software from Axon Instruments. The pipette solution used for all patch recordings contained the following (in mmol/L): KCl 140. HEPES 10. $MgCl_2$ 1.4. EGTA 1. glucose 10. The pH of the solution was adjusted to 7.4 with KOH. This solution was also used in the recording chamber to superfuse the cells for experiments using symmetrical [K^+]. Sensitivity to barium. a K-channel pore blocker. was verified to indicate identity of the K^+ channel. The addition of 2 mM $BaCl_2$ caused the blockade of current through the channel assuring the identity of the recorded channel. No other barium sensitive currents were observed in these cells.

Calcium Overload Measurements

Right ventricular myocytes from rat were superfused with the experimental solution. Cells were loaded for 15 min with the esterified form of the Ca^{2+}-sensitive fluorescent probe. FURA-2-AM (5 μmol/L. dissolved in dimethylsulfoxide plus 40 % v/v pluronic acid: Molecular Probes. OR. USA.) Once loaded. cells were washed and placed on a cover slip for observation at 200X magnification under an inverted microscope (Zeiss Axiovert 100). A Photon Technology International (PTI. NJ. USA) imaging system using PTI Imagemaster software was used for data acquisition and analysis. FURA-2 was excited with alternating 340 nm and 380 nm wavelengths of light and the emitted light at 520nm was digitized and stored. An estimate of intracellular calcium was obtained from the calculated 340/380 nm ratio (experimental range of non-normalized ratios was 1.07-

1.24 under normoxic (control) conditions). In experiments on recombinant sarcK_{ATP} channels. the calcium-sensitive dye FLUO-3-AM (Molecular Probes) was used in conjunction with expression of the blue-fluorescent protein (BFP) marker. The use of BFP instead of GFP was necessary to prevent any overlap of the absorption/emission spectra of the fluorescent moieties. Intracellular calcium was estimated. using the PTI Imagemaster software. from the change in intensity of the 525 nm light emission during excitation with 488 nm light. Normalized values were obtained from the equation F(test)/F(control). where F(test) = fluorescence intensity at a given time point and F(control) is the mean fluorescence measured during the stable one minute pre-CIH normoxic period.

Myocyte Viability Assay

Freshly isolated populations of single myocytes from rat right ventricle were used for this study and were stored in the experimental solution containing (in mmol/L): NaCl 136.5. KCl 5.4. $CaCl_2$ 1.8. $MgCl_2$ 0.53. glucose 5.5. HEPES-NaOH 5.5. pH 7.4 at 20-22 °C. Myocyte viability was assessed in each group after a 5 min exposure to chemically-induced hypoxia (CIH) and then 3 min reoxygenation at 37 °C. For the induction of CIH. 2 mmol/L NaCN and 5 mmol/L 2-deoxyglucose were added to the experimental solution. Myocytes were gently pelleted in a microfuge for 30 s at 2.000 rpm and solutions then changed. All normoxic controls underwent the same handling procedure to minimize experimental artifacts. A modified Tyrode solution containing (in mmol/L) KCl 2.68. $CaCl_2$ 1.8. NaH_2PO_4 0.42. $NaHCO_3$ 11.9. $MgSO_4$ 0.83. glucose 5.55. and amylbarbitone 3.0. 0.5% v/v glutaraldehyde. and 0.5 % w/v trypan blue was then applied prior to

assessment of myocyte viability. Under these conditions, dead cells stain blue, whereas viable cells do not stain. Approximately 200 myocytes per sample were counted in <10 min from each sample. >1000 myocytes were sampled in each experimental group in total. From these data the percentage of dead cells was calculated and compared between experimental groups. All results were normalized to control data.

Experimental Compounds

ATP (as MgATP, Sigma) was added as required from a 10 mmol/L stock, which was prepared immediately before use. 5-hydroxydecanoate (5-HD, Sigma/RBI, MA, USA) and HMR 1098 (sodium salt of HMR 1883, Aventis, Frankfurt, Germany) were dissolved as stock solutions in distilled water. 2-Chloro-N^6-cyclopentyladenosine (CCPA, Sigma/RBI) and 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, Sigma/RBI) were made up as stock solutions (100μM) in dimethylsulfoxide. The phorbol esters 4-β-PMA (Sigma) and 4-α-PDD (Calbiochem, San Diego, CA) were stored in ethanol at a concentration of 100 μmol/L at –20 °C. Chelerythrine chloride (Calbiochem) was stored at 5 mmol/L in distilled H_2O at –20 °C. Stock solutions were diluted to the required concentration immediately before use. Statistical significance was evaluated using Student's paired t test, as appropriate. Differences with values of probability ($p < 0.05$) were considered to be significant. All values in the text are mean ± S.E.M.

RESULTS

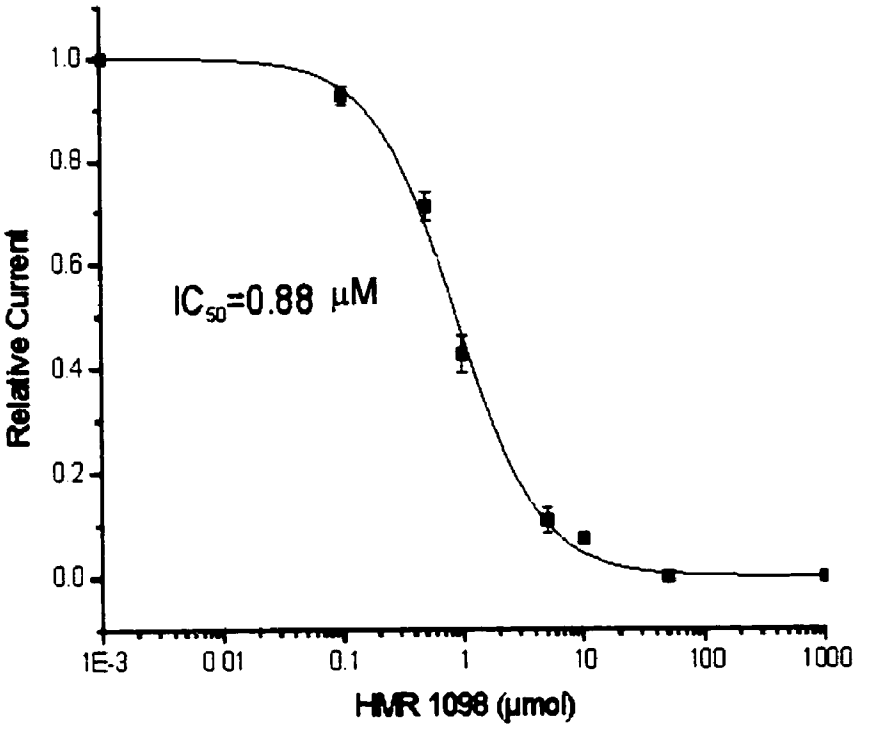
HMR 1098 is an Effective Blocker of *sarc*K_{ATP} Channels

This study of K_{ATP} channel involvement was dependent upon selective targeting of the sarcK_{ATP} and mitoK_{ATP} channels during CIH. HMR 1098 is a relatively new sulfonylurea derivative that is suggested to be selective for the sarcK_{ATP} channel; however, no studies have been conducted at the single-channel level to conclusively support this. Therefore, it was imperative that we first characterize the effects of this drug to properly understand its effects for implementation in the study. Single channel inside-out patch clamp studies were performed on native K_{ATP} channels from rat right ventricular myocytes. Currents were measured at 1 nmol/L, 0.1, 0.5, 1, 5, 10, 50 and 1000 μmol/L HMR 1098. HMR 1098 was an effective inhibitor of sarcK_{ATP} channels in a dose dependent manner (see figure 7). Fitting of the HMR 1098 dose-response relationship determined the IC_{50} and Hill coefficient to be 0.88 μmol/L and 1.27, respectively. Accordingly, an HMR 1098 concentration of 10 μmol/L was used to block >90% of the sarcK_{ATP} channel current in subsequent experiments. Analysis of the single-channel properties illustrates that HMR 1098 reduces the open probability of the sarcK_{ATP} channels in the patch, with no effect on unitary current amplitude (~3.5 pA, see figure 8, table 2). The effect of the drug was partially reversible on immediate washout and fully reversible after an extended wash out period (5 min, unpublished data).

Figure 7. The effects of HMR 1098 on native *sarc*K_{ATP} channels

A. Dose-response curve and IC_{50} of HMR 1098, obtained from single-channel inside-out patch recordings of sarcK_{ATP} channels in rat ventricular myocytes. The IC_{50} estimate was obtained by grouping data from 3-9 patches at each HMR 1098 concentration. ATP-sensitivity was assessed prior to HMR 1098 application (in presence of 10 μmol/L internal ATP). Data were fitted to the equation $I_{rel}= 1/[1+([ATP]/IC_{50})^n]$, where I_{rel} is the current relative to the maximal current observed in the absence of HMR 1098 and n is the Hill coefficient. Fitting of the HMR 1098 dose-response relationship determined the IC_{50} and Hill coefficient to be 0.88 μmol/L and 1.27 respectively. The patches were excised, held at -50 mV in symmetrical K^+ (140 mmol/L) and exposed to HMR 1098 concentrations of 1nmol/L, 0.1, 0.5, 1, 5, 10, 50, and 1000 μmol/L. **B.** Representative recording of an inside-out membrane patch containing at least 5 *sarc*K_{ATP} channels in response to application of 1 μmol/L HMR 1098. The dotted line denotes zero current level.

A


1.0
0.8
0.6
0.4
0.2
0.0
Relative Current
IC50=0.88 µM
1E-3
0.01
0.1
1
10
100
1000
HMR 1098 (µmol)

B

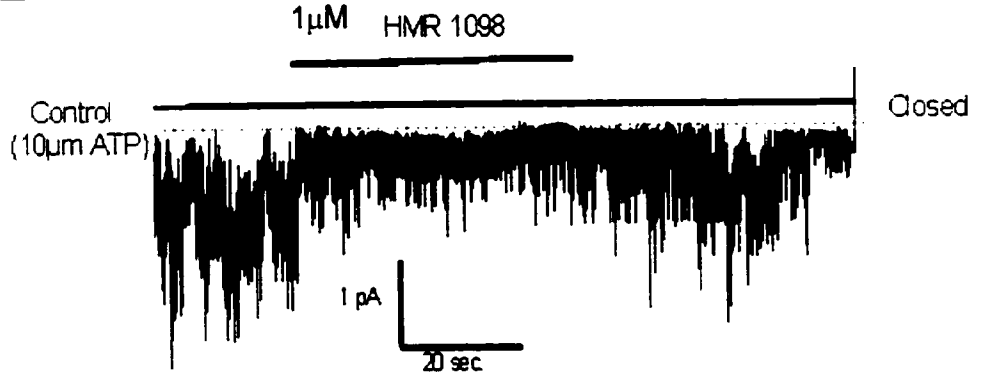
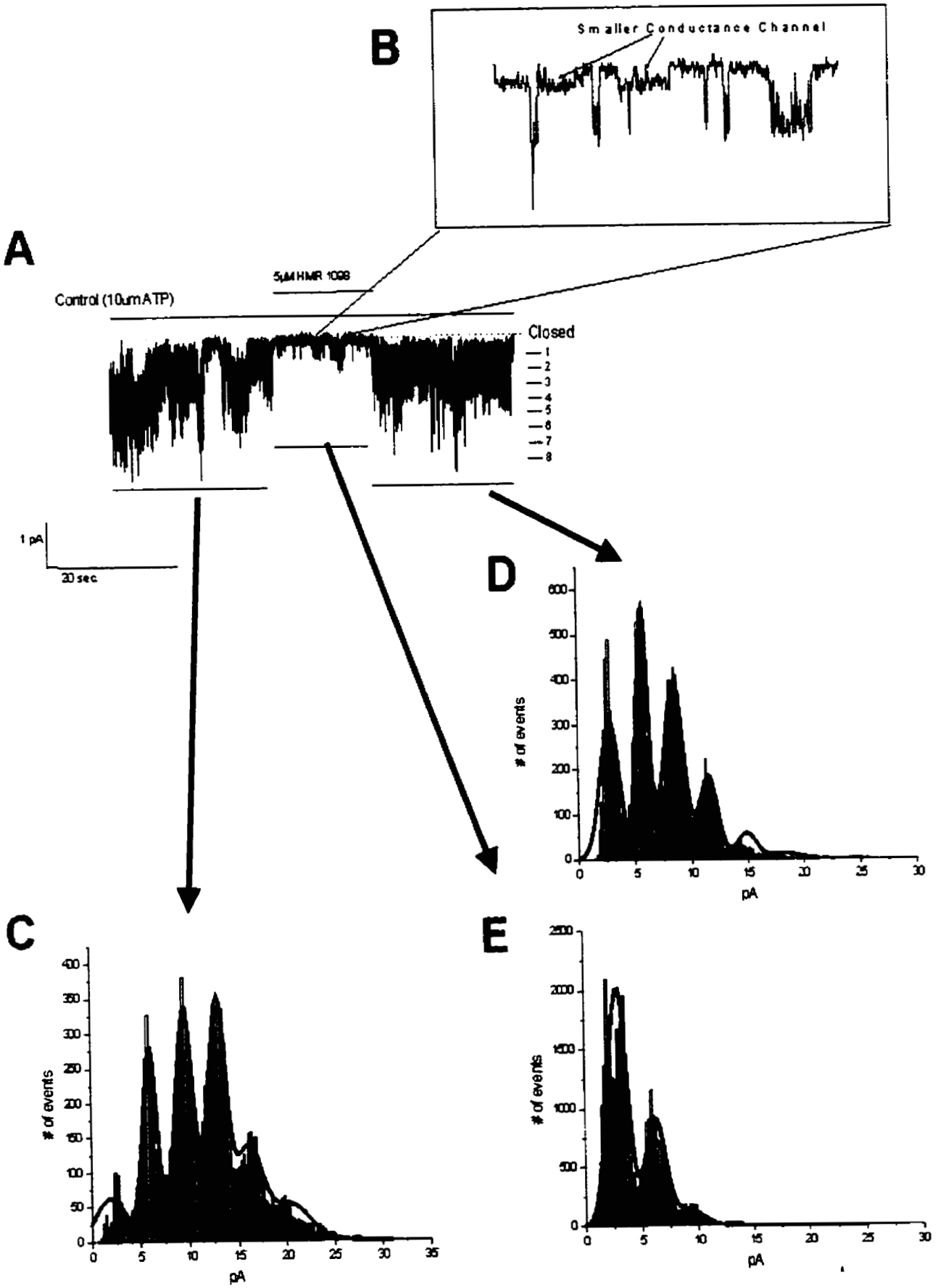

1µM HMR 1098
Control
(10µm ATP)
Closed
1 pA
20 sec

Figure 8. Single channel analysis of channels in response to HMR 1098

A. Representative recording of an inside-out membrane patch containing at least 7 sarcK_{ATP} channels from right ventricular myocytes in response to application of 5 μmol/L HMR 1098. Holding potential was maintained at –50 mV. dashed line denotes zero current level. **B.** An expanded section of the above trace illustrating the presence of a smaller conductance channel likely to be a small inward rectifier. **C.** Current-amplitude histogram from the control section of the above-mentioned trace. Approximately 50% of the channels are in the open configuration. **D.** Current-amplitude histogram while 5 μmol/L HMR 1098 was applied to the patch. Shift in the open probability caused closure of most channels. Note the 2 base peaks as a result of the closed state of the K_{ATP} channels and the open and closed states of the smaller conductance channel as illustrated in panel B. **E.** Current-amplitude histogram of the patch after wash out. Channels respond by opening: single channel unitary conductance is maintained in comparison to control.


B
Smaller Conductance Channel
A
5μM HMR 1098
Control (10um ATP)
Closed
1
2
3
4
5
6
7
8
1 pA
20 sec
D
# of events
pA
C
# of events
pA
E
# of events
pA

	Control (C)	5 μM HMR 1098 (D)	Wash (E)
Peak 1 (pA)	2.58 ± 0.084	2.72 ± 0.114	2.37 ± 0.105
Peak 2 (pA)	5.98 ± 0.096	6.32 ± 0.05	5.92 ± 0.096
Difference b/w 1-2	3.4	3.6	3.55
Peak 3 (pA)	9.41 ± 0.113	9.8 ± 0.085	9.01 ± 0.116
Difference b/w 2-3	3.43	3.48	3.09
Peak 4 (pA)	12.96 ± 0.128	-	12.14 ± 0.104
Difference b/w 3-4	3.55		3.13
Peak 5 (pA)	17.16 ± 0.087	-	15.13 ± 0.102
Difference b/w 4-5	4.2		2.99
Peak 6 (pA)	21.2 ± 0.092	-	-
Difference b/w 5-6	4.04		
nP_o	5.393	1.025	3.206
Relative Current	1	0.19	0.59

Table 2. Summary of single channel analysis in response to HMR 1098

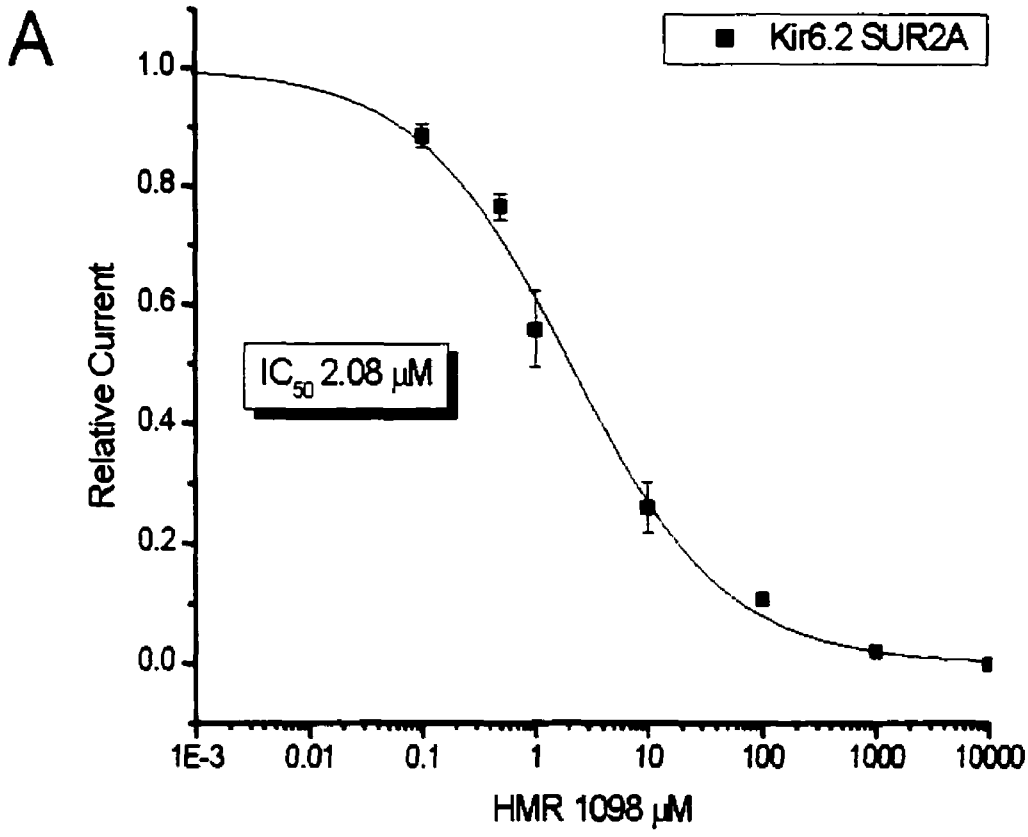
Summary of data describing current peaks from the least squares fit of all-points histograms during control, 5 μmol/L HMR, and wash out. Normalized open probability (nP_o) was calculated by the equation, nPo = (mean current/unitary current amplitude). Relative Current was calculated by, $I_{rel} = nP_o test / nP_o control$.

The effect of HMR 1098 on whole-cell current studies yielded similar results. HMR 1098 inhibited current in a dose dependent manner when administered to cells expressing the cardiac K_{ATP} channel isoform (see figure 9). Whole-cell patch clamp studies were performed on tsA201 cells transiently expressing the Kir6.2/SUR2A channel. Currents were measured at 0.1. 0.5. 1. 10. 100. 1000 and 10000 μmol/L HMR 1098. Fitting of the HMR 1098 dose-response relationship determined the IC_{50} and Hill coefficient to be 2.08 μmol/L and 0.68 respectively. This study gave an IC_{50} value for the $K_{IR}6.2$/SUR2A channel at approximately two-fold that of the native channel.

The effect of HMR 1098 was also studied on the β-cell K_{ATP} isoform. Kir6.2/SUR 1. The responsiveness of this channel to the drug was much than that of Kir6.2/SUR2A. However. current was still inhibited in a dose dependent manner. Whole-cell voltage clamp studies were performed on tsA201 cells transiently expressing the Kir6.2/SUR1 channel. Currents were measured with 0. 0.01. 0.05. 0.5. 1. 10. and 100 mmol/L HMR 1098 present. Fitting of the HMR 1098 dose-response relationship determined the IC_{50} and Hill coefficient to be 0.86 mmol/L and 0.83. respectively. The Kir6.2/SUR1 channelappears to be almost ~1000X less sensitive to HMR 1098 than the native sarcK_{ATP} channel and ~400X less sensitive than to the Kir6.2/SUR 2A channel (see figure 10).

Figure 9. The effects of HMR 1098 on Kir6.2/SUR2A channels.

A. Dose-response curve and IC_{50} of HMR 1098, obtained from whole-cell recordings of Kir6.2/SUR2A, the cardiac K_{ATP} isoform. The IC_{50} estimate was obtained by grouping data from 4-6 cells at each HMR 1098 concentration. Barium sensitivity was assessed prior to HMR 1098 application (in presence of 2 mmol/L $BaCl_2$). Data were fitted to the equation $I_{rel}= 1/[1+ ([ATP]/IC_{50})^n]$, where I_{rel} is the current relative to the maximal current observed in the absence of HMR 1098 and n is the Hill coefficient. Fitting of the HMR 1098 dose-response relationship determined the IC_{50} and Hill coefficient to be 2.08 μmol/L and 0.68, respectively. The whole cell currents were measured while voltage was stepped to -50 mV for 200 ms in symmetrical K^+ (140 mmol/L) and exposed to HMR 1098 concentrations of 0.1, 0.5, 1, 10, 100, 1000, and 10000 μmol/L. **B.** Representative whole cell current trace of Kir6.2 SUR2A under influence of several concentrations of HMR 1098. Current is inhibited in a dose dependent manner.


A
Kir6.2 SUR2A
Relative Current
1.0
0.8
0.6
0.4
0.2
0.0
IC50 2.08 µM
1E-3
0.01
0.1
1
10
100
1000
10000
HMR 1098 µM

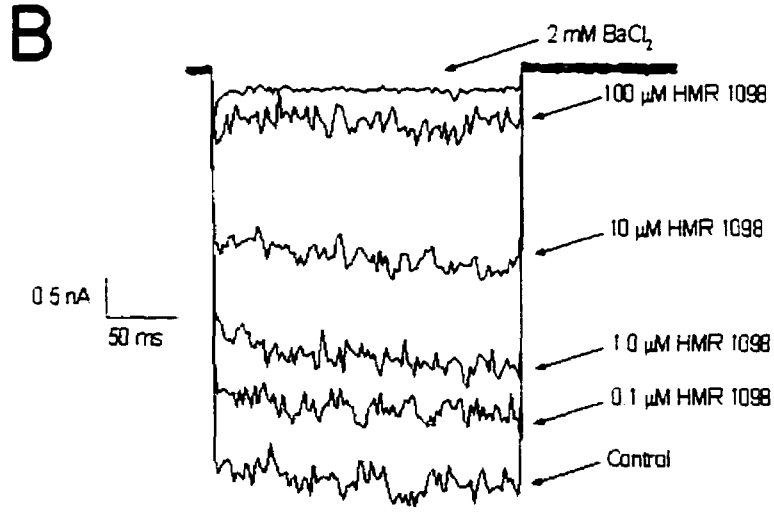
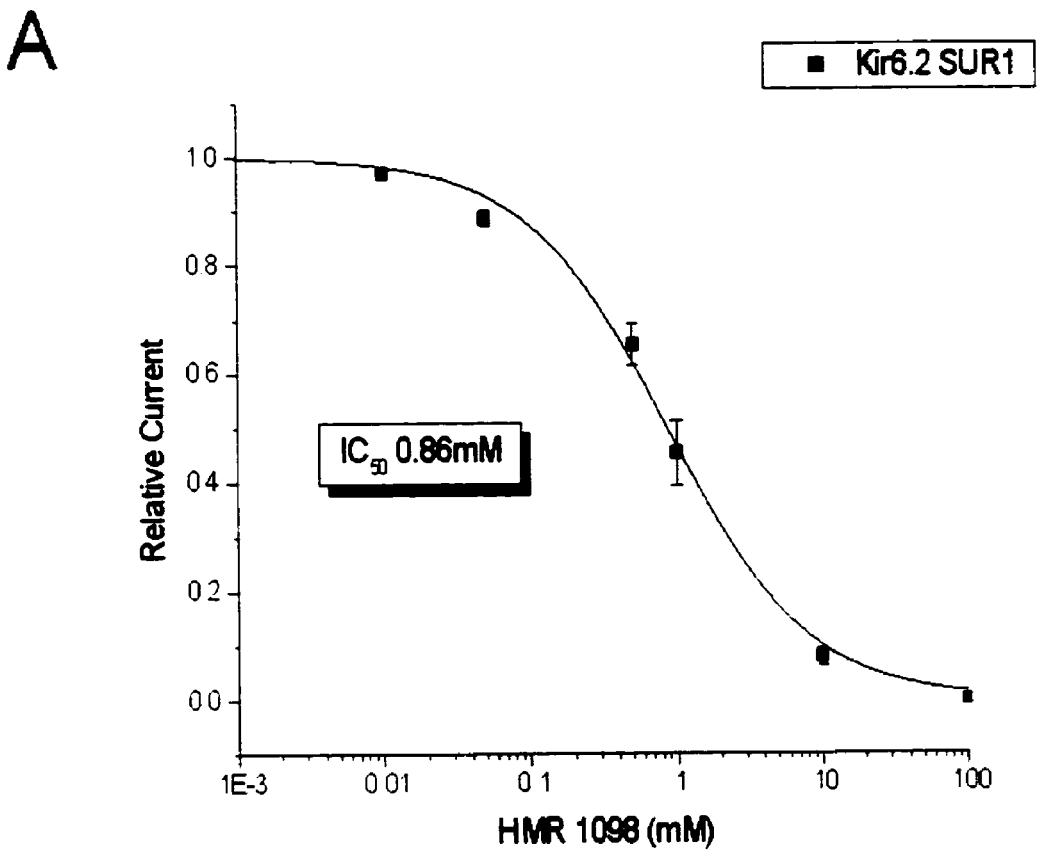
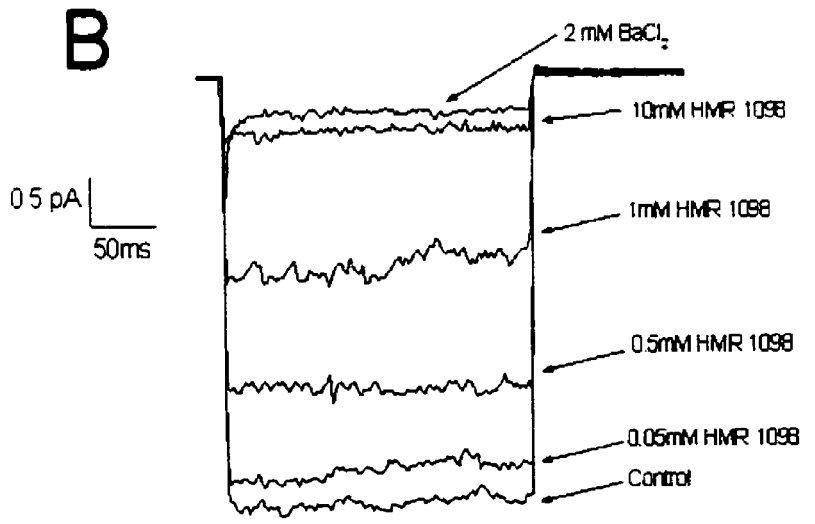

B
2 mM BaCl2
100 µM HMR 1098
10 µM HMR 1098
1.0 µM HMR 1098
0.1 µM HMR 1098
Control
0.5 nA
50 ms

Figure 10. The effects of HMR 1098 on Kir6.2/SUR1 channels.

A. Dose-response curve and IC_{50} of HMR 1098, obtained from whole-cell recordings of *Kir6.2/SUR1*, the β-cell K_{ATP} isoform. The IC_{50} estimate was obtained by grouping data from 5-8 cells at each HMR 1098 concentration. Barium sensitivity was assessed prior to HMR 1098 application: 2 mmol/L $BaCl_2$ blocked >95% of the total current. Data were fitted to the equation $I_{rel} = 1/[1 + ([ATP]/IC_{50})^n]$, where I_{rel} is the current relative to the maximal current observed in the absence of HMR 1098 and n is the Hill coefficient. Fitting of the HMR 1098 dose-response relationship determined the IC_{50} and Hill coefficient to be 0.86 mmol/L and 0.83, respectively. The whole cell currents were measured while voltages were stepped from 0 to -50 mV for 200 ms in symmetrical K^+ (140 mmol/L) and exposed to HMR 1098 concentrations of 0.01, 0.05, 0.5, 1, 10, and 100 mmol/L. **B.** Representative whole cell current trace of Kir 6.2/SUR1 under influence of several concentrations of HMR 1098. Current is inhibited in a dose dependent manner.


A
Kir6.2 SUR1
Relative Current
IC50 0.86mM
HMR 1098 (mM)
1E-3
0.01
0.1
1
10
100
0.0
0.2
0.4
0.6
0.8
1.0

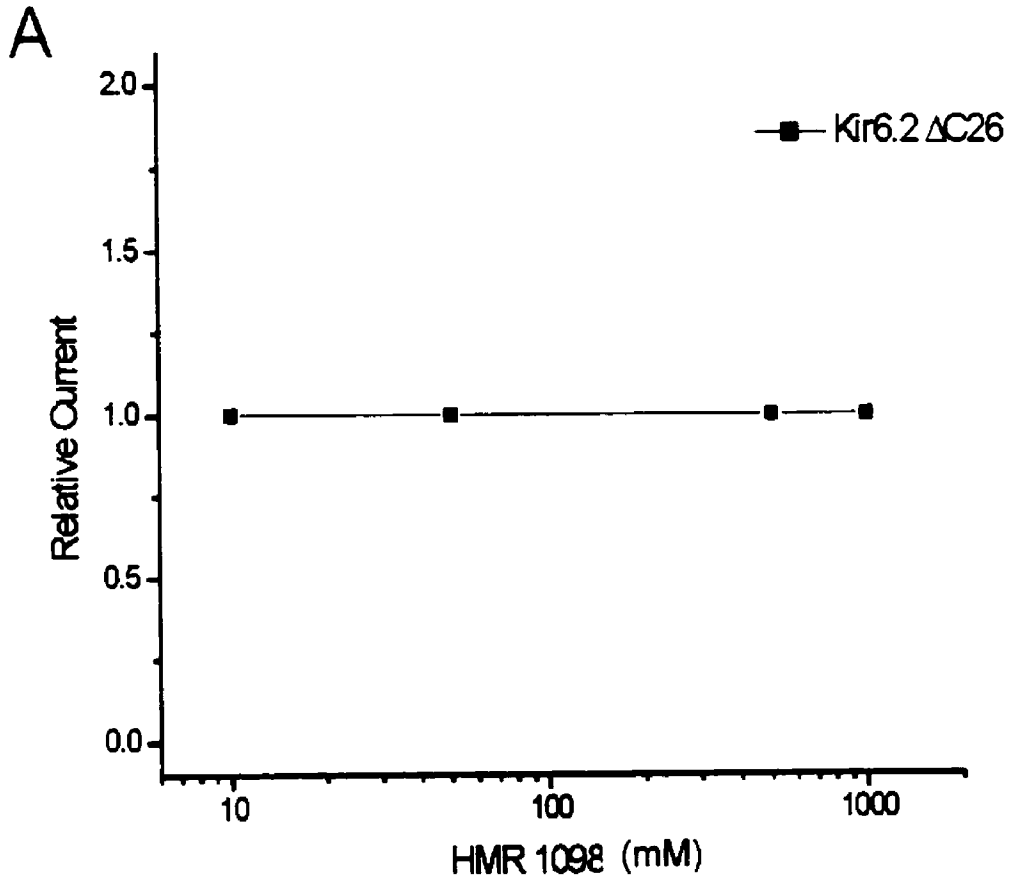

B
2 mM BaCl2
10mM HMR 1098
1mM HMR 1098
0.5mM HMR 1098
0.05mM HMR 1098
Control
0.5 pA
50ms

To investigate the site of action for HMR 1098, whole-cell patch recordings were performed on Kir6.2 ΔC26. The missing 26 residues atthe carboxy-terminus of this channel enable its expression and functioning without the SUR subunit being present (Aguilar-Bryan and Bryan, 1999). Whole cell-currents were measured with application of 0, 10, 50, 500 and 1000 μmol/L HMR 1098. Current was not affected by these concentrations of HMR 1098 tested and thus Kir6.2 ΔC26 was deemed insensitive to HMR 1098 (see figure 11). This is consistent with the idea that the drug acts via the SUR subunit.

In addition, Kir2.1 was studied under the influence of HMR 1098. This channel is a strong inward rectifier cloned from a macrophage cell line (Kubo et al., 1998). It is a small conductance, voltage-gated K^+ channel that is constructed of only two transmembrane domains which compose the pore of the channel. This channel does not need, nor does it associate with, the SUR to form a functional channel. Whole cell-currents were measured and the effects of 0, 10, 50, 500,1000 and 10000 μmol/L HMR 1098 were tested. Current was not altered by the addition of HMR 1098 up to 10 mmol/L. This K^+ channel isoform is not responsive to HMR 1098 (see figure 12).

Figure 11. The effects of HMR 1098 on Kir6.2 ΔC26 channels.

A. Dose-response curve for HMR 1098, obtained from whole-cell recordings of Kir6.2 ΔC26. K_{ATP} channel mutant construct able to form a functional channel without associating with the SUR subunit. Data were averaged from 6-9 cells at each HMR 1098 concentration. Barium sensitivity was assessed prior to HMR 1098 application (in presence of 2 mmol/L external $BaCl_2$). No significant block was observed for concentrations up to 1 mmol/L. The whole cell currents were measured while voltage was stepped to -50 mV in symmetrical K^+(140 mmol/L) for 200 ms and exposed to HMR 1098 concentrations of 10, 50, 500, and 1000 μmol/L. **B.** Representative whole cell current trace of Kir6.2 ΔC26 under influence of several concentrations of HMR 1098. Current was not altered by HMR 1098 application.


A
Kir6.2 ΔC26
Relative Current
2.0
1.5
1.0
0.5
0.0
10
100
1000
HMR 1098 (mM)

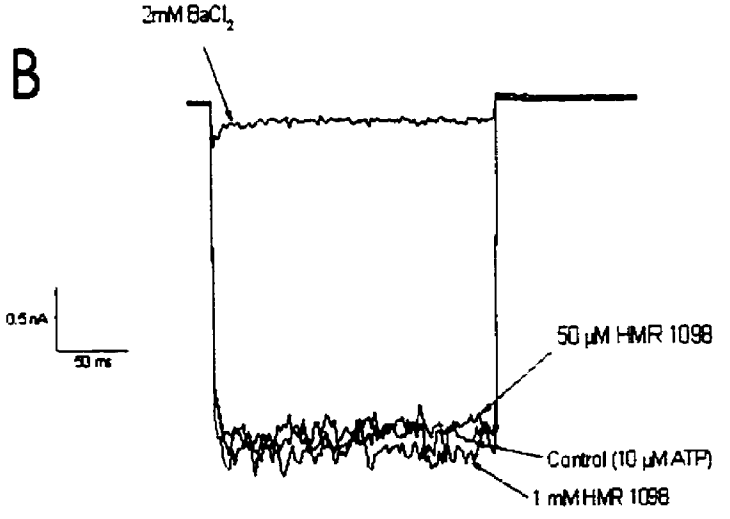
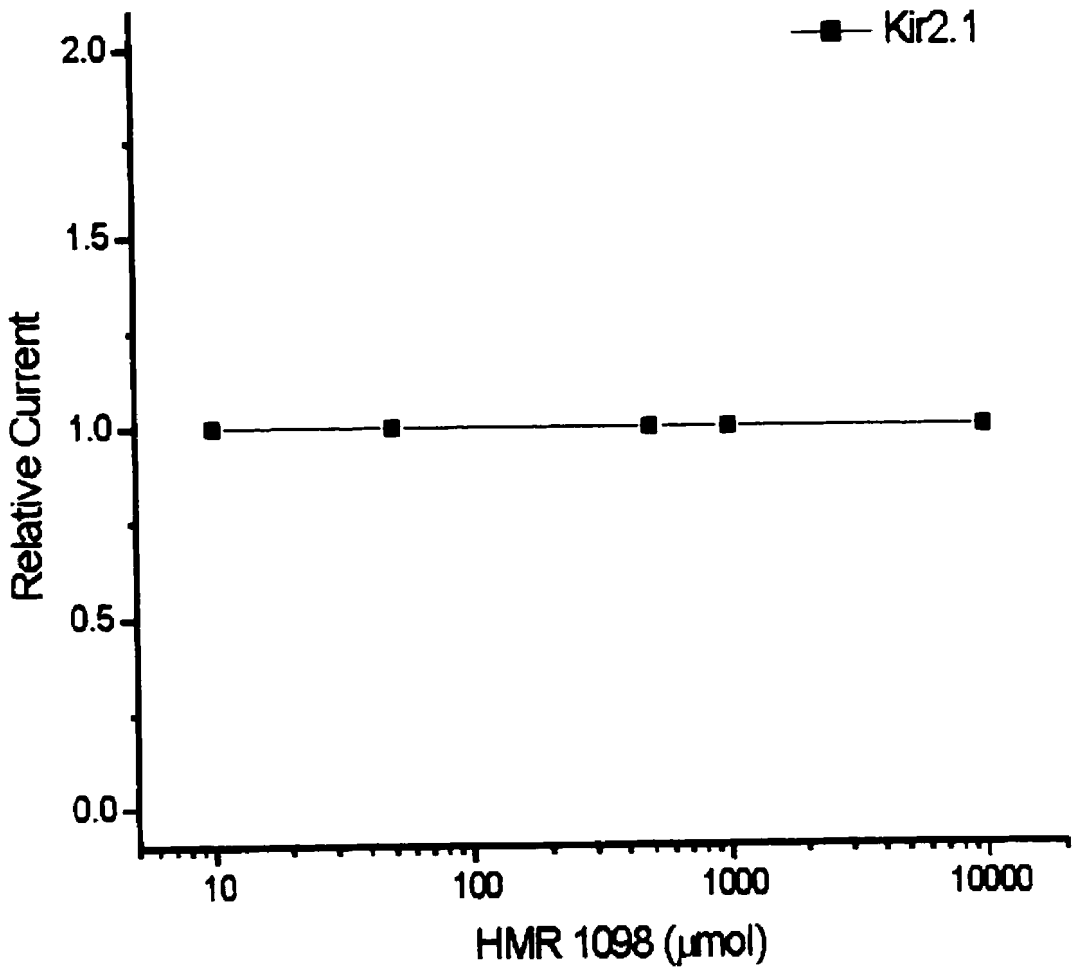

B
2mM BaCl2
0.5 nA
50 ms
50 μM HMR 1098
Control (10 μM ATP)
1 mM HMR 1098

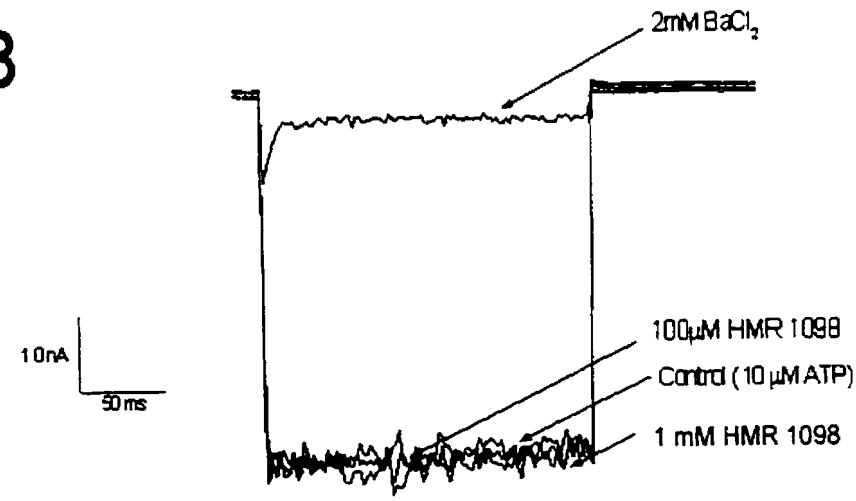
Figure 12. The effects of HMR 1098 on Kir2.1 channels.

A. Dose-response curve and IC_{50} of HMR 1098, obtained from whole-cell recordings of *Kir2.1*, an inward rectifying K^+ channel that is capable of forming a functional channel without the SUR subunit. Barium sensitivity was assessed prior to HMR 1098 application (in presence of 2 mmol/L external $BaCl_2$, near complete block was achieved). No significant block was observed for HMR 1098 concentrations up to 10 mmol/L. The whole cell currents were measured while voltage was stepped from 0 to -50 mV for 200 ms in symmetrical K^+ (140 mmol/L) and exposed to HMR 1098 concentrations of 10, 50, 500 µmol/L, 1 and 10 mmol/L. **B.** Representative whole cell current trace of Kir2.1 in the absence and presence of the indicated concentrations of HMR 1098. Current was not altered by HMR 1098 administration.

A


Kir2.1
2.0
1.5
1.0
0.5
0.0
Relative Current
10
100
1000
10000
HMR 1098 (µmol)

B


2mM BaCl2
100µM HMR 1098
Control (10 µM ATP)
1 mM HMR 1098
10nA
50ms

PKC, K_{ATP} Channels and Ca^{2+}- Overload

It has been reported that sustained elevation and overload in $[Ca^{2+}]_i$ are indicative of cell morbidity and damage. Rises in intracellular calcium levels are thought to be responsible for initiating cell death and apoptosis (for review see Bolli and Marban. 1999). Thus. as a real-time assessment of cellular damage we implemented a model measuring changes in internal calcium levels. Cells were loaded with the FURA-2 AM dye and assayed for cytoplasmic calcium during chemically induced hypoxia and reoxygenation. This model was reported by Jovanovic et al. as an effective method to investigate the role of the K_{ATP} channel during hypoxia and reoxygenation (Jovanovic et al.. 1999). It was therefore used as the model in this study to assess the protective effects of the native and recombinant K_{ATP} channel opening in moderating hypoxic injury.

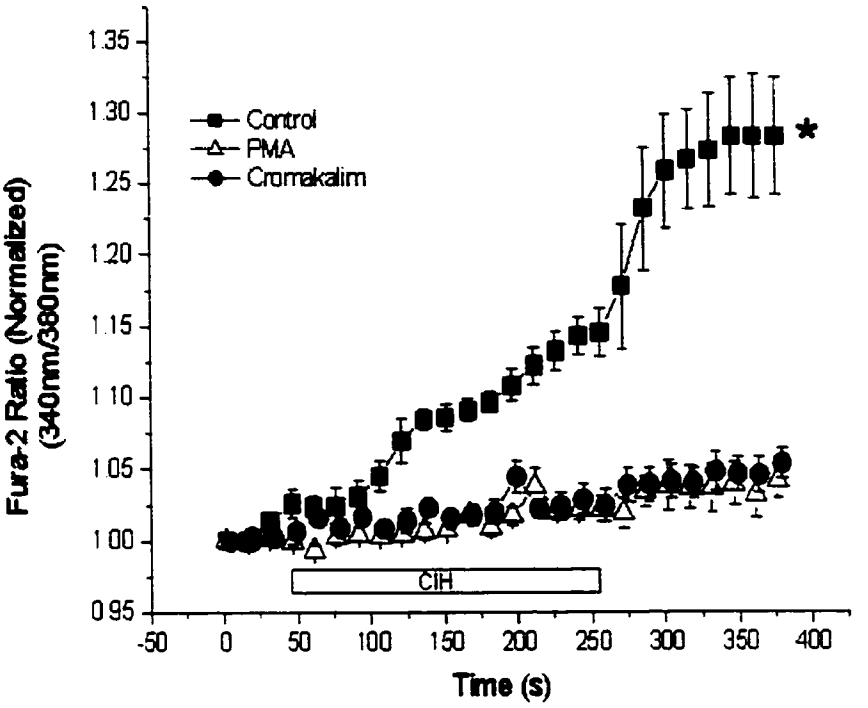
The purpose of our study was to investigate the role of the sarc and the mito K_{ATP} channel during hypoxia and reoxygenation. Moreover. effort was focused on when during hypoxia and reoxygenation the channels were activated and how the activation affected injury. The first step in this process was dissecting the nature of injury itself. This was accomplished by analyzing injury reflected by Ca^{2+} loading during hypoxia and reoxygenation. Under normoxic conditions no change in $[Ca^{2+}]_i$ was observed. However. superfusion with the chemically-induced hypoxia (CIH) solution caused a steadyrise in $[Ca^{2+}]_i$ (9.1 ± 0.75% as denoted by a rise in the FURA-2 fluorescence ratio after 3 min CIH) that was accelerated upon reoxygenation with normoxic solution for 2 min (37.6 ± 4.6%. $p<0.001$. see Fig. 13A). The time course of injury reflected by the rise in $[Ca^{2+}]_i$

was therefore seen to be biphasic, characterized by a moderate rise during hypoxia and more severely during reoxygenation. In order to test how activation of K_{ATP} channels affects the observed loading during CIH we pharmacologically activated the channels throughout hypoxia and reoxygenation. Interestingly, addition of levcromakalim (20 μmol/L), a non-specific K_{ATP} channel opener, prevented Ca^{2+} loading both during CIH and reoxygenation (5.3 ±1.0%, see figure 13A). Thse data inspired testing of the ability of up-stream activators of the K_{ATP} channel as having the ability to produce similar results. Indeed, pretreatment with the PKC-activating 4-β-phorbol 12-myristate, 13-acetate (PMA, 100 nmol/L) completely abolished the increase in $[Ca]_i$ induced by the CIH (2.1 ± 1.0%) and reoxygenation (3.0 ± 1.2%, see Fig 13A.). In comparison to the response observed while cells were treated with levcromakalim, the effect of PMA was virtually identical. As controls, neither the inactive phorbol ester PDD alone, nor PMA in the presence of the PKC inhibitor chelerythrine (100 nmol/L) protected against the calcium loading observed during CIH/reoxygenation (see figure 13 B).

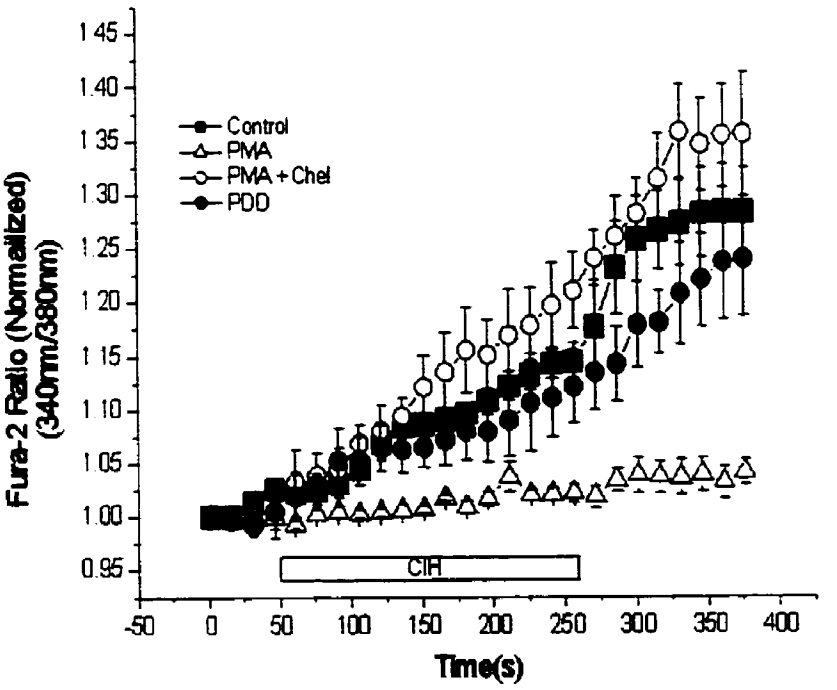
Figure 13. The effects of opening K_{ATP} channels on calcium overload during CIH/reoxygenation in ventricular myocytes.

A. Calcium measurements using Fura-2 AM ratiometric dye before, during and after 3 min exposure to chemically induced hypoxia (CIH). Administration of 2 mmol/L NaCN and 5 mmol/L 2-deoxyglucose produced CIH. PMA (100 nmol/L), a PKC activator, or levcromakalim (20 μmol/L), a non-selective K_{ATP} channel opener, were applied throughout the experimental protocol. Activation of sarcolemmal K_{ATP} channels by these agents impeded calcium overload. **B.** Under the same protocol, chelerythrine (100 nmol/L), a selective PKC blocker, in the presence PMA, or the inactive analogue of PMA, PDD (100 nmol/L), were used as controls (* = <0.001). Each group is a mean of +- 9 separate experiments (sample size of 20-50 individual myocytes per group in total).

A


Control
PMA
Cromakalim
*
CIH
Fura-2 Ratio (Normalized)
(340nm/380nm)
Time (s)
1.35
1.30
1.25
1.20
1.15
1.10
1.05
1.00
0.95
-50
0
50
100
150
200
250
300
350
400

B


Control
PMA
PMA + Chel
PDD
CIH
Fura-2 Ratio (Normalized)
(340nm/380nm)
Time(s)
1.45
1.40
1.35
1.30
1.25
1.20
1.15
1.10
1.05
1.00
0.95
-50
0
50
100
150
200
250
300
350
400

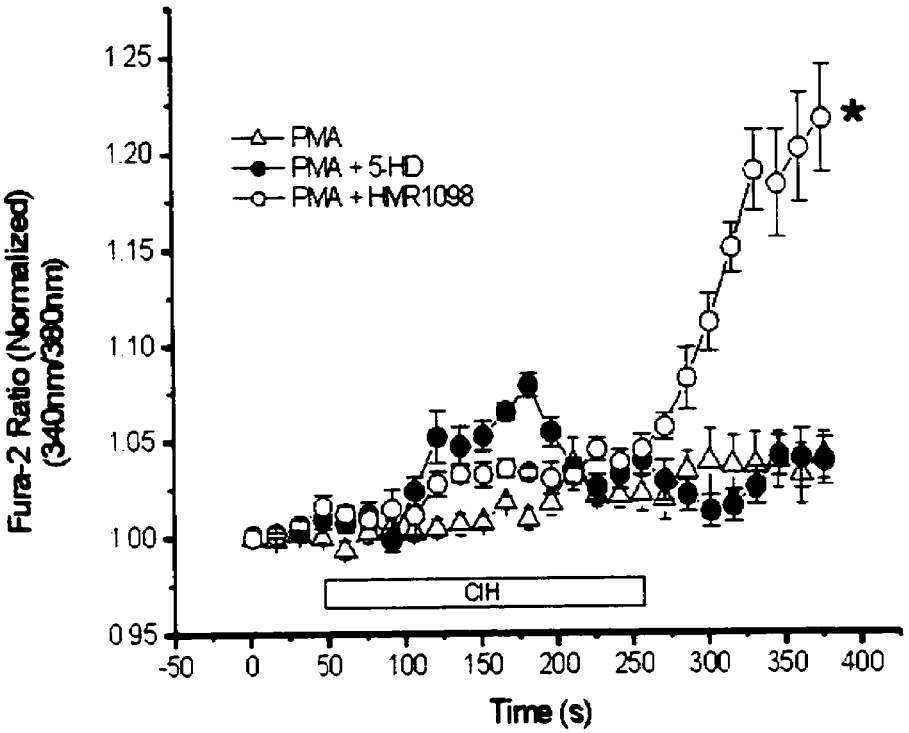
To further decipher if the mito or sarc K_{ATP} channels were responsible for the protection observed, channels were activated by the PKC pathway and selected pharmacologically. As described, HMR 1098 effectively inhibits the sarcK_{ATP} channels, while 5-HD blocks the mitoK_{ATP} channels. Using these agents, the biphasic calcium overload observed under control CIH was dissociated by selective inhibition of the sarc and mito K_{ATP} channels.

This result was achieved by continuous treatment of myocytes with the specific mitoK_{ATP} channel blocker, 5-hydroxydecanoate (5-HD, 100 μmol/L), which relieved some of the protection afforded by PMA during the CIH period (see figure 14 A) but did not hinder the PMA-induced protection from Ca^{2+} loading during reoxygenation (2.5 ± 0.9%). Conversely, exposure to HMR 1098 (10 μmol/L), had little effect on the protection afforded by PMA immediately following CIH onset but resulted in the reappearance of calcium overload during reoxygenation (21.3 ± 2.7%, $p<0.001$, see Fig 14A). Therefore, by the administration of mito or sarc K_{ATP} channel blockers in addition to PMA, the injury and Ca^{2+}-overload associated with CIH onset or reoxygenation was abated, respectively. The addition of both 5-HD and HMR 1098 in the presence of PMA completely restored the observed calcium overload during both the CIH (12.1 ± 1.6%) and reoxygenation period (38 ± 4.6%, $p<0.001$) to levels even higher than observed during CIH/reoxygenation alone (see Fig 14 B). These results support the involvement of both the sarc and mito K_{ATP} channels in impeding the Ca^{2+} overload and injury observed during CIH and reoxygenation.

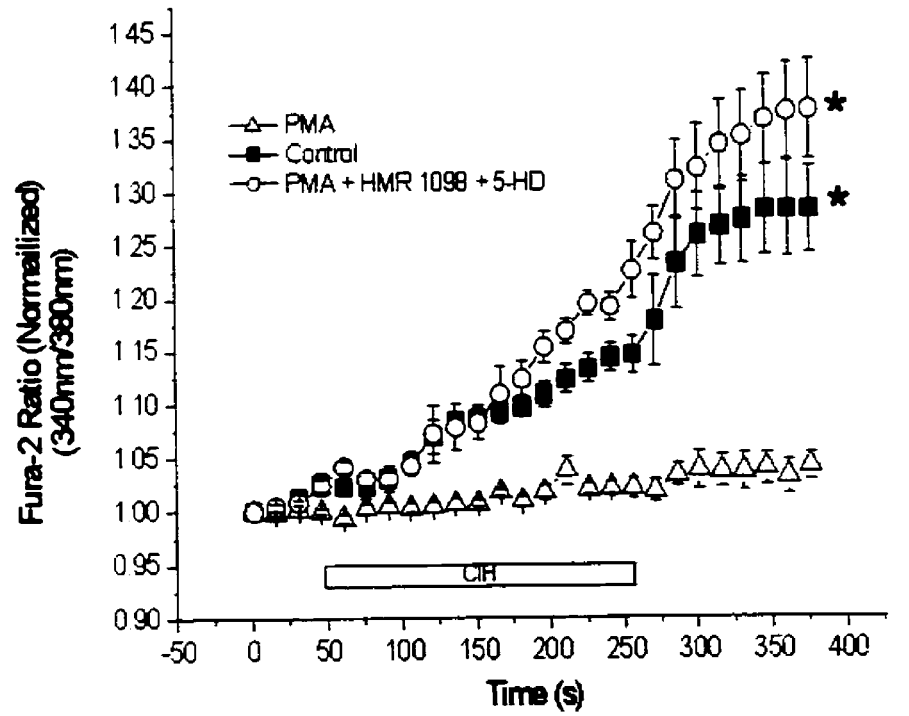
Figure 14. Contribution of the sarcK_{ATP} and mitoK_{ATP} channels on calcium overload during hypoxia and reoxygenation

A. The effects of 5-hydroxydecanoate (5-HD, 100 μmol/L) a selective mitoK_{ATP} channel blocker, or HMR 1098 (10 μmol/L), a sarcK_{ATP} channel blocker, on the calcium-loading response during CIH and reoxygenation (* = $p<0.001$ from PMA alone). **B.** Calcium-overload experiments in rat ventricular myocytes during CIH and reoxygenation in control and treatment with both HMR 1098 (10 μmol/L) and 5-HD (100 μmol/L. * = $p<0.001$). Each group is a mean of 5-9 separate experiments (sample size of 20-50 individual myocytes per group in total).

A


PMA
PMA + 5-HD
PMA + HMR1098
CIH
Fura-2 Ratio (Normalized)
(340nm/380nm)
Time (s)
1.25
1.20
1.15
1.10
1.05
1.00
0.95
-50
0
50
100
150
200
250
300
350
400
*

B


PMA
Control
PMA + HMR 1098 + 5-HD
CIH
Fura-2 Ratio (Normalized)
(340nm/380nm)
Time (s)
1.45
1.40
1.35
1.30
1.25
1.20
1.15
1.10
1.05
1.00
0.95
0.90
-50
0
50
100
150
200
250
300
350
400
*
*

K_{ATP} Channels and Myocyte Viability

Although calcium overload has been reported to be indicative of cellular morbidity, it does not provide a direct assay of cell death. It was therefore of importance to compare the results of Ca^{2+} flux and cell death for the experiments described. Association between the two strengthens the data and puts Ca^{2+} overload into context, validating its usefulness as an assay. These data also make clearer the respective roles of the two K_{ATP} channel isoforms in ischemic damage protection.

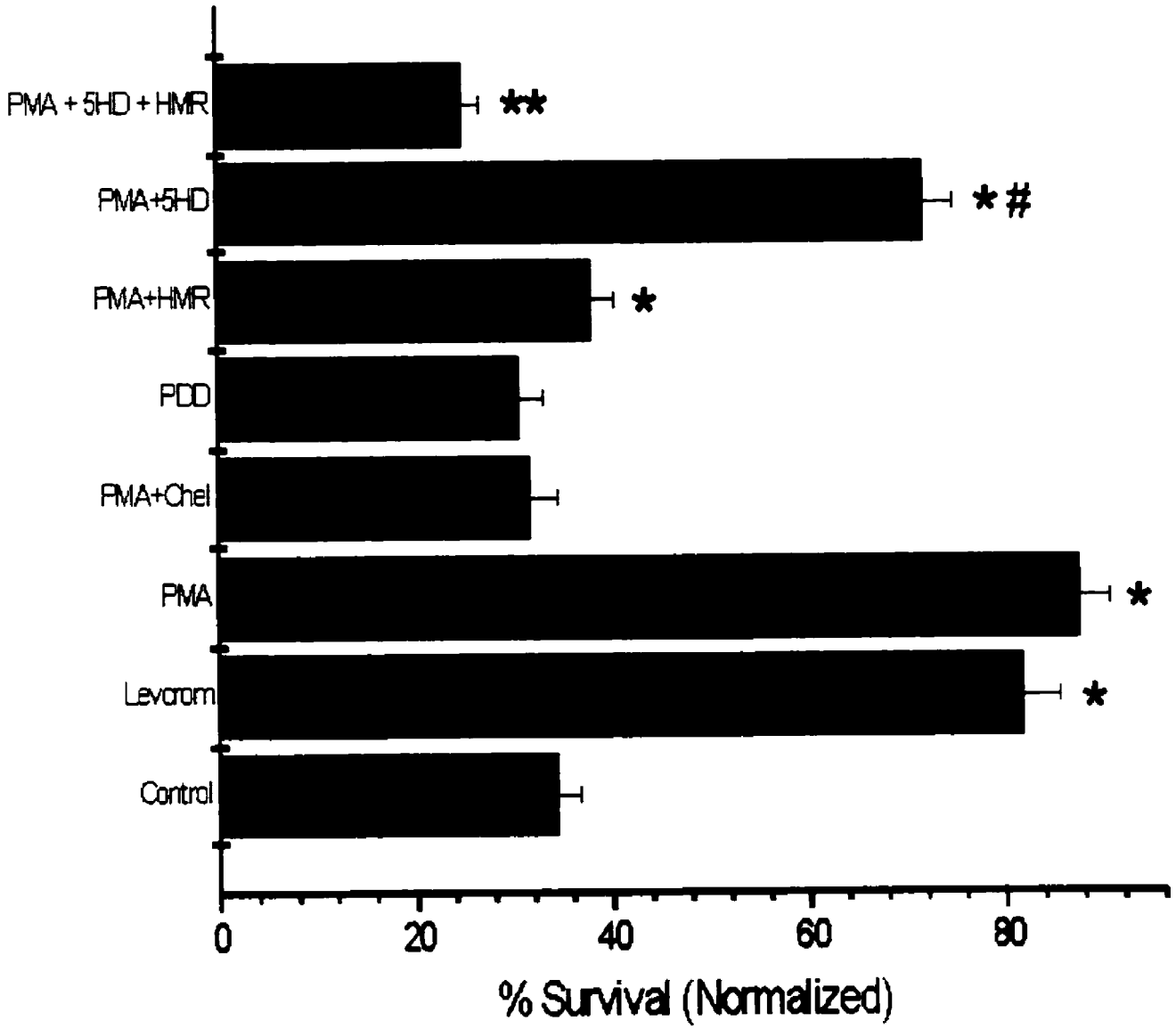
In order to evaluate the roles of both K_{ATP} channel populations on myocyte survival, a series of experiments was performed in which cell viability was assessed using the trypan blue exclusion assay. After a 5 min CIH and 3 min reoxygenation period at 37 ^{0}C, myocyte survival was reduced to 34.3 ± 2.2% ($p<0.001$, normalized to normoxic control, see figure 15). In contrast, treatment with PMA (100 nmol/L), or the K_{ATP} channel opener levcromakalim (20 μmol/L), increased cell survival to values of 87.6 ± 3.1% and 81.4 ± 4.1%, respectively (compared to control). These results correlate well with the Ca^{2+} loading experiments that suggested protective effects of the K_{ATP} channels following their activation by PMA and levcromakalim.

The above-mentioned experiments illustrate the roles of the K_{ATP} channel in the presence of PKC; however, a question remained as to the direct involvement of the sarc and mito K_{ATP} channels in preventing injury. The Ca^{2+} measurement experiments suggested that

both play an important role, but the extent remained unknown. Therefore, to better understand the extent of injury that the sarc and mito K_{ATP} channels moderated, each was selectively inhibited. It was observed that the protection afforded by PMA was significantly reduced by the addition of HMR 1098 (10 μmol/L) with only 38.3 ± 1.4% of cells surviving ($p<0.001$, compared to PMA alone). The addition of 5-hydroxydecanoate (5-HD) alone reduced the protection afforded by PMA (71.2 ± 2.3% survival, $p<0.05$ compared to PMA alone), though not to the same extent as HMR 1098. Simultaneous administration of both HMR 1098 (10 μmol/L) and 5-HD (100 μmol/L) entirely abolished the protective effects of PMA (25.4 ± 1.1%, $p<0.001$ from PMA alone). This value is also significantly lower than control ($p<0.01$), suggesting that basal activity of K_{ATP} channels may contribute to cell survival during CIH/reoxygenation in the absence of PKC activation (see figure 15).

Figure 15. Trypan blue exclusion viability assay on rat ventricular myocytes under influence of K_{ATP} channel activators/inhibitors

Trypan blue viability assay of rat ventricular myocytes subjected to various pharmacological agents during 5 min of CIH and 3 min reoxygenation. Control = CIH alone: Levcrom = levcromakalim, non-selective K_{ATP} channel opener (20 μmol/L): PMA = 4-β-PMA (100 nmol/L), a PKC activator and known K_{ATP} channel opener: Chel = chelerythrine (100 nmol/L), selective PKC antagonist: PDD = 4-α-PDD (100 nmol/L), inactivate phorbol ester: HMR = HMR 1098 (10 μmol/L), sarcK_{ATP} channel blocker: 5-HD = 5-hydroxydecanoate (100 μmol/L), selective mitoK_{ATP} channel blocker. (* $p< 0.001$ from CIH. ** $p< 0.01$ from CIH. # $p<0.01$ from PMA). More than 1000 cells were sampled per group.


PMA + 5HD + HMR
PMA+5HD
PMA+HMR
PDD
PMA+Chel
PMA
Levorom
Control
**
*#
*
*
*
0
20
40
60
80
% Survival (Normalized)

Adenosine A_1 Agonists, Calcium-Overload, and Myocyte Viability

In the next series of experiments we set out to determine if both K_{ATP} channel populations are involved in the protection afforded by adenosine as a mediator upstream from PKC. In studying this mechanism of action we had hoped to identify the major pieces to construct a plausible pathway for the activation of K_{ATP} channels during ischemia and reoxygenation. There is strong evidence to suggest that adenosine, acting via the A_1 receptor, is a major physiological trigger of IPC in the mammalian heart (Cohen et al., 2000a; Miura et al., 2000). The signaling pathways downstream of A_1 receptor stimulation likely involve the activation and translocation of several PKC isoforms as well as activation of K_{ATP} channels (Downey and Cohen, 1997). However, the respective contributions of the sarc and mito K_{ATP} channel populations as mediators of adenosine-induced protection remain to be determined.

Pretreatment of ventricular myocytes with the A_1 receptor selective agonist CCPA (Lohse et al., 1988) (1 μmol/L) before and during the CIH protocol induced protection from calcium-overload to a similar extent to that observed with PKC-activation after CIH (2.9 ± 0.3%) or during reoxygenation (4.1 ± 0.3%, Figure 16 A). In looking more specifically at the contribution of the sarcK_{ATP} channel, the CCPA-mediated protection from Ca^{2+} loading was significantly reduced by the addition of HMR 1098 (10 μmol/L, 22.4 ± 3.3% increase after reoxygenation, $p<0.001$ compared to CCPA alone). When similar experiments were conducted with 5-HD to inhibit the mitoK_{ATP} channel alone

(100 μmol/L). the CCPA-mediated protection was maintained during reoxygenation (3.2 ± 0.4%. n = 15. Fig. 16 A).

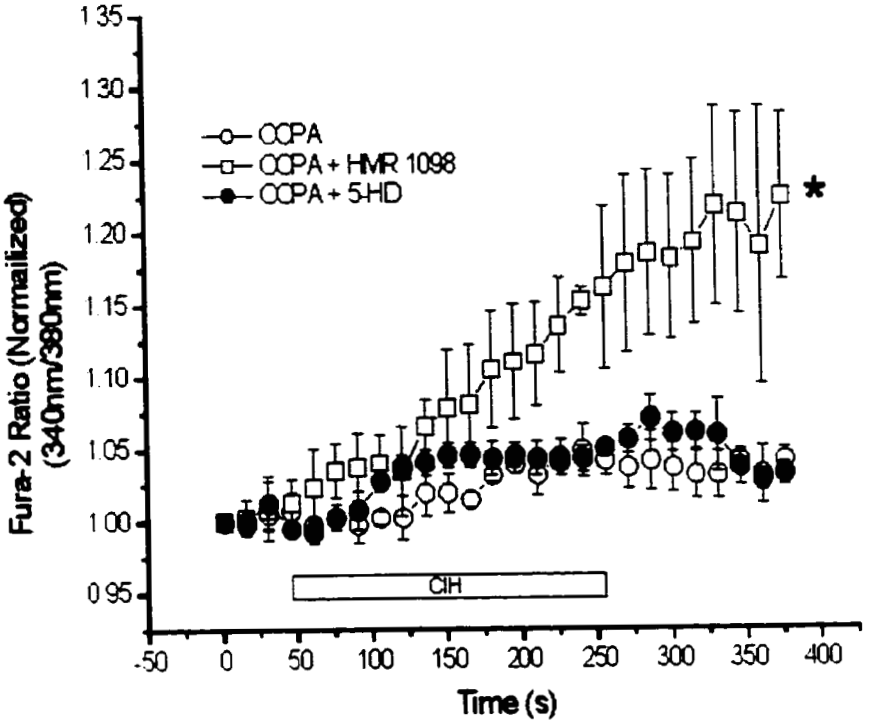
We then antagonized the A_1 receptor agonist to test if the protection was entirely due to the action of this receptor coupled pathway. This was accomplished by the addition of 8-cyclopentyl-1.3-dipropylxanthine (DPCPX. 10 μmol/L). a selective A_1 receptor antagonist (Coates et al.. 1994). which prevented the CCPA-induced Ca^{2+} overload protection and thus resulted in calcium-loading during CIH (9.3 ± 2%) and reoxygenation (22.6 ± 2%. $p< 0.001$ from CCPA).

In order to determine if A_1 receptor activation was coupled to PKC. a study looking more closely at the inhibition of PKC during A_1 receptor coupled protection was conducted. The addition of a selective PKC blocker. chelerythrine (100 nmol/L). to CCPA prevented its protection from calcium-overload (8.2 ± 1.3%. during CIH and 15.4 ± 0.5%. during reoxygenation $p<0.01$ from CCPA). These data suggest that the effect of A_1 receptor stimulation was mediated at least in part by the activation of PKC and subsequent opening of K_{ATP} channels (see Figure 16 B).

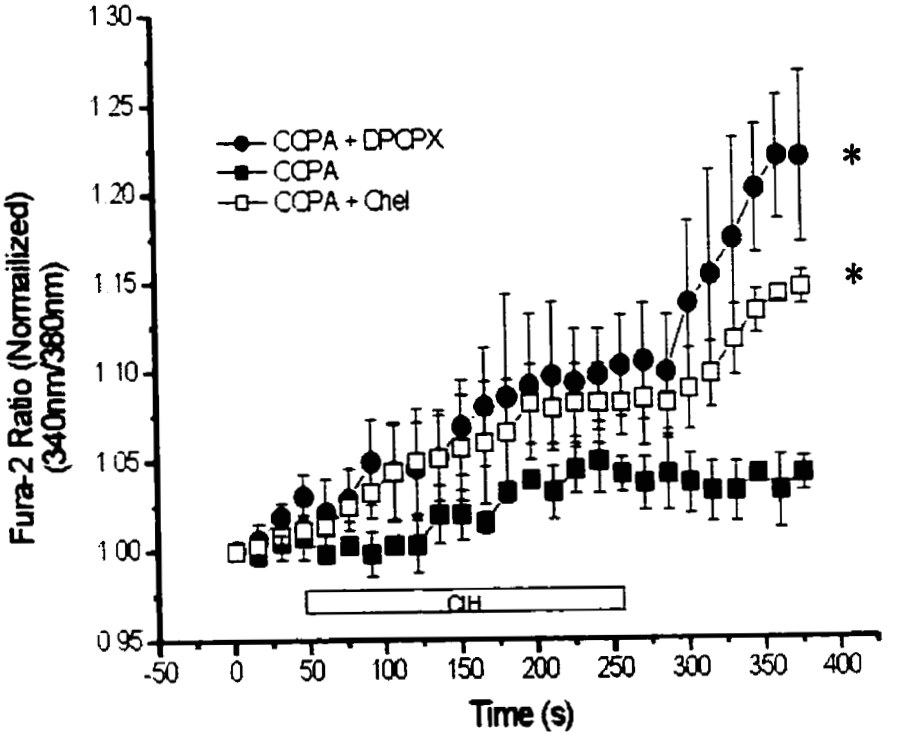
Figure 16. Effects of A_1-receptor agonist in modulating Ca^{2+} overload during hypoxia and reoxygenation

A. The effects of A_1-receptor agonist CCPA (2-chloro-N^6-cyclopentyladenosine. 1 μmol/L) on calcium overload during CIH and reoxygenation. To study the role of *mito* and *sarc* K_{ATP} channels during A_1-receptor mediated protection. 5-HD (100 μmol/L) and HMR 1098 (10 μmol/L) respectively were present throughout the experimental protocol. **B.** The CCPA effect was antagonized by the co-treatment of CCPA with the selective A_1-receptor antagonist 8-cyclopentyl-1.3-dipropylxanthine (DPCPX. 10 μmol/L) or chelerythrine (100 nmol/L). a selective PKC blocker. (* = $p < 0.001$ from CCPA)

A


CCPA
CCPA + HMR 1098
CCPA + 5-HD
Fura-2 Ratio (Normalized)
(340nm/380nm)
CIH
Time (s)
*

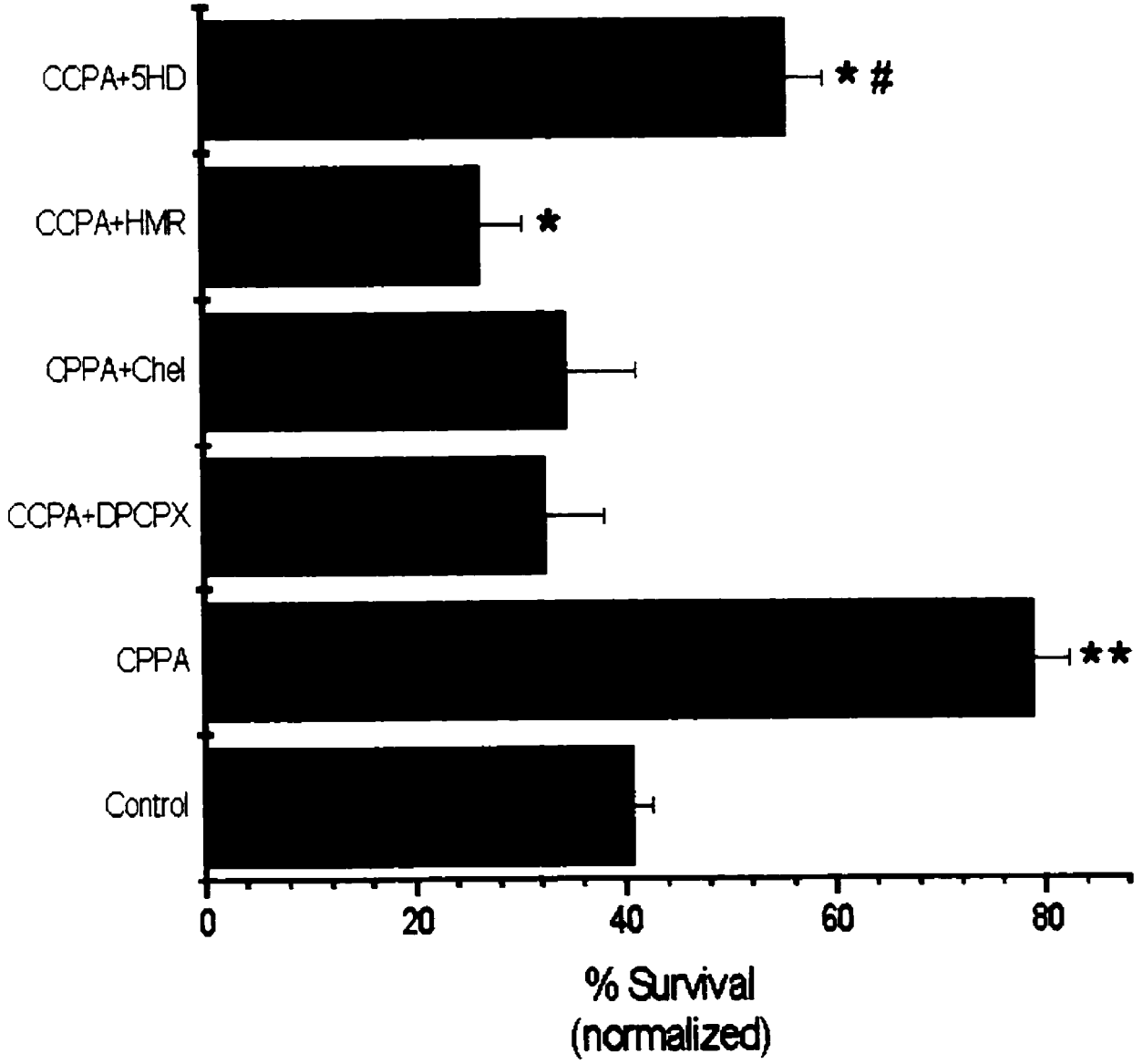
B


CCPA + DPCPX
CCPA
CCPA + Chel
Fura-2 Ratio (Normalized)
(340nm/380nm)
CIH
Time (s)
*
*

The protective effects of activating the A_1 receptor coupled pathway were also confirmed using the trypan blue viability assay to correlate calcium-overload and cell death. After 5 min of CIH and 3 min reoxygenation, survival was reduced to 40.4 ± 1.4%. The pretreatment of myocytes with CCPA (1 μmol/L) during this protocol significantly increased cell survival to 79.6 ± 2.2% ($p< 0.001$). The CCPA-mediated protection was abolished in the presence of either DPCPX (10 μmol/L) or chelerythrine (100 nmol/L), resulting in cell survival of 32.6 ± 4.0% and 34.3 ± 5.1%, respectively. In addition, HMR 1098 prevented the protection afforded by CCPA resulting in only 26.3 ± 3.1% of cells surviving ($p<0.01$ from control). 5-HD (100 μmol/L) also significantly reduced the number of cells surviving in the presence of CCPA; however not as effectively as HMR 1098 (55.6 ± 2.2%, see figure 17 B. $p<0.001$ from CCPA, $p<0.01$ from CIH).

Figure 17. Trypan blue exclusion viability assay on rat ventricular myocytes under influence of K_{ATP} channel activators/inhibitors

The effects of CCPA (1 μmol/L) on myocyte cell survival after 5 min CIH and 3 min reoxygenation. Either A_1 receptor antagonist 8-cyclopentyl-1.3-dipropylxanthine (DPCPX. 10 μmol/L) or chelerythrine (10 nmol/L) was added to antagonize protection and to test the involvement of A_1 receptor coupled and PKC mediated protection. respectively. To test involvement of sarc and mito K_{ATP} channels. either HMR 1098 (10 μmol/L) or 5-HD (100 μmol/L) was separately administered during the entire protocol. >1000 myocytes in total were sampled for each group (* = $p<0.01$ from CIH. ** = $p<0.001$ from CIH. # = $p<0.01$ from CIH +CCPA).


CCPA+5HD
CCPA+HMR
CPPA+Chel
CCPA+DPCPX
CPPA
Control
* #
*
**
0
20
40
60
80
% Survival
(normalized)

A Single Residue Substitution in the *sarc*K_{ATP} Channel Prevents the PKC-Mediated Reduction in Calcium Overload Following Reoxygenation.

The recent cloning of the sarcK_{ATP} channel subunits has provided useful tools with which to probe the involvement of K_{ATP} channels in mediating the protective effects by phosphorylation at the molecular level. Previous studies have demonstrated that PKC activates the sarcK_{ATP} channel at physiological levels of ATP by altering the Hill coefficient for ATP binding (Light et al., 1996a). In addition, it has been shown that a single threonine residue at position 180 in the pore-forming Kir6.2 subunit is the target for the functional PKC-mediated phosphorylation of the sarcK_{ATP} channel complex (Light et al., 2000). Substitution of this T180 residue with an alanine (T180A) prevents PMA-induced activation of the sarcK_{ATP} channel. Therefore, in the next series of experiments we set out to determine whether the PKC-mediated reduction in calcium-overload observed during reoxygenation can be prevented by this single residue substitution in a model of hypoxia/reoxygenation utilizing recombinant K_{ATP} channels.

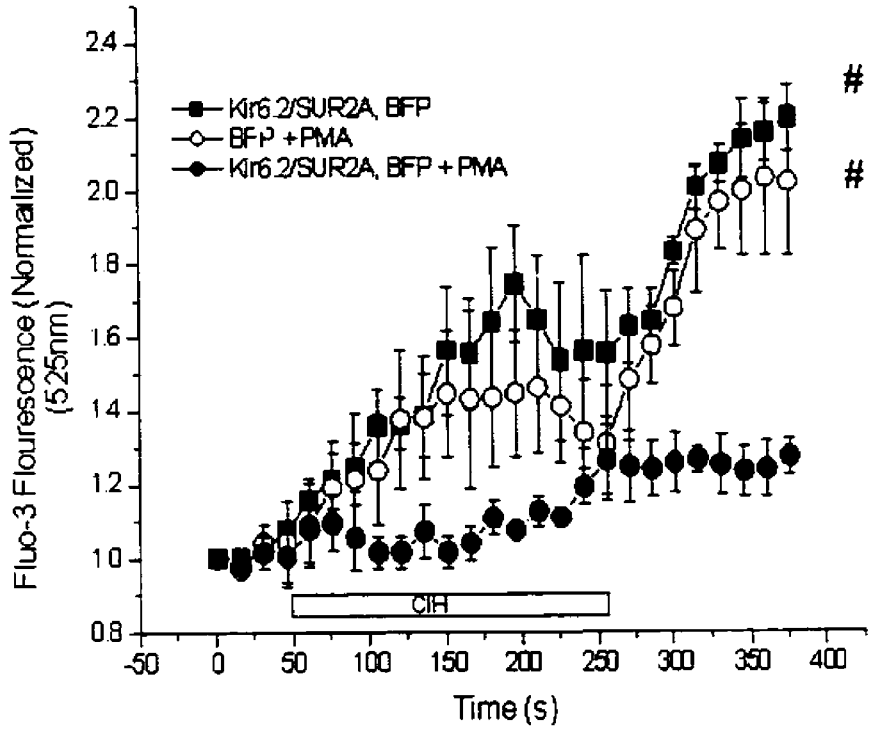
In tsA201 cells transiently expressing the blue fluorescent marker protein alone, a biphasic calcium-overload was observed during CIH/reoxygenation, similar to that observed with native ventricular myocytes. Treatment with PMA (100 nmol/L) alone had no effect on calcium-overload in cells expressing only BFP (119.0 ± 8.9% increase in Fluo-3 fluorescence, see Fig 18 A.). It has previously been shown that pharmacological activation of expressed recombinant K_{ATP} channels is required to elicit protection from

CIH/reoxygenation-induced calcium overload (Jovanovic et al., 1999). In accordance with these findings we show that transient expression of the recombinant sarcK_{ATP} channel subunits in the absence of activation only slightly reduces the level of calcium-overload observed (102.1 ± 20.3%) compared to BFP alone. However, in cells expressing the sarcK_{ATP} channel subunits when treated with PMA (100 nmol/L) significantly reduced the level of calcium overload during the reoxygenation period (27.4 ± 5.0%, $p<0.001$, compared to the result in the absence of PMA, see Fig 18 A). Moreover, expression of the SUR2A and mutant Kir6.2.T180A subunits significantly attenuated the PMA-mediated reduction in calcium overload (65.3 ± 5.0%, $p<0.01$, see Fig 18 B). These data demonstrate the functional importance of this residue in mediating the effects of PKC and protection from Ca^{2+} overload.

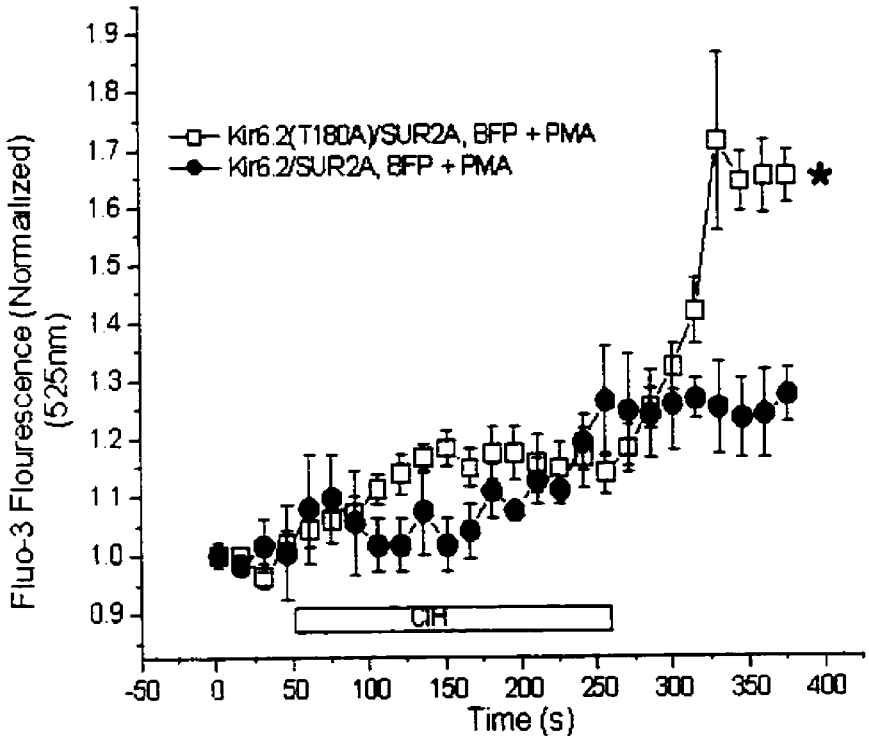
Figure 18. Intracellular calcium changes during CIH/reoxygenation in transiently transfected tsA201 cells using Fluo-3 fluorescence

A. Calcium dependent fluorescence in cells transfected with the cardiac K_{ATP} isoform. Kir6.2/SUR2A. Blue Fluorescent Protein (BFP) marker was used in order to identify transfected cells. Cells transfected with Kir6.2/SUR2A were subjected to CIH followed by reoxygenation while treated with and without PMA (100 nmol/L). BFP was also transfected without the channel and tested under the same conditions as a control. **B.** tsA201 cells were transfected with Kir6.2(T180A), the pore-forming subunit in which the PKC phosphorylation site for functional channel activation has been removed (Light et al., 2000). The cells were subjected to CIH and reoxygenation in an identical manner as ventricular myocytes while continuously treated with PMA (100 nmol/L). Protection was observed only when the wild-type Kir6.2/SUR2A, cardiac K_{ATP} isoform, was activated by PMA during CIH and reoxygenation. (# = $p < 0.001$ from Kir6.2/SUR2A, BFP; * = $p < 0.001$ from Kir6.2/SUR2A, BFP + PMA)

A


Kir6.2/SUR2A, BFP
BFP + PMA
Kir6.2/SUR2A, BFP + PMA
#
#
CIH
Fluo-3 Flourescence (Normalized)
(525nm)
Time (s)

B


Kir6.2(T180A)/SUR2A, BFP + PMA
Kir6.2/SUR2A, BFP + PMA
*
CIH
Fluo-3 Flourescence (Normalized)
(525nm)
Time (s)

DISCUSSION:

This study probed the role of the sarc and mito K_{ATP} channels in hypoxia and reoxygenation with the hope to better understand the mechanisms that result in ischemic preconditioning. Using a pharmacological approach the targeting of this channel is possible and selective activation of each channel population helped decipher the respective participation in managing ischemic injury.

HMR 1098: An Analysis of Target Channel Specificity

The novel drug, HMR 1098, was reported to be selective for the sarcK_{ATP} channel (Liu et al., 2001). In order to test this assertion and characterize its effects, patch clamp studies were conducted at both the single channel and whole cell level in native and recombinant cells, respectively. Analysis of the single channel patch clamp studies on native cardiac myocytes suggests that this compound does in fact inhibit native sarcK_{ATP} channels in a dose dependent manner. During the 2000 American Heart Association meeting in New Orleans Liu et al. reported on the pharmacology of the mitoK_{ATP} channel, concluding that it most closely resembles the SUR1 subunit (Liu et al., 2001). Therefore, in light of this finding, we tested HMR 1098 on two K_{ATP} isoforms, the cardiac Kir6.2/SUR2A and β-cell Kir6.2/SUR1. The whole-cell current studies on these recombinant cells were consistent with the previous single-channel patch clamp studies conducted on native myocytes. The β-cell channel was resistant to inhibition by HMR 1098 and was almost

1000 times less sensitive than the native myocyte channel and 400 times less sensitive than the recombinant Kir6.2/SUR2A. Assuming that the pharmacology of the mitoK$_{ATP}$ channel is similar to the β-cell isoform as suggested by Liu et al., it is reasonable to conclude that HMR 1098 is selective for the sarcK$_{ATP}$ channel. Therefore to minimize non-specific effects on the mitochondrial channel we applied a concentration of 10 μmol/L HMR 1098 in our experiments which should accordingly inhibit >90% of sarcK$_{ATP}$ channels and <10% of mitoK$_{ATP}$ channels.

Comparison of the dose-response curve constructed from the single-channel patch clamp experiments on native sarcK$_{ATP}$ channels and that from the whole-cell recordings on Kir6.2/SUR 2A shows a ~2 fold discrepancy between the IC$_{50}$ values. One would expect the IC$_{50}$ values for the native sarcK$_{ATP}$ and the Kir6.2/SUR2A to be virtually identical. The source of this discrepancy might be related to the method of current recording. The single channel recordings from native myocytes were done on patches that were subjected to several concentrations of the drug directly on the internal side of the patch by way of the perfusion pipette. The whole cell recordings required that the drug permeate through the membrane to act on the channel. HMR 1098, being very water soluble, may have impaired solubility across the cellular membrane unlike the more lipophilic sulfonylureas such as glibenclamide. This characteristic may be the reason for the disparity in IC$_{50}$ values. Preliminary patch-clamp studies on isolated patches appear to be closer to the native IC$_{50}$ value and therefore confirm that this is the likely reason for the discrepancy observed.

Further studies were conducted on two K-channels, namely, the Kir6.2 ΔC26 mutant construct and the Kir2.1. Both these channels form functional channels without association with the SUR subunit. The ΔC26 channel is a modified Kir6.2 pore-forming unit that has been truncated 26 residues at the carboxy terminus. This channel has significantly lowered sensitivity to ATP and does not exhibit any apparent SUR-related pharmacology. The Kir2.1 channel is a lower conductance, strong inward rectifier and also forms a functional channel in the absence of the SUR subunit (Kubo et al., 1998). These channels were selected to investigate the target site of HMR 1098. Since neither of these channels showed sensitivity to the drug, it is concluded that HMR 1098 acts on the SUR subunit to alter channel activity.

Analysis of the single-channel recordings also yields some information as to the action of HMR 1098. It is evident from the current amplitude histogram (see Figure 8) that unitary current amplitude is unchanged before, during and after drug application. The change in normalized open probability suggests that HMR 1098 alters the open probability to favor a closed state. In addition, re-establishment of current after washout demonstrates that the effects of the drug are partially reversible.

The data support the conclusion that HMR 1098 is a selective, reversible inhibitor of sarcK_{ATP} channels. This drug effectively blocks current by acting on the SUR subunit and can be used as an agent to selectively target the sarcK_{ATP} channel over the mitoK_{ATP} channel.

PKC and the Protection Pathway

This study focused on the involvement of the sarc and mito K_{ATP} channels during hypoxia and reoxygenation. As mentioned earlier, there exists a wealth of data that supports the involvement of the K_{ATP} channel as an effector or at the very least a contributor to IPC (Menasche et al., 1995; Tomai et al., 1999). The data presented here are in agreement with recent studies implicating the K_{ATP} channels. What is most stricking is the demonstration of both channels affording some level of protection at different, well defined stages in the response to metabolic stress. The data suggest that the mito and sarc channels play distinct roles in moderating cellular injury. An analysis of the Ca^{2+} loading experiments shows that calcium loading is biphasic. The initial loading correlates with the onset of hypoxia and the latter follows reoxygenation. This biphasic loading was dissociated by selective activation of the sarc or mitoK_{ATP} channels. By using a selective pharmacological approach, targeting of either the sarc or mitoK_{ATP} channels led us to conclude that both have an important but distinct role. When the mitoK_{ATP} channels were selectively activated, the initial calcium loading associated with the onset of ischemia was relieved, but injury during reoxygenation was still prominent. Conversely, selective activation of the sarcK_{ATP} channel caused the opposite effect, resulting in ablation of the loading observed during reoxygenation without affecting the initial calcium-loading event. It is therefore suggested that the mitoK_{ATP} channel is responsible in some way to moderate insult associated with the onset of ischemia and the sarcK_{ATP} channel is responsible for minimizing insult during reoxygenation.

In an attempt to understand the protection pathway, PKC was studied as a mediator of protection. Several studies have indicated that activation of PKC is important for preconditioning (Liu et al., 1994; Speechly-Dick et al., 1994). Activation of PKC during hypoxia and reoxygenation reduced the level of injury in comparison to control. Both the initial and reoxygenation loading were abolished, resulting in a calcium loading response that paralleled loading observed under the influence of levcromakalim. PKC is known to activate both the mito and sarc K_{ATP} channels (Hu et al., 1999; Light et al., 2000; Sato et al., 1998b). Our data are consistent with these observations and now suggest that PKC stimulation is also able to mediate this effect via activation of the sarc and mito K_{ATP} channel populations. PMA induced protection was challenged by the addition of the PKC antagonist, chelerythrine, confirming again the important role of PKC in mitigating injury.

The cell viability data demonstrate that the PKC-induced increase in cell survival after hypoxia/reoxygenation is affected by both sarcK_{ATP} and mitoK_{ATP} channel populations. In a recent whole-heart study, it was suggested that the mitoK_{ATP} channels act to regulate infarct size, while activation of sarcK_{ATP} channels modulates functional recovery of the surviving tissue (Toyoda et al., 2000). Our data suggest that PKC-induced activation of sarcK_{ATP} channels is an important pathway by which myocytes can limit increases in intracellular calcium during reperfusion. The exact mechanism(s) by which the sarcK_{ATP} channel elicits these protective effects remains to be determined. In addition, the data suggest that the mechanism by which PKC elicits protection via the mitoK_{ATP} channel

population is unlikely to involve the ability of myocytes to handle cytosolic calcium during the reoxygenation period. Recent reports suggest that the activation of mitoK_{ATP} channels may result in a transient increase in free radical species that in turn mediates the protection observed during IPC (Gross and Fryer, 2000; Pain et al., 2000), probably via activation of a MAP kinase signaling cascade (Cohen et al., 2000b; Nakano et al., 2000).

Activation of adenosine A_1 receptors can elicit opening of K_{ATP} channels, possibly through a G protein-mediated mechanism (Ito et al., 1994; Kirsch et al., 1990). This led us to study the involvement of A_1 receptor activation, stimulation of PKC and opening of the K_{ATP} channels as a common signaling pathway, all leading to the activation of the K_{ATP} channel as the final effector. The PKC hypothesis is attractive because PKC translocation or protein phosphorylation could facilitate the interaction between A_1 receptors and K_{ATP} channels. The data collected provide pharmacological evidence that the protection from adenosine receptor activation is associated with the opening of K_{ATP} channels. The activation of these channels occurs via PKC activation and chelerythrine, a selective PKC blocker, caused almost complete inhibition of protection during A_1 receptor activation. The calcium loading experiments suggest that adenosine receptor activation facilitated the action of the sarcK_{ATP} channel in suppressing injury. However, the viability studies implicate both the sarc and mitoK_{ATP} channels in mitigating injury. Though the exact mechanism is still in question, the sarc and mitoK_{ATP} channel maintained the recently proposed exclusive roles in this set of experiments as mediators of protection during ischemia and reoxygenation, respectively.

Though chelerythrine blocked the action of A_1-receptor activation, it did not completely relieve protection from calcium loading. The calcium loading did not revert to control levels as expected, suggesting that A_1 receptor activation may elicit a secondary pathway in addition to PKC activation that impedes calcium loading and contributes to protection.

The data clearly implicate the A_1 receptor, PKC and K_{ATP} channels, both mito and sarc, in a single protective pathway. Initiation of the pathways still remains in question; however, the data delineate a logical pathway of events that may lead to protection during ischemia.

The data support the role of the mitoK_{ATP} channel as mediating injury during ischemia and the sarcK_{ATP} channel during reoxygenation. A good correlation was derived between the calcium loading protocol and the viability assays, further supporting calcium overload as a precursor of cell death. Recent studies by Light et al. demonstrated that phosphorylation of a single residue on the sarcK_{ATP} channel at the T180 site is responsible for activation of the channel, and mutation of this site abolished all sensitivity to PKC (Light et al., 2000). Employing this finding, a mutation of the T180 residue to alanine was performed to impair phosphorylation and thus activation of the channel. Calcium overload experiments on cells expressing the mutant channel confirmed that the sarcK_{ATP} is primarily responsible for reducing calcium overload and insult during reoxygenation. Treatment of cells expressing the mutant channel with PMA did not prevent calcium overload as was the case with the wild-type channel. These data very convincingly put great importance on the sarcK_{ATP} channel as a potent protective influence against reoxygenation injury.

Sarc vs. Mito K_{ATP} - Which is Responsible for Protection?

Both the mito and sarc K_{ATP} channels have been implicated as necessary contributors in a series of events leading to protection from ischemia. How do we reconcile these data with the recent emphasis on the mitoK_{ATP} channel as the sole mediator of IPC? In a report published by D'hahan et al., the role of diazoxide as a selective activator of the mitoK_{ATP} was challenged (D'hahan et al., 1999). It was illustrated that the effect of diazoxide on the sarcK_{ATP} channel is enhanced by increased concentrations of ADP in the cell, as is the case during ischemia. It seems likely therefore that some of the cardioprotective effect of diazoxide may be mediated via its ability to open sarcK_{ATP} channels. These recent data have caused much uncertainty with regards to data collected illustrating the mitochondrial channel as an effector by diazoxide activation. Gross et al. recently reported that HMR 1098 completely blocked the protective effects of diazoxide to reduce infarct size in rats (Gross, 2000). Since it has been shown here that HMR 1098 is selective for the sarcK_{ATP} channel it is reasonable to conclude that diazoxide is not acting solely via the mitoK_{ATP} channel. In light of this recent finding, the reports placing the mitoK_{ATP} channel at the helm of protection are under debate. Although the mitoK_{ATP} channel is still thought to play a significant role, my results strongly support the idea that the sarcK_{ATP} channel is a key player in the response to hypoxia and reoxygenation.

Sarc AND mitoK_{ATP} – How is Protection Afforded?

The data collected clearly stipulate different roles for the sarc and mito channels. In addition, A_1 receptor activation and PKC appear to be critical upstream activators of these channels. The sarcK_{ATP} channel is crucial in handling calcium overload during the reoxygenation phase. The calcium influx may result from osmotic disturbances given that, during ischemia, there is an intracellular accumulation of metabolites, such as lactate, that leads to an increase in cytoplasmic osmolarity (Piper et al., 1998b). Therefore, during reperfusion there is an inward movement of water resulting in increased cell volume. The calcium movement appears to be a restoring element in reoxygenation. During experiments performed by Jovanovic et al., it was reported that the calcium resulting in a rise in intracellular levels was from extracellular sources. Furthermore, inhibition of L-type calcium channels did not affect the calcium overload observed, suggesting that the loading occurred as a result of a leaky membrane or other calcium permeable pores (Jovanovic et al., 1998b). Reperfusion or reoxygenation therefore results in swelling or stretching of ventricular myocytes: this in turn is known to activate the K_{ATP} channels. If stretching and increasing ADP under hypoxic conditions initiate the activation of the K_{ATP} channel then this channel is very susceptible to opening when challenged by ischemia. Opening of the channel would result in the outward flux of K^+ ions resulting in shortening of the action potential duration and reduction in Ca^{2+} influx and contractility, which in turn would result in the conservation of energy (D'hahan et al., 1999; Gross, 2000). This "Action Potential" hypothesis for protection has been refuted by evidence that protection is observed in the absence of APD shortening

(Rees et al., 1995). How is it then that extrusion of K^+ ions is beneficial to the survival of the cell? Knowing now that K_{ATP} channels function to relieve Ca^{2+} overload during reoxygenation, it is possible that the K_{ATP} channel may be working to relieve some of the ionic stress associated with ischemia and reperfusion. In addition there is evidence that stretch-activated chloride channels may also open when the cell is swollen (Diaz et al., 1999). Therefore, opening of the stretch activated chloride channel and K_{ATP} channel in concert during reoxygenation may serve as an osmo-protective mechanism to counter the flow of water and Ca^{2+} into the cell. The extrusion of both these ions results in an electroneutral ionic flux that may relieve some of the osmotic stress caused by the build-up of metabolites during ischemia and the disparity during reoxygenation. The data presented in this study, in addition to the known activity of the stretch-activated chloride channel, provide a strong foundation to support this mechanism of protection. This mechanism of protection is still devoid of a very important element – the mitoK_{ATP} channel. Though it is apparent from the data that the sarcK_{ATP} channel has greater bearing on cell survival in this model, there still appears to be an important role for the mitoK_{ATP} channel as a mediator of protection. At the present time we are limited by the obscure pharmacology of this channel.

Activation of the mitoK_{ATP} channel leads to enhanced cell survival under hypoxia and reoxygenation; however, the question of how this protection is afforded is still elusive. Mitochondria are essential in the normal functioning and survival of a cell. In addition to the responsibilities of ATP production and calcium homeostasis, mitochondria have been recently implicated as mediators of ischemic preconditioning. However, recent

pharmacological findings have challenged this idea. It is still believed however that the mitoK$_{ATP}$ channel plays a significant, if not imperative, role in mitigating injury (Gross, 2000). Our studies have shown that the mitoK$_{ATP}$ channel may be involved in modulating Ca^{2+} entry during ischemia, though it is not known if this is a correlative or causal effect. How then is this channel working to assuage injury? This question has plagued researchers from the time that mitoK$_{ATP}$ channels were introduced into discussions of the IPC phenomenon.

It has been shown, in isolated cardiac mitochondria, that K$_{ATP}$ channel openers depolarize the matrix membrane, accelerate respiration, slow ATP production, release accumulated Ca^{2+}, produce swelling, and stimulate efflux of intermembrane proteins (Holmuhamedov et al., 1998). It has been proposed that the increased K^+ conductance may reduce mitochondrial Ca^{2+} influx through the calcium uniporter, which would blunt mitochondrial Ca^{2+} overload. In addition, calcium accumulation has been shown to destabilize the inner mitochondrial membrane, causing the inner membrane pore to open, which permits further movement of cations across the mitochondrial membrane (Frolkis et al., 1988). The opening of these pores renders the mitochondrion incapable of synthesizing ATP and has been suggested to be an important event in the process leading to myocardial cell death (McCormack and Denton, 1990). In addition to mediating mitochondrial calcium handling, opening mitoK$_{ATP}$ channels may cause mild uncoupling and oxidation of flavoproteins induced by diazoxide resulting in increased free radical production in the mitochondria and changes in mitochondrial membrane potential that

could alter glycolytic pathways during ischemia in favor of myocyte survival (Sato and Marban, 2000).

The widely accepted notion that opening of mitoK$_{ATP}$ channels impedes calcium entry into the cell was recently challenged in a report by Garlid et al. Their data convincingly suggest that opening of the mitoK$_{ATP}$ channel does not cause uncoupling and that reports of uncoupling and inhibition of Ca^{2+} uptake are the result of using toxic concentrations of K$_{ATP}$ channel openers, far greater than the concentration used in our experiments (Garlid, 2000). However the influx of K^+ ions is believed to result in mitochondrial matrix volume changes that are suggested to have significant bioenergetic consequences for energy coupling in the cell. K^+ influx is driven by an equal amount of H^+ ejection by the electron transport system (ETS). This in turn reduces the electrical potential (Garlid, 2000). It was also ascertained that preconditioning causes a moderate increase in production of reactive oxygen species (ROS). This report suggests that ROS production is upregulated by increased intermitochonderial volume, possibly deriving from activation of the ETS. Elevated concentrations of ROS have been shown to trigger gene transcription and therefore may play a role in the mechanism of action of mitoK$_{ATP}$ channels in triggering IPC (Chandel et al., 1998; Xie et al., 1999).

The data presented in our study illustrate that opening of the mitoK$_{ATP}$ channel impedes a rise in intracellular Ca^{2} at the onset and during ischemia. Thus, opening of the mitochondrial channel may serve to enhance the sequestering of cytosolic calcium in some form. This observation was consistent during the activation of mitoK$_{ATP}$ channels

by PMA: however, A_1 receptor pathway activation and opening of mitoK_{ATP} channels did not "significantly" alter intracellular calcium during ischemia, though there was an observed influx. This discrepancy of observations suggests that calcium handling by activation of mitoK_{ATP} channel opening may not be responsible for the protection observed. During both PKC and A_1 receptor mediated activation of the mitoK_{ATP} channels, similar protection from cell death was afforded. These data clearly substantiate an important role for the mitoK_{ATP} channel in mitigating injury; however, the mechanism by which opening of the mitoK_{ATP} channel produces cardioprotection remains unclear. The data refute the hypothesis that the mitoK_{ATP} channel is responsible for depressing calcium entry into the mitochondria and furthermore are consistent with the recent observation that mitoK_{ATP} opening does not prevent calcium overload in the mitochondria. Therefore the mitoK_{ATP} channel may furnish protection through another pathway related to increased cellular viability.

It is possible that the mitoK_{ATP} channel may be working as a trigger for the second window of protection. Studies have shown that mitoK_{ATP} channels have a functional role in both early (classical) and late phase (second window) of cardioprotection (Baines et al., 1998; Fryer et al., 2000). Opening of the mitoK_{ATP} channel is known to result in increased production of ROS, a putative initiator of gene transcription (Baines et al., 1999; Miura et al., 2000). Downey et al. in a recent report endorse the hypothesis that the mitoK_{ATP} channels may mediate injury by production of freeradicals (Pain et al., 2000). The data presented in their report propose that the mitoK_{ATP} channels are not the end effectors of protection, but rather their opening during ischemia generates free radicals

that trigger entrance into a preconditioned state and activation of kinases such as MAPK and p38. These kinases are thought to mediate protection by activation of gene transcription that in turn leads to strengthened membrane integrity by protein production and resilience to insult.

How is Preconditioning Activated? Putting Together the Pieces

The data presented in our study describe a plausible series of events to explain in part the pathway of IPC. The mechanism of IPC is emerging as a complex chain of interactions among receptors, protein kinases, and ion channels. The purpose of this study was not to determine the entire mechanism for IPC, but reasonably to investigate the possible signal transduction pathways regulating the K_{ATP} channel. The observations link several disparate elements that are known to play important roles in IPC. The data confirm a role for adenosine and PKC in relieving injury. During ischemia it is known that there is a large amount of adenosine release by the myocardium (Liu et al., 1991). It is reasonable to assume that during ischemia the release of adenosine may work in an autocrine manner to initiate a series of events that may lead to injury suppression. To recapitulate, the data clearly illustrate that activation of the A_1 receptor pathway involves signal transduction by PKC. In addition, Light et al. have illustrated how phosphorylation of the sarcK_{ATP} by PKC increases its open probability. Experiments on the Kir6.2(T180A) mutant sarcolemmal channel, incapable of phosphorylation, argue that the sarcK_{ATP} channel is a critical effector mitigating reoxygenation calcium overload.

Though the mechanism of IPC is complicated and a clear understanding of the entire pathway is still a topic of intense debate, findings in this study help to clarify some of the key elements. My data suggest that ischemic preconditioning may be initiated by adenosine release and that this pathway is mediated by PKC. In addition, it was demonstrated that the activation of sarcK_{ATP} channels is protective during reoxygenation and activation of mitoK_{ATP} channels protects during the initial onset of hypoxia. It is proposed, based on the data collected, that the sarcK_{ATP} channel might relieve the osmotic stress from the build-up of metabolites during hypoxia. In addition, the role of the sarcK_{ATP} channel was demonstrated to be more influential in mitigating cell death than the mitoK_{ATP} channel. The mitoK_{ATP} channel appears to play a role in protection, though the mechanism remains unclear. Recent reports suggest that the mitoK_{ATP} channel may be more inclined to modulate the second window of protection by transcription of protective elements. Our observations suggest, however, that there is a role for the mitoK_{ATP} channel in the acute response to hypoxia and reoxygenation. The findings reported here shed new light on previously suggested mechanisms in IPC. The sarcK_{ATP} channel has proven to be a strong mediator, with additional influence by the mitoK_{ATP} channel. The complete pathway of activation is still uncertain but a plausible mechanism of action has been proposed. These results bring us closer to understanding the mysterious cardioprotective effect of ischemic preconditioning. The potential ability to induce protection in a clinical setting drives the pursuit to understand these mechanisms in their entirety (see figure 19).

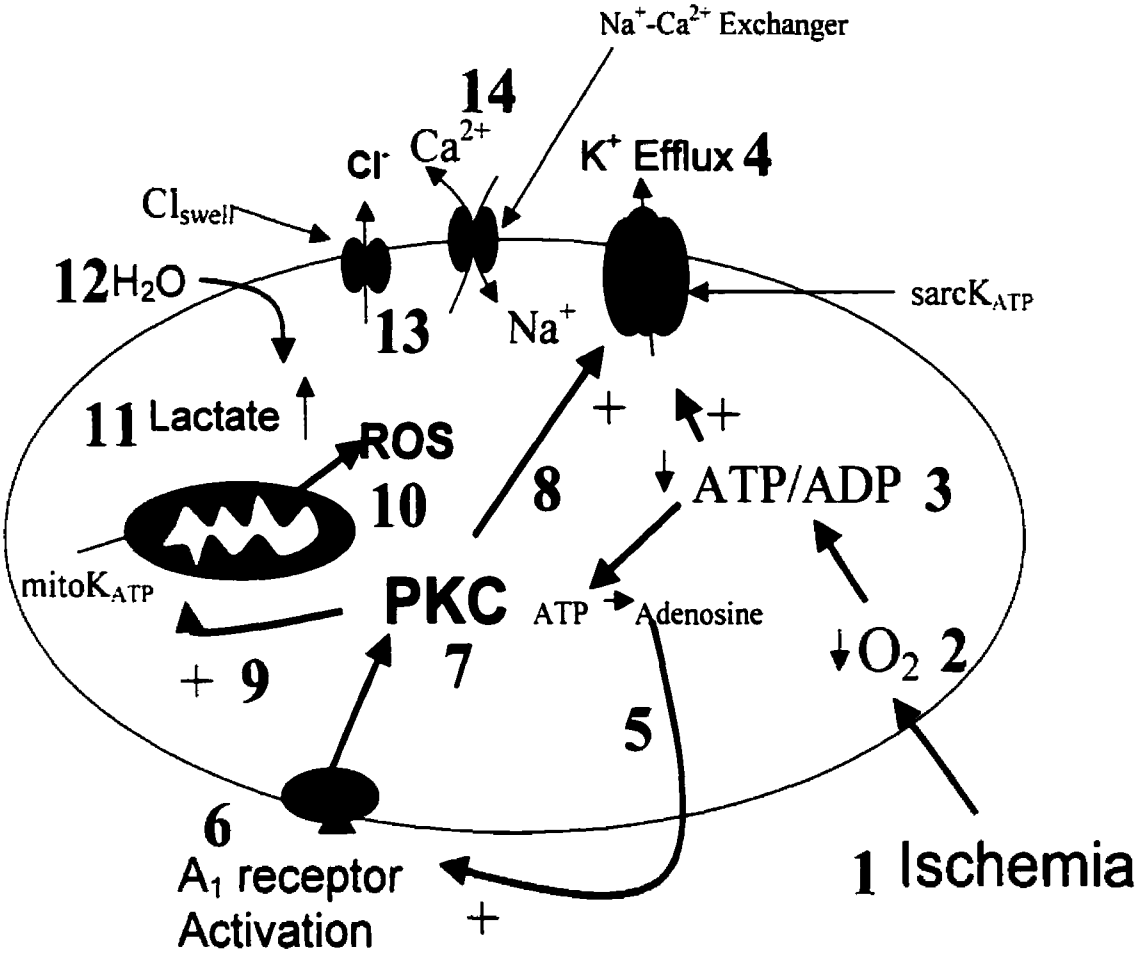


Figure 19. A schematic diagram of the proposed series of events thought to take place during Ischemia and reperfusion. Hypoxia results in lowering of the intracellular [ATP/ADP] ratio causing the opening of the $sarcK_{ATP}$ channel. The K^+ efflux through the $sarcK_{ATP}$ channel is thought to mediate protection by shortening the APD or maintaining ion homeostasis. The use and dephosphorylation of ATP to ADP results in the release of adenosine acting in an autocrine manner to initiate the A_1 receptor coupled pathway. Furthermore, this pathway is thought to activate PKC which "primes" the sarc and $mitoK_{ATP}$ channels by phosphorylation. During Ischemia there is also a build-up of lactate and metabolites, which acts as a driving force for water to enter into the cell. Swell activated chloride channels are thought to open as a result, releasing chloride into the extracellular space and in turn equilibrating the ionic disparity. Lastly, the sodium calcium exchanger may also contribute to the extrusion of Ca^{2+}. As a summary these series of events ultimately lead to strengthened membrane integrity during hypoxia and reoxygenation.

Future Directions:

Experiments substantiating the exact role of both the sarc and mito K_{ATP} channels are needed in order to understand the mechanisms behind IPC. To induce protection in a clinical setting, the question of "how" protection is afforded should be addressed. A proposed pathway for the role of the sarcK_{ATP} channel in modulating reperfusion injury was presented. Experiments on osmotic swelling and relief by K_{ATP} channel stimulation would help to determine whether this channel does in fact mitigate injury by an osmotic mechanism.

Cloning of the mitoK_{ATP} channel would help to characterize this channel and identify its physiological function. Studies investigating its role as a mediator of both classical and late conditioning need to be conducted. A particular focus on the involvement of ROS as produced by mitochondrial activation may be instructive. Experiments testing activation of the sarcK_{ATP} channel by ROS would be useful in determining if mitoK_{ATP} channel activation results in sarcK_{ATP} channel opening thereby providing a link between the two. In addition, a more complete, quantitative description of the pharmacology of the mitoK_{ATP} by direct patch clamp experiments needs to be obtained. Using the mitoplast preparation, patch clamp studies on mitoK_{ATP} channels should be performed as a direct measure of activity and pharmacological properties.

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