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Cardioprotective effects of K_{ATP} channel activation during hypoxia in goldfish *Carassius auratus*

Jerri Chen, Julia X. Zhu, Ingred Wilson and John S. Cameron*
Department of Biological Sciences, Wellesley College, Wellesley, MA 02481, USA
*Author for correspondence (e-mail: jcameron@wellesley.edu)

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Summary

The activation of ATP-sensitive potassium (K_{ATP}) ion channels in the heart is thought to exert a cardioprotective effect under low oxygen conditions, possibly enhancing tolerance of environmental hypoxia in aquatic vertebrates. The purpose of this study was to examine the possibility that hypoxia-induced activation of cardiac K_{ATP} channels, whether in the sarcolemma ($sarcK_{ATP}$) or mitochondria ($mitoK_{ATP}$), enhances viability in cardiac muscle cells from a species highly tolerant of low oxygen environments, the goldfish *Carassius auratus*. During moderate hypoxia (6–7 kPa), the activation of $sarcK_{ATP}$ channels was indicated by a reduction in transmembrane action potential duration (APD). This response to hypoxia was mimicked by the NO-donor SNAP (100 $\mu mol\ l^{-1}$) and the stable cGMP analog 8-Br-cGMP, but abolished by glibenclamide or L-NAME, an inhibitor of NO synthesis. The $mitoK_{ATP}$ channel opener diazoxide did not affect APD. Isolated ventricular muscle cells were then incubated under normoxic and hypoxic conditions. Cell viability was decreased in hypoxia; however, the negative effects of low oxygen were reduced during simultaneous exposure to SNAP, 8-Br-cGMP, and diazoxide. The cardioprotective effect of diazoxide, but not 8-Br-cGMP, was reduced by the $mitoK_{ATP}$ channel blocker 5-HD. These data suggest that hypoxia-induced activation of $sarcK_{ATP}$ or $mitoK_{ATP}$ channels could enhance tolerance of low-oxygen environments in this species, and that $sarcK_{ATP}$ activity is increased through a NO and cGMP-dependent pathway.

Key words: hypoxia, ATP-sensitive K^+ channels, K_{ATP}, nitric oxide, cardioprotection, goldfish, *Carassius auratus*.

Introduction

Many studies suggest that the activation of cardiac muscle ATP-sensitive potassium (K_{ATP}) channels can play a beneficial role during hypoxia and ischemia in mammals (Gross and Peart, 2003; Grover and Garlid, 2000; O'Rourke, 2000). This conclusion is in accord with the original hypothesis of Noma (1983), who was the first to characterize sarcolemmal K_{ATP} ($sarcK_{ATP}$) channels. These are activated when intracellular oxygen levels and ATP/ADP ratios are low, although the precise function of $sarcK_{ATP}$ channels in cardiac muscle is unknown (Flagg et al., 2004). Since the subsequent discovery that K_{ATP} channels are also located in the mitochondrial inner membrane ($mitoK_{ATP}$; Inoue et al., 1991), there has been considerable debate as to the relative roles played by $sarcK_{ATP}$ *vs* $mitoK_{ATP}$ channels in cardioprotection and ischemic preconditioning (Gross and Peart, 2003; Toyoda et al., 2000). K_{ATP} channel activation under depleted oxygen conditions is often linked to the prior production of nitric oxide (NO), which acts through a cGMP-dependent mechanism (Dawn and Bolli, 2002; Wang et al., 2001).

The observation that hypoxia-induced K_{ATP} channel activation is beneficial in mammals raises the possibility that a similar response in aquatic ectotherms could promote tolerance of environmental hypoxia. $SarcK_{ATP}$ channel currents have been recorded in isolated myocytes from the hearts of several teleost species (Ganim et al., 1998; Paajanen and Vornanen, 2002), and there is evidence for the existence of cardiac $mitoK_{ATP}$ channels in fish (MacCormack and Driedzic, 2002). In addition, nitric oxide synthase (NOS) has been localized in the hearts of goldfish (Bruning et al., 1996), and NO has been shown to play a critical role, *via* cGMP, in regulating the heart and peripheral vasculature in a variety of teleost species (Imbrogno et al., 2003; Jennings et al., 2004; Pellegrino et al., 2003). In goldfish, NO synthesized in cardiac myocytes plays a role in $sarcK_{ATP}$ channel activation during moderate hypoxia (Cameron et al., 2003). Although it has been proposed that hypoxia-induced K_{ATP} channel activation, whether in the heart or in the brain, contributes to the enhanced tolerance of environmental oxygen depletion exhibited by many ectothermic vertebrates (Cameron and Baghdady, 1994; Pek-Scott and Lutz, 1998), this possibility has not been directly tested in fish.

The purpose of the present study, then, was to determine whether $sarcK_{ATP}$ or $mitoK_{ATP}$ channel activation exerts a cardioprotective effect during hypoxia in goldfish. This species is highly tolerant of depleted environmental oxygen; indeed, the congeneric crucian carp *Carassius carassius* retains normal

cardiac function for 5 days of complete anoxia (Stecyk et al., 2004). A cellular model of environmental hypoxia was used to examine the effects of altered channel activity on isolated myocytes. Our hypothesis was that the NO- and cGMP-dependent activation of cardiac K_{ATP} channels would promote survival of isolated cells, thereby contributing to a suite of mechanisms that dramatically enhance tolerance of hypoxia in these fish. Preliminary results of this work have appeared in abstract form (Zhu et al., 2004).

Materials and methods

Goldfish *Carassius auratus* L. were obtained from local suppliers and acclimated at 21°C in filtered, aerated 200 liter aquaria under a 12 h:12 h light:dark cycle. On the day of an experiment, goldfish of either sex were anesthetized by immersion in a solution of MS-222 (tricaine methanesulfonate, 0.2 g l^{-1}; pH 7.6). The heart was rapidly excised and prepared for standard intracellular recording using the intact ventricle, or for enzymatic dispersion of individual myocytes.

Intracellular recording

Animals used for conventional intracellular recording ranged in size from 9.5–28.1 g (mean ± S.E.M., 18.1±4.8 g). Procedures have been previously described (Cameron et al., 2003); briefly, the entire heart was mounted in a tissue bath and superfused with normoxic saline solution at a rate of 15 ml min^{-1}. This solution contained (in mmol l^{-1}) 150 NaCl, 5 KCl, 1.2 NaH_2PO_4, 1.2 $MgSO_4$, 1.8 $CaCl_2$, 10 Hepes and 10 glucose; pH was maintained at 7.6 and temperature held at 21±1°C.

Standard glass microelectrodes filled with 3 mol l^{-1} KCl were connected to a differential amplifier (WPI, Sarasota, FL, USA), and spontaneous intracellular action potentials (APs) were recorded from the most superficial layer of ventricular muscle. APs were digitized (PowerLab; ADInstruments, Mountain View, CA USA) and analyzed using appropriate software (Chart, Peak Parameters; ADI). Parameters measured were action potential amplitude, resting membrane potential, slope of action potential upstroke phase and action potential duration at various levels of repolarization.

Oxygen partial pressure in the tissue bath was monitored using an oxygen meter (Cameron Instruments, Port Aransas, TX, USA) while moderate hypoxia (6.1±0.2 kPa) was induced by switching the superfusate from air-bubbled saline (20 kPa) to a glucose-free solution gassed with 100% N_2. The new condition was reproducible, reversible, and achieved in the tissue bath within 1 min. Electrophysiological responses to the acute onset of hypoxia and to reoxygenation were first assessed in the absence of any drug. Recordings were collected after 10–30 min exposure to hypoxia, and again 20 min after a return to normoxic solution.

Drugs

Drugs, including the nitric oxide (NO) donor S-nitroso-*N*-acetylpenicillamine (SNAP; 100 μmol l^{-1}), the nitric oxide synthase (NOS) inhibitor *N*ω-Nitro-L-arginine methyl ester (L-NAME; 50 μmol l^{-1}), the K_{ATP} channel antagonist glibenclamide (5 μmol l^{-1}), the mitoK_{ATP} channel agonist 3-methyl-7-chloro-1,2,4-benzothiadiazine-1,1-dioxide (diazoxide; 100 μmol l^{-1}), the stable and cell-permeable cGMP analog 8-bromoguanosine 3′:5′-cyclic monophosphate (8-Br-cGMP; 100 μmol l^{-1}), and the mitoK_{ATP} channel inhibitor 5-hydroxydecanoic acid (5-HD; 100 μmol l^{-1}), were purchased from Sigma (St Louis, MO, USA). Diazoxide was first dissolved in a drop of DMSO. Following the initial period of equilibration in normoxic saline, the effects of SNAP, 8-Br-cGMP and diazoxide on ventricular action potential parameters were recorded after 10 min exposure. In other experiments, glibenclamide or L-NAME was made up in hypoxic solution and applied to the tissue bath as described above. Dosages were chosen from among those recently employed in comparable studies.

Cellular model of environmental hypoxia

Isolation of cardiac myocytes

Animals used for this procedure ranged in size from 23.7 to 64.9 g (mean ± S.E.M., 43.3±12.4 g). Cell isolation procedures were modified from those of Karttunen and Tirri (1986), as previously described (Cameron et al., 2003). Upon removal, the heart was bathed in a chilled Ca^{2+}-containing saline buffer (μmol l^{-1}): 137 NaCl, 4.6 KCl, 3.5 NaH_2PO_4, 1.2 $MgCl_2$, 11 glucose, 10 Hepes and 0.025 $CaCl_2$. The atrium was tied off and an olive-tipped cannula (27 gauge) was inserted into the ventricle through the opening of the bulbus arteriosus. The ventricle was then perfused with an identical but nominally Ca^{2+}-free saline solution for 5 min, followed by 25 min perfusion with an enzyme buffer containing 50 ml Ca^{2+}-free saline, 35 mg collagenase (Type I), 25 mg trypsin and 25 mg bovine serum albumin (Fraction V). The heart was removed from the perfusion apparatus and carefully torn into small pieces. After 5 min of swirling agitation followed by 5 min of gentle trituration with a Pasteur pipette, the supernatant containing single free-floating cells was removed. Fresh buffer was added, and the procedure repeated to obtain a second and third fraction of cells. This procedure produced a high yield (80–90%) of viable, elongated myocytes.

Cellular model

The method used to assess cellular viability in hypoxia was derived from the *in vitro* model of cellular ischemia developed by Vander Heide et al. (1990), as modified by Sato et al. (2000). Portions of the myocyte suspension (0.5 ml) were pipetted into 5 ml test tubes, and 0.5 ml of saline buffer or drug solution was added. The hypoxic condition was induced in some tubes by layering an additional 0.5 ml of mineral oil on top of the myocyte suspension, preventing gaseous diffusion of oxygen. After 60 min incubation, 0.5 ml of myocytes were sampled from each tube and mixed with 0.5 ml of staining solution containing 0.5% gluteraldehyde and 0.5% Trypan Blue in saline buffer. The resulting ml of suspended cells was placed on a slide and examined by phase contrast microscopy

Fig. 1. Isolated ventricular myocytes from goldfish heart after exposure to 60 min hypoxia in a solution containing Trypan Blue. Most cells retained the elongated shape of those maintained under normoxic conditions, but some were unable to exclude the dye (left), indicating cellular damage. Scale bar, 50 μm.

at 100×. Elongated myocytes with a normal morphological appearance were included in the analysis, while dead, 'rounded-up' cells were excluded (Vander Heide et al., 1990). These dead cells were always blue, and were presumed to have been damaged during the enzymatic isolation procedure. Viability of the elongated cells was assessed by their capacity to exclude Trypan Blue (Fig. 1); the percentage staining clearly after 3 min exposure to the staining solution was recorded for each experimental condition. Myocytes staining dark blue were considered irreversibly damaged. Drugs and concentrations were identical to those used in the intracellular experiments; they were made up in saline buffer on the day of an experiment and were present throughout the period of *in vitro* hypoxia.

Data analysis

Data obtained from the intracellular and cellular hypoxia experiments were transferred to Microsoft Excel spreadsheets; *t*-tests and analysis of variance for repeated measures (ANOVA with *post-hoc* Fisher PLSD and Scheffe *F*-test; Statview) were used to determine statistical significance ($P<0.05$) among group means; results were expressed as means ± S.E.M.

Results

Intracellular recordings

Effects of hypoxia

Transmembrane intracellular recording techniques were used to monitor the effects of moderate hypoxia (6.1±0.2 kPa)

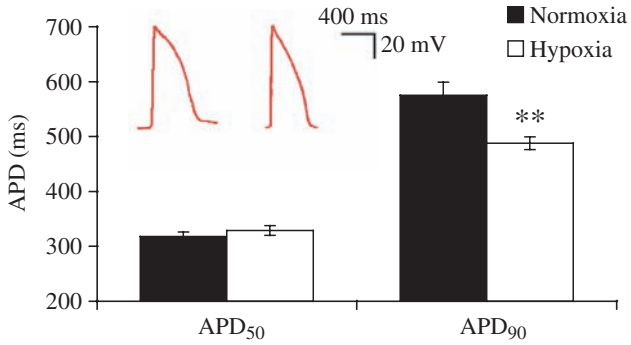

Fig. 2. Effects of moderate, substrate-free hypoxia on ventricular action potential duration (APD) in the isolated goldfish heart. APD in hypoxia was significantly shortened at 90% of full repolarization, but not at 50% ($N=5$). Inset shows representative action potential configuration in one experiment (normoxia at left). Values are means ± 1 S.E.M. **Significantly different from values recorded under normoxic conditions ($P<0.01$).

on the configuration of ventricular action potentials in goldfish heart. Cardiac action potential duration (APD) was decreased in all experiments ($N=5$). As previously observed (Cameron et al., 2003), there was a significant ($P<0.01$) reduction in APD at 90% of full repolarization (APD_{90}) after 10 min of exposure to substrate-free hypoxia (Fig. 2). Mean APD_{90} under normoxic conditions was 575±24 ms; after exposure to hypoxia, this value fell to 488±12 ms, a decrease of 15.1%. APD at 50% of full repolarization (APD_{50}) was not significantly reduced. There were no significant effects of hypoxia on any other electrophysiological parameters, including resting membrane potential, action potential amplitude or slope of action potential upstroke.

Glibenclamide and L-NAME in hypoxia

To examine the possibility that APD shortening in hypoxia was caused by the activation of K_{ATP} channels, the effects of the channel antagonist glibenclamide (5 μmol l^{-1}) on hypoxia-induced shortening were monitored in isolated hearts. Simultaneous exposure to both glibenclamide and hypoxia

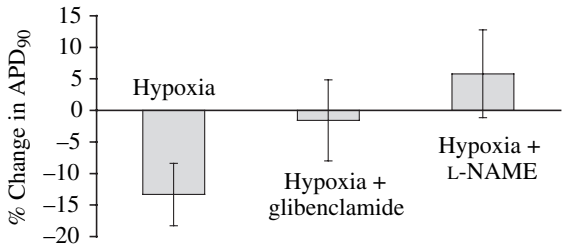

Fig. 3. Effects of glibenclamide (5 μmol l^{-1}), a K_{ATP} channel blocker, and L-NAME (50 μmol l^{-1}), an inhibitor of nitric oxide synthase (NOS), on the percentage change in action potential duration at 90% repolarization (APD_{90}) induced by hypoxia in goldfish ventricle ($N=4$). Hypoxia-induced APD_{90} shortening was eliminated by simultaneous exposure to either glibenclamide or L-NAME. Values are mean change ± 1 S.E.M.

abolished the reduction in APD characteristic of hypoxia alone (N=3; Fig. 3).

To determine whether NO plays a role in cellular responses to hypoxia in goldfish, the effects of the NOS inhibitor L-NAME were studied in isolated hearts and in ventricular myocytes. Hypoxia-induced shortening of APD_{90} was eliminated by previous exposure to 50 μmol l^{-1} L-NAME (N=4; Fig. 3).

Effects of K_{ATP} activators under normoxic conditions

To determine whether NO and cGMP could mimic cellular responses to hypoxia, the effects on action potential parameters of the NO donor SNAP and the stable cGMP analog 8-Br-cGMP were monitored under normoxic conditions. At a concentration of 100 μmol l^{-1}, SNAP significantly (P<0.05) reduced APD_{90} after 10 min exposure (N=4; Fig. 4). With prolonged exposure to SNAP, APD was further reduced; at 60 min, both APD_{50} and APD_{90} were significantly decreased. There were no significant effects of SNAP on any other action potential parameter.

At a concentration of 200 μmol l^{-1}, 8-Br-cGMP also shortened ventricular APD under normoxic conditions (P<0.05). APD_{90} was reduced by 17.6% (N=3; Fig. 4), although APD_{50} remained unchanged. At a concentration of 100 μmol l^{-1}, diazoxide had no significant effect on ventricular APD_{90} (N=6; Fig. 4).

Cellular viability in vitro

A cellular model of environmental hypoxia was used to assess the potential cardioprotective effects of K_{ATP} channel activation. Under normoxic conditions, 37.8±4.0% of elongated, isolated ventricular myocytes from goldfish were unable to exclude Trypan Blue, suggesting cellular injury (N=6; Fig. 5). After 60 min hypoxia, however, the incidence of Trypan Blue-positive cells was significantly (P<0.001) increased compared to that observed in normoxia. The percentage of stained cells in hypoxia was 81.2±2.6, a 115% increase. More than 500 myocytes were individually evaluated.

Following simultaneous exposure of isolated myocytes to L-NAME and 60 min hypoxia, the percentage of Trypan Blue-stained cells was moderately increased relative to that seen with hypoxia alone (Fig. 6). After 60 min exposure of isolated myocytes to hypoxia plus SNAP, however, the percentage of Trypan Blue-stained cells was markedly reduced compared to that observed with hypoxia alone. To determine whether this cardioprotective action of SNAP was linked to the hypoxia-induced production of NO in muscle cells, the effect of SNAP in combination with L-NAME also was tested. Blockade of NO synthesis by L-NAME entirely eliminated the reduction in stained cells observed with SNAP and hypoxia alone (Fig. 6).

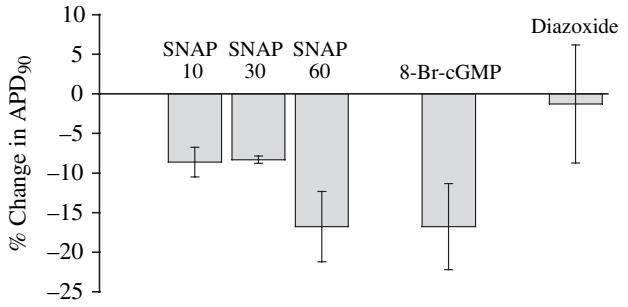

Fig. 4. Effects of presumed K_{ATP} activators on the percentage change in ventricular action potential duration (APD) under normoxic conditions. APD_{90} was shortened by 10, 30 and 60 min exposure to SNAP (100 μmol l^{-1}), a nitric oxide (NO) donor (N=4), and by 10 min exposure to 8-Br-cGMP (200 μmol l^{-1}), a stable cGMP analog (N=6). Diazoxide (50 μmol l^{-1}), a specific mitochondrial K_{ATP} (mitoK_{ATP}) channel opener, did not significantly affect APD (N=6). Values shown are mean changes ± 1 S.E.M.

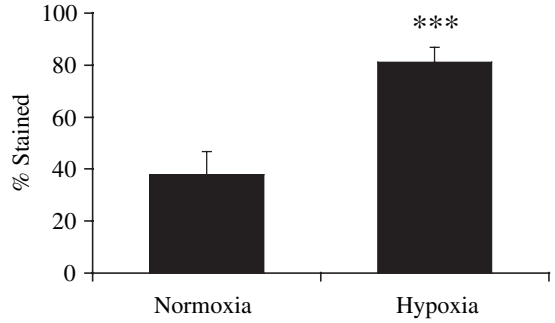

Fig. 5. Effect of 60 min hypoxia *in vitro* on the incidence of cell staining by Trypan Blue. Under hypoxic conditions, a significantly greater percentage of cells was unable to exclude the stain compared to that observed in normoxia, suggesting cellular injury. A total of more than more than 500 myocytes were individually evaluated during six experiments; values are mean changes ± 1 S.E.M. ***Significantly different from values recorded in normoxia (P<0.001).

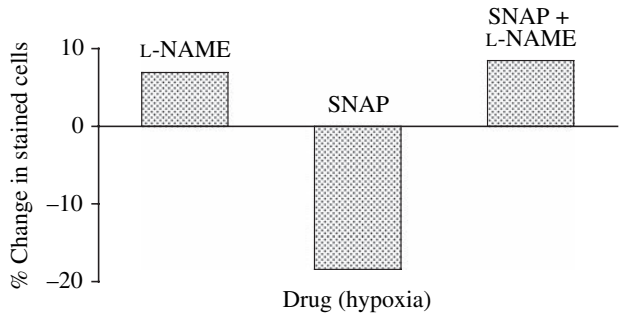

Fig. 6. Cardioprotective effect of SNAP (100 μmol l^{-1}) on the hypoxia-induced damage incurred by isolated myocytes *in vitro*. Figs 6 and 7 show the percent change in the total fraction of stained cells after 60 min hypoxia in the presence of various agents, compared to that recorded after hypoxia alone. Under hypoxic conditions, SNAP reduced the percentage of stained cells. In contrast, L-NAME, which blocks hypoxia-induced synthesis of NO, increased the percentage of stained cells when given alone, and eliminated the cardioprotective influence of SNAP when the two drugs were administered simultaneously.

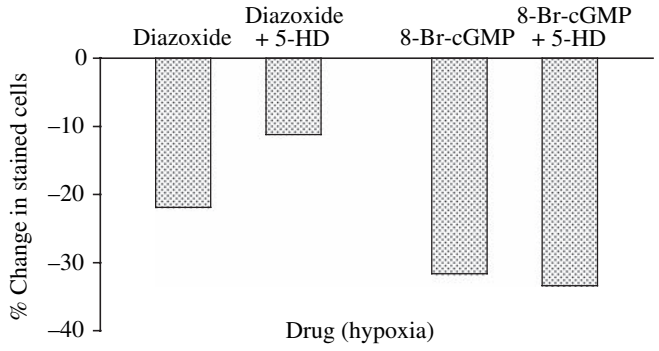

Fig. 7. Cardioprotective influence of the mitoK_{ATP} opener diazoxide (50 μmol l^{-1}) and the stable cGMP analog 8-Br-cGMP (200 μmol l^{-1}) on the hypoxia-induced damage incurred by isolated cells. This figure illustrates the percentage change in the total fraction of stained cells after 60 min hypoxia in the presence of various agents, compared to that recorded after hypoxia alone. Under hypoxic conditions, both diazoxide and 8-Br-cGMP decreased the percentage of stained cells *vs* that seen with hypoxia alone. Blockade of mitoK_{ATP} channel activity with 5-HD (100 μmol l^{-1}) reduced but did not eliminate the beneficial effect of diazoxide, and did not affect the response to 8-Br-cGMP.

Selective activation of sarcK_{ATP} vs *mitoK_{ATP} channels*

In isolated cells exposed to hypoxia, diazoxide exerted a marked cardioprotective influence. Isolated myocytes exposed to diazoxide during 60 min hypoxia survived in greater numbers than did those affected by hypoxia alone (Fig. 7).

In mammals, diazoxide and 5-HD have been shown to be selective for mitoK_{ATP} over sarcK_{ATP} channels — the former as an activator and the latter as an inhibitor (Mannhold, 2004). In goldfish, the beneficial effects of diazoxide in preserving isolated myocytes was reduced, but not eliminated, by 5-HD (Fig. 7). In contrast, 5-HD did not alter the cardioprotective effect of 8-Br-cGMP; the reduction in the percentage of stained cells in hypoxia that was produced by 8-Br-cGMP alone was not affected when this agent was given in combination with the presumed mitoK_{ATP} blocker.

Discussion

The present findings support the hypothesis that hypoxia-induced activation of cardiac sarcolemmal or mitochondrial K_{ATP} channels in goldfish protects muscle cells during metabolic stress, possibly enhancing whole-animal tolerance of environmental hypoxia. The cardioprotective activation of K_{ATP} channels in this species, whether in whole hearts or isolated myocytes, is linked to the production of nitric oxide, which acts through a cGMP-dependent mechanism.

Electrophysiology

In the present study we confirm our previous observation (Cameron et al., 2003) that transmembrane action potential duration is significantly shortened during moderate hypoxia in intact, isolated goldfish heart (Fig. 2). In mammals, cardiac APD shortening in hypoxia is thought to arise through the activation of sarcK_{ATP} channels (Flagg et al., 2004; Shigematsu and Arita, 1997; Suzuki et al., 2002). Patch clamp studies also have revealed a hypoxia-induced increase in the activity of sarcK_{ATP} channels in both mammals (Budas et al., 2004; Liu et al., 2001) and goldfish (Cameron et al., 2003). We now show that in fish, the APD shortening can be eliminated by simultaneous exposure of the tissue glibenclamide (Fig. 3), an antagonist of both sarc- and mitoK_{ATP} channels. The hypoxia-induced reduction in APD was also abolished by L-NAME, an inhibitor of NO synthase, suggesting an upstream role for NO in the activation of sarcK_{ATP} channels (Fig. 3). Further, APD shortening can be similarly induced by the NO donor SNAP, as well as by a stable analog of cGMP (Fig. 4). NO has previously been shown to activate sarcK_{ATP} channels in mammalian cardiac muscle (Chen et al., 2000; Moncada et al., 2000), and this response has been linked to cGMP (Chen et al., 2000; Han et al., 2001). In contrast, the mitoK_{ATP} activator diazoxide had no significant effect on APD.

Cardioprotection in vitro

In an attempt to demonstrate that hypoxia-induced and NO-dependent activation of K_{ATP} channels promotes the survival of ventricular myocytes, we used an *in vitro* model of environmental hypoxia. Similar methods have been used to simulate the effects of ischemia on isolated mammalian cells, and to demonstrate, for example, the cardioprotective activation of mitoK_{ATP} channels by diazoxide (Sato et al., 2000) or volatile anesthetics (Zaugg et al., 2002). In each study, the viability of apparently intact, elongated myocytes was assessed by their capacity to exclude Trypan Blue dye.

The present results indicate that ventricular myocytes from goldfish are damaged after 60 min of *in vitro* hypoxia under mineral oil. As illustrated in Fig. 5, cells exposed to the low-oxygen environment were significantly more often positive for Trypan Blue than those maintained under normoxic conditions. However, the cellular damage caused by hypoxia could be reduced by agents thought to activate K_{ATP} channels. Cells exposed to hypoxia in the presence of SNAP or 8-Br-cGMP, both of which reduced APD in the intact heart, were much more likely to survive and exclude the dye than were cells exposed to hypoxia alone (Figs 6, 7). In contrast, the NOS inhibitor L-NAME, which prevented hypoxia-induced APD shortening, increased the numbers of stained, damaged cells. Perhaps surprisingly, L-NAME also eliminated the cardioprotective influence of SNAP, suggesting that SNAP promotes the endogenous synthesis and release of NO from cardiac muscle cells. Confounding this analysis is the fact that hypoxia itself may increase the NO concentration in cardiac muscle through activation of NOS isoforms (which vary from one species to the next), or through NOS-independent pathways (Schulz et al., 2004). SNAP has been shown to increase the concentration of cGMP in mammalian myocardium (Qin et al., 2004).

The present data strongly suggest that NO, in its capacity to activate sarcK_{ATP} channels in goldfish, promotes survival of cardiac muscle cells during hypoxia. The cardioprotective,

cGMP- and K_{ATP}-dependent influence of NO in hypoxia has been recently reviewed (Kolar and Ostadal, 2004; Schulz et al., 2004). In mammalian myocytes, NOS is upregulated during ischemia (Wang et al., 2002), and a similar enhancement of NOS activity occurs in response to hypoxia in the vasculature of an elasmobranch fish (Renshaw and Dyson, 1999).

SarcK$_{ATP}$ vs *mitoK$_{ATP}$*

While a cardioprotective role for K_{ATP} channel activation has been described by many investigators, there remains some question as to the relative roles of sarcolemmal *vs* mitochondrial channels (Gross and Peart, 2003). In the present study, the cellular damage induced by hypoxia could be reduced by agents thought to specifically activate *either* sarcK_{ATP} or mitoK_{ATP} channels. SNAP and 8-Br-cGMP, which reduce APD through activation of sarcK_{ATP} channels, also promote tolerance of hypoxia in isolated cells (Figs 6, 7). Diazoxide, which is thought to act primarily at mitoK_{ATP}, also decreased the numbers of stained, damaged myocytes (Fig. 7). In the present study, diazoxide did not affect APD (Fig. 4), supporting the view that its effect is primarily at the mitochondrial channel. However, a recent study involving rat ventricular myocytes during metabolic inhibition indicates that this agent can open sarcK_{ATP} channels as well, probably by an indirect mechanism (Rodrigo et al., 2004). Finally, 5-HD, a specific inhibitor of mitoK_{ATP} in mammals, was found to reduce (but not eliminate) the cardioprotective influence of diazoxide, but to have no effect on the beneficial response to 8-Br-cGMP.

Data arising from mammalian studies of hypoxia and ischemic preconditioning also suggest that activation of either sarcK_{ATP} or mitoK_{ATP} channels can be cardioprotective (Sanada et al., 2001; Tanno et al., 2001; Toyoda et al., 2000). Studies of transgenic mice expressing a mutant K_{ATP} channel with reduced ATP-sensitivity suggest that the sarcolemmal channel, at least in mammals, is required for optimal response to ischemia (Rajashree et al., 2002). In pharmacological studies, sarcK_{ATP} activation protects against ischemic injury (Suzuki et al., 2002), and the present data suggest a similar action against hypoxia in fish (Fig. 6). In contrast, Sato et al. used an *in vitro* model of cellular ischemia, very similar to that employed in the present study, to show that activation of mitoK_{ATP} channels, and not sarcK_{ATP} channels, was responsible for ischemic cardioprotection in rabbit myocytes (Sato et al., 2000). Indeed, many recent studies, most involving diazoxide, 5-HD and other agents presumed to be selective for cardiac sarcK_{ATP} *vs* mitoK_{ATP} channels, implicate the latter subtype in protection against ischemia (Murata et al., 2001; Oldenburg et al., 2002; Uchiyama et al., 2003).

There has been considerable speculation as to the specific benefits provided to the myocardium by sarcK_{ATP} or mitoK_{ATP} channel activation (Gross and Fryer, 1999; Oldenburg et al., 2002). Recent evidence suggests that NO/cGMP-dependent cardioprotection involves mitoK_{ATP} channels, not as an end-effector, but as an element in a cascade of events leading to the production of reactive oxygen species (ROS; Lebuffe et al., 2003; Qin et al., 2004; Xu et al., 2004).

One factor that hinders the interpretation of data in studies such as these is that many of the pharmacological agents typically used, including several of those employed in the present study, may protect the heart by means unrelated to ion channel activation. It has been reported that the cardioprotective influence of SNAP, for example, may involve the mitochondria and be unrelated to its capacity to affect sarcK_{ATP} channels (Rakhit et al., 2001). Both diazoxide and 5-HD can have beneficial but channel-independent effects on mitochondrial metabolism (Dzeja et al., 2003; Hanley et al., 2002), leading at least one group to challenge the existence of diazoxide- and 5-HD-sensitive K_{ATP} channels in mammalian mitochondria (Das et al., 2003). In addition, preliminary data from our laboratory suggest that 5-HD eliminates the reduction in APD characteristic of hypoxia, suggesting that this agent is not specific to mitoK_{ATP} channels in fish, or that other channels are affected. The present data cannot exclude the possibility that diazoxide benefits goldfish myocytes by means other than mitoK_{ATP} channel activation. This could underlie our observation that 5-HD reduced, but did not completely eliminate, the cardioprotective effects of diazoxide (Fig. 7).

It is now widely accepted that the NO- and cGMP-dependent activation of sarcK_{ATP} and/or mitoK_{ATP} channels is cardioprotective under conditions of metabolic stress in mammals, and that channel activity underlies the phenomenon of ischemic preconditioning (Gross and Peart, 2003; Kolar and Ostadal, 2004). Aquatic ectotherms may be regularly exposed to acute or chronic environmental hypoxia. Moreover, both sarcK_{ATP} (Cameron et al., 2003; Paajanen and Vornanen, 2002) and mitoK_{ATP} (MacCormack and Driedzic, 2002; MacCormack et al., 2003) channels exist in the hearts of teleost fish, and the former are specifically activated in hypoxia by a NO-dependent mechanism (Cameron et al., 2003). The present findings support the hypothesis that sarcK_{ATP} or mitoK_{ATP} channel activation promotes the survival of ventricular myocytes exposed to conditions of depleted oxygen *in vitro*. A similar channel activation in critical tissues *in vivo* may extend whole-animal tolerance of hypoxia.

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