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A role for nitric oxide in hypoxia-induced activation of cardiac K_{ATP} channels in goldfish (*Carassius auratus*)

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Summary

Hypoxia-induced shortening of cardiac action potential duration (APD) has been attributed in mammalian hearts to the activation of ATP-sensitive potassium (K_{ATP}) channels. Since K_{ATP} channels are also present at high densities in the hearts of vertebrate ectotherms, speculation arises as to their function during periods of reduced environmental oxygen. The purpose of the present study was to determine whether nitric oxide (NO) plays a role in cardiac sarcolemmal K_{ATP} channel activation during hypoxia in a species with a high degree of tolerance to low oxygen environments: the goldfish (*Carassius auratus*). Conventional intracellular and patch-clamp recording techniques were used to record responses from excised ventricles or isolated ventricular myocytes and inside-out patches, respectively, from fish acclimated at 21°C. During moderate, substrate-free hypoxia (6.1±0.2 kPa), ventricular APD was significantly shortened at 50% and 90% of full repolarization, a response that was reversible upon reoxygenation and blocked by the K_{ATP} channel antagonist BDM. Under normoxic conditions, APD was also reduced in the presence of the NO-donor SNAP (100 μmol l^{-1}). In cell-attached membrane patches, sarcolemmal K_{ATP} channel activity was enhanced after 10 min hypoxia, an effect that was reduced or eliminated by simultaneous exposure to BDM, to the guanylate cyclase inhibitor ODQ or to the NO synthase inhibitor L-NAME. In cell-free patches, K_{ATP} channel activity was abolished by 2 mmol l^{-1} ATP but increased by SNAP; the cGMP analog 8-Br-cGMP (200 μmol l^{-1}) also enhanced activity, an effect that was eliminated by BDM. Our data indicate that NO synthesized in cardiac myocytes could enhance sarcolemmal K_{ATP} channel activation during moderate hypoxia in goldfish. This response may serve a cardioprotective role by helping to conserve ATP or by reducing intracellular Ca^{2+} accumulation.

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

Introduction

ATP-sensitive potassium (K_{ATP}) channels were initially described in the mammalian heart by Noma (1983) and have since been shown to play critical regulatory roles in a variety of other tissues (Ashcroft and Ashcroft, 1990). Despite intensive work, however, the functional consequences of sarcolemmal channel activation in cardiac muscle are still unknown (Flagg and Nichols, 2001). K_{ATP} channels are activated when intracellular oxygen levels and ATP/ADP ratios are low, thereby contributing to hypoxia-induced shortening of action potential duration (APD; Nakaya et al., 1991; Shigematsu and Arita, 1997).

Mounting evidence suggests that K_{ATP} channels play a beneficial role in cellular response to hypoxia and ischemia in mammals (Cohen et al., 2000; Grover and Garlid, 2000; O'Rourke, 2000; Yamada et al., 2001). The precise mechanism by which cardiac K_{ATP} channel activation protects the heart is unclear, and the question is complicated by the fact that the relative roles of sarcolemmal *vs* mitochondrial K_{ATP} channels in cardioprotection are still being debated (Gross and Fryer, 1999; Rajashree et al., 2002; Sato et al., 2000). These observations, however, invite speculation as to the role potentially played by these channels in the hearts of aquatic ectotherms, which may be regularly exposed to environmental hypoxia. Sarcolemmal K_{ATP} channels are found at high densities in the hearts of goldfish (Ganim et al., 1998), and those of the closely related crucian carp *Carassius carassius* are activated during chemical anoxia and metabolic inhibition (Paajanen and Vornanen, 2002). In addition, mitochondrial K_{ATP} channels have been shown to influence cardiac performance during anoxia in the yellowtail flounder (*Limanda ferruginea*; MacCormack and Driedzic, 2002). It has been hypothesized that K_{ATP} channel activation under metabolically stressful conditions, whether in the heart or in the brain, could contribute to the enhanced tolerance of environmental hypoxia exhibited by many ectothermic vertebrates (Cameron and Baghdady, 1994; Pek-Scott and Lutz, 1998).

To the extent that K_{ATP} channels are acknowledged to play a cardioprotective role during hypoxia in mammalian myocardium, identification of the signaling pathway by which channel activation occurs has become a priority. Within the past ten years, investigators have begun to appreciate the diversity of roles played by nitric oxide (NO) in a number of physiological processes within the heart (Kelly et al., 1996). NO is thought to exert a cardioprotective effect during ischemia in mammals (Dawn and Bolli, 2002; Rakhit et al., 2001). In addition, NO has been reported to activate K_{ATP} channels in mammalian ventricular myocytes (Moncada et al., 2000; Sasaki et al., 2000), and the protective effects of NO have been linked directly to mitochondrial K_{ATP} activation (Ockaili et al., 1999; Wang et al., 2001).

Among non-mammalian vertebrates, information on the distribution of NO and its importance to cardiovascular function has begun to emerge. Nitric oxide synthase (NOS) has been localized in the hearts of teleost fish, including the goldfish (Bruning et al., 1996). There is evidence that NO acts as a modulator of peripheral circulation in zebrafish (*Danio rerio*; Fritsche et al., 2000), eel (*Anguilla anguilla*; Pellegrino et al., 2002) and most recently in the icefish *Chionodraco* (Pellegrino et al., 2003). NO has been found to play a major role in regulating ventricular performance in the working eel heart, acting through a cGMP-dependent mechanism (Imbrogno et al., 2001). Finally, in relation to hypoxia, microvascular NOS production was found to be enhanced after exposure to low oxygen conditions in the epaulette shark (*Hemiscyllium ocellatum*; Renshaw and Dyson, 1999).

The purpose of this study, then, was to examine the potential role of NO in cardiac K_{ATP} channel activation during hypoxia in a teleost species known to be particularly tolerant of depleted environmental oxygen. Our hypothesis was that the hypoxia-induced release of NO from cardiac myocytes or vascular endothelium activates sarcolemmal K_{ATP} channels in goldfish, a response that may enhance tolerance of hypoxia in this species. Preliminary results of this work have appeared in abstract form (Zia et al., 2001).

Materials and methods

Goldfish (*Carassius auratus* L.) ranging in size from 16 g to 38 g (mean ± S.E.M., 31.1±3.2 g) were obtained from local suppliers. They were acclimated at 21°C in filtered, aerated 132 or 208 liter aquaria on a 12 h:12 h light:dark cycle and were maintained for at least two weeks prior to experiments. 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 measurements using the intact ventricle or for enzymatic dispersion of individual myocytes and single-channel recording.

Intracellular studies

For conventional intracellular recording, hearts were quickly removed, mounted in a tissue bath and superfused with normoxic saline solution at a rate of 15 ml min^{-1}. This solution contained: 150 mmol l^{-1} NaCl, 5 mmol l^{-1} KCl, 1.2 mmol l^{-1} NaH_2PO_4, 1.2 mmol l^{-1} $MgSO_4$, 1.8 mmol l^{-1} $CaCl_2$, 10 mmol l^{-1} K-Hepes and 10 mmol l^{-1} 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); spontaneous intracellular action potentials recorded from the most superficial layer of ventricular muscle were digitized (PowerLab; ADInstruments, Castle Hill, NSW, Australia) and analyzed using appropriate software (Chart, Peak Parameters; ADInstruments). Parameters measured were action potential amplitude, resting membrane potential, slope of action potential upstroke phase and action potential duration at various levels of repolarization. Data were exported to a Microsoft Excel spreadsheet for further analysis (see below).

Oxygen partial pressure in the temperature-controlled tissue bath was monitored with an electrode (DO-051; Cameron Instruments, Port Aransas, TX, USA) connected to an OM-200 oxygen meter. Moderate, substrate-free hypoxia (6.1±0.2 kPa) was induced by switching the superfusion solution 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 were assessed first in the absence of any drug. Isolated hearts were allowed to equilibrate in the bath for 20 min prior to control recordings; experimental recordings were collected after 10 min exposure to hypoxia and again 20 min after a return to normoxic solution.

Drugs

Drugs, including the K_{ATP} channel antagonist 2,3-butanedione monoxime (BDM; 60 μmol l^{-1}) and the nitric oxide (NO) donor *S*-nitroso-*N*-acetylpenicillamine (SNAP; 100 μmol l^{-1}), were purchased from Sigma-RBI (St Louis, MO, USA). BDM has been shown to block surface K_{ATP} channels directly, by mechanisms independent of channel dephosphorylation, and without affecting current amplitude (Smith et al., 1994). Following the initial period of equilibration in normoxic saline, the effects of 5 min exposure to SNAP on ventricular action potential parameters were recorded. In other experiments, BDM was made up in hypoxic solution and applied to the tissue bath; recordings were made after 10 min, as described above. Dosages were chosen from among those recently employed in comparable studies using mammalian or teleost tissues.

Single-channel studies

Isolation of cardiac myocytes

Cell isolation procedures were extensively modified from those of Karttunen and Tirri (1986), as previously described (Ganim et al., 1998). All solutions used in this procedure were kept on ice at pH 7.6 and bubbled with 100% O_2; all reagents were obtained from Sigma.

After removal from the animal, the heart was bathed

in a Ca^{2+}-containing saline buffer: 137 mmol l^{-1} NaCl, 4.6 mmol l^{-1} KCl, 3.5 mmol l^{-1} NaH_2PO_4, 1.2 mmol l^{-1} $MgCl_2$, 11 mmol l^{-1} glucose, 10 mmol l^{-1} K-Hepes and 0.025 mmol l^{-1} $CaCl_2$. 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) and 25 mg bovine serum albumin (Fraction V). The heart was then removed from the perfusion apparatus and carefully torn into small pieces. After 10 min of swirling agitation, the supernatant containing single free-floating cells was removed. Fresh buffer was added, and agitation continued for an additional 10 min. After removal of the second supernatant fraction, the remaining tissue was gently triturated with a Pasteur pipette and agitated for 5 min, leaving a third fraction from which most of the cells used in this study were obtained. This procedure produced a high yield (80–90%) of viable, spindle-shaped myocytes.

Patch clamp

Isolated single myocytes were placed into a 7.5 ml tissue bath (21±1°C) mounted on the stage of an inverted microscope (Nikon) and superfused with normal saline or a pipette solution containing: 140 mmol l^{-1} KCl, 5 mmol l^{-1} K-Hepes and 1 mmol l^{-1} EGTA. Single-channel recording was carried out using the cell-free (inside-out) and cell-attached patch-clamp techniques described by Hamill et al. (1981), as modified in our laboratory for use with teleost myocytes (Ganim et al., 1998). Recording electrodes of 2–4 MΩ resistance were prepared using 1.6 mm-diameter borosilicate glass capillary tubing (Drummond Scientific, Broomall, PA, USA), pulled with a Sutter P-97 micropipette puller, heat-polished on a custom microforge and positioned with a three-dimensional hydraulic micromanipulator (Narishige, Tokyo, Japan). Appropriate suction (10–30 cm water) to form a 1–3 GΩ seal was applied with a 60 ml syringe. A List EPC-7 amplifier was used to monitor channel activity; output currents were low-pass filtered at 1 kHz. The cell-free patches were bathed symmetrically in equimolar potassium solution ($[K^+]_i=[K^+]_o=$ 140 mmol l^{-1}; $E_K=0$). In order to avoid channel rundown, most experiments were conducted within 10–20 min of patch excision.

Data analysis

All single-channel data were stored using a Dagan DAS-900 videocassette recorder. In most cases, data were also digitized on line using an ITC-18 computer interface (Instrutech, Port Washington, NY, USA), then filtered and stored using commercial software (Acquire; Bruxton Corp., Seattle, WA, USA). Subsequent analysis of stored records was accomplished using TAC/TACFit software for Macintosh (Bruxton Corp.); event detection was carried out over the first 10 s of data recorded after the switch from 0 voltage to a new holding potential. The degree of channel activity before and after experimental manipulation was assessed and plotted through determination of the overall number of active channels in the patch, the overall probability that a channel would be found in the open state (P_o; Ganim et al., 1998) and through an analysis of channel closed- and open-lifetime distributions. For this latter determination, a duration histogram was used to display the relative frequency of dwell times between the transition from any one current level within the recording to any other. Most patches contained at least 3–10 active channels, as reflected by multiple discrete levels of current within the patch-clamp record.

Drug protocols

All pharmacological agents were obtained from Sigma-RBI and included ATP (2 mmol l^{-1}), BDM (60 μmol l^{-1}), SNAP (100 μmol l^{-1}), the stable and cell-permeable cGMP analog 8-bromoguanosine 3′,5′-cyclic monophosphate (8-Br-cGMP; 200 μmol l^{-1}), an inhibitor of NO-sensitive guanylate cyclase [1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ); 20 μmol l^{-1}] and the NO synthase inhibitor *N*-nitro-L-arginine methyl ester (L-NAME; 50 μmol l^{-1}).

Drugs were made up in the bath solution and administered either to the intracellular side of the membrane patch in the cell-free configuration or to the extracellular milieu in the case of cells studied using the cell-attached configuration. In most cases, drugs were made up as stock solutions and frozen in aliquots maintained at –70°C until just before use. These were added directly to the tissue bath. Other agents, in particular those made up in hypoxic solution (see below), were contained in separate reservoirs and entered the bath through a multi-barrel rapid infusion system (Warner Instruments, Hamden, CT, USA).

As in the intracellular experiments, ion channel responses to the acute onset of moderate hypoxia were first determined in the absence of any drug. After a brief period of equilibration under normoxic conditions (>20 kPa), hypoxic solution (6.0±0.3 kPa; N_2-gassed and pH=7.6, as above) was rapidly infused into the tissue bath, and K_{ATP} channel activity over a range of depolarizing and hyperpolarizing potentials was assessed after 10 min, 20 min and 30 min using the cell-attached patch configuration. After each experimental challenge, the myocytes were again exposed to normoxic solution to test for current run-down, myocyte damage and reproducibility of results.

To determine their effects on the K_{ATP} channel response to hypoxia in intact cells, BDM, ODQ and L-NAME were made up in the N_2-gassed hypoxic solution and were administered to the tissue as described for hypoxic solution alone. In other experiments, cells were exposed to each of these drugs under normoxic conditions. Channel activity in response to a range of holding potentials was recorded at 10 min, 20 min and 30 min after each experimental manipulation.

To help characterize the K_{ATP} channels under study and to determine their response to agents thought to influence nitric oxide pathways, we also recorded channel response to ATP, SNAP, 8-Br-cGMP and 8-Br-cGMP + BDM in cell-free,

inside-out patches. Thus, these agents were applied directly to the intracellular surface of the patch. In each case, the initial 15 min stabilization period was followed by administration of the drug directly to the tissue bath; channel responses were recorded within 3 min and again at 10 min after drug exposure.

Statistical analysis

Data obtained from all experiments were transferred to Microsoft Excel spreadsheets; paired *t*-tests and analysis of variance for repeated measures (ANOVA with *post-hoc* Fisher PLSD and Scheffé 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 studies

Effects of hypoxia

Intracellular recording techniques were used to determine the effects of moderate hypoxia (6.1±0.2 kPa) on the configuration of transmembrane cardiac action potentials in goldfish. In all preparations ($N=8$), low oxygen decreased cardiac action potential duration (APD). After 10 min of hypoxia, there were significant ($P<0.05$) reductions in APD at 50% and 90% of full repolarization (APD_{50} and APD_{90}, respectively; Fig. 1). Mean APD_{90} under normoxic conditions was 642±14 ms; after exposure to hypoxia, this value fell to 579±17 ms, a decrease of 9.8%. APD_{50} declined by 13.6%. There were no significant effects of hypoxia on any other parameter, including resting membrane potential, action potential amplitude or slope of action potential upstroke.

The effect of hypoxia on APD was reversible upon the reintroduction of normoxic solution (20 kPa) to the tissue bath (Fig. 1, washout). Values for APD were collected 20 min after washout of the hypoxic solution; oxygen levels had returned to pre-experimental levels within 3 min.

To determine whether the hypoxia-induced reduction in myocardial APD was due to the activation of K_{ATP} channels, isolated hearts were exposed to hypoxia in the presence of the K_{ATP} antagonist 2,3-butanedione monoxime (BDM). At the concentration tested (60 μmol l^{-1}), this agent prevented hypoxia-related shortening of APD (Fig. 2; $N=6$).

Effects of NO

To indirectly assess the potential for NO to activate K_{ATP} channels in goldfish myocardium, the effects of the NO donor SNAP on action potential parameters were monitored in isolated hearts. At a concentration of 100 μmol l^{-1}, SNAP significantly reduced both APD_{50} (by 26.9%) and APD_{90} (by 12.1%) (Fig. 3; $N=8$). There were no significant effects of SNAP on any other action potential parameter.

Single-channel studies

Characterization of goldfish cardiac K_{ATP} channels

Sarcolemmal K_{ATP} channel activity was first observed in isolated myocytes using single-channel recording techniques in the cell-free (inside-out) configuration (Hamill et al., 1981). The channels showed some inward rectification, and the current/voltage (*I*/*V*) relationship reflected a linear slope conductance of 61 pS, a value similar to that previously reported by us for goldfish (Ganim et al., 1998) and by others for K_{ATP} channels in mammalian heart (Noma, 1983; Kakei et al., 1985). Channel activity was entirely abolished by the application of 2 mmol l^{-1} ATP.

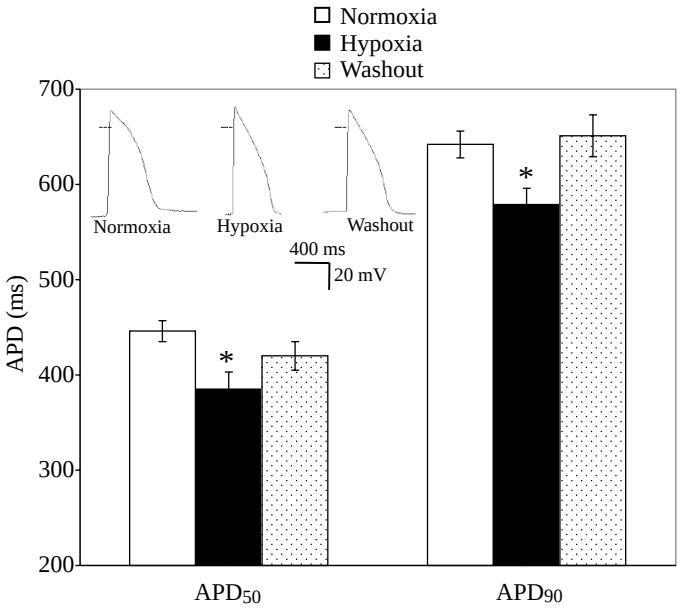

Fig. 1. Effects of hypoxia on action potential duration at 50% (APD_{50}) and 90% (APD_{90}) of full repolarization ($N=8$). Action potentials recorded in isolated ventricles were significantly shortened after 10 min hypoxia (6.1±0.2 kPa), an effect that was reversible upon a return to normoxic solution (washout). Inset shows representative action potential configuration in one experiment. Values are means ± 1 S.E.M. *****, significantly different from values recorded under normoxic conditions ($P<0.05$).

Cell-attached patch-clamp experiments

Effects of hypoxia. Cardiac K_{ATP} channel activity was also monitored using the cell-attached, patch-clamp configuration (Hamill et al., 1981). Following an initial equilibration period, intact, individual myocytes were exposed in the tissue bath to moderately hypoxic solution (6.0±0.3 kPa), and channel activity was recorded at 10 min intervals over the following 30 min. In response to hypoxia, a slowly developing increase in K_{ATP} channel activity was observed in seven of eight preparations. Specific hypoxia-induced changes included an increase in the frequency of K_{ATP} current transitions within 10 s of the application of depolarizing potentials (Fig. 4), an increase in the number of active channels in the patch (Fig. 5, inset), as well as elevated open-state probability (P_o; Fig. 5, normoxia *vs* hypoxia). Current amplitude and conductance were not affected. The overall increase in channel activity was maximal after 20 min exposure to hypoxia and did not increase

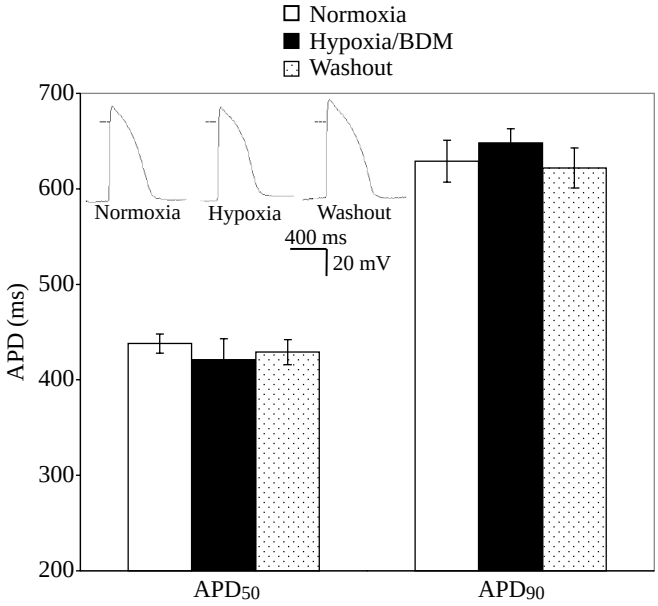

Fig. 2. Effects of hypoxia with simultaneous application of the K_{ATP} channel antagonist BDM (60 μmol l^{-1}) on cardiac muscle action potential duration (APD). No significant change in APD_{50} or APD_{90} was recorded when BDM was present during hypoxia (N=6). Values are means ± 1 S.E.M.

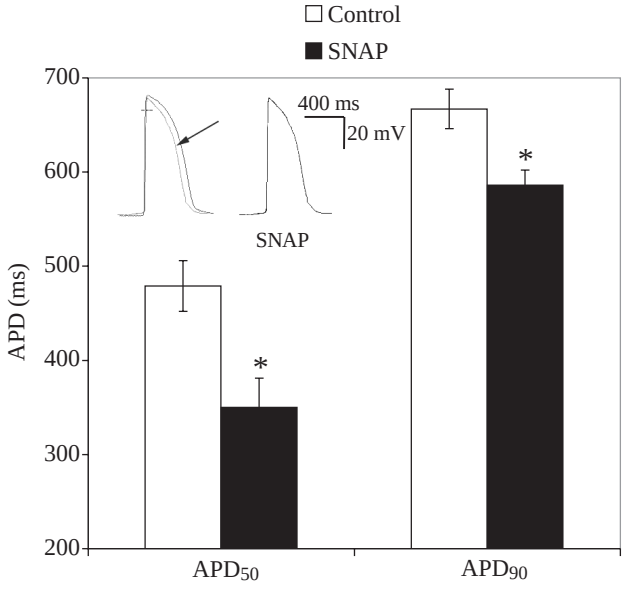

Fig. 3. Effect of SNAP (100 μmol l^{-1}), an NO donor, on cardiac muscle action potential duration (APD) in goldfish ventricle (N=8). Under normoxic conditions, APD_{50} and APD_{90} were significantly shortened upon 3 min exposure to SNAP. Inset shows a control action potential (left) and one recorded in the presence of SNAP (right, and superimposed at arrow). Values are means ± 1 S.E.M. *****, significantly different from control (P<0.05).

significantly thereafter. The effect of hypoxia on the channels was reversible, in that K_{ATP} activity declined again upon return to the normoxic solution (Fig. 5, washout).

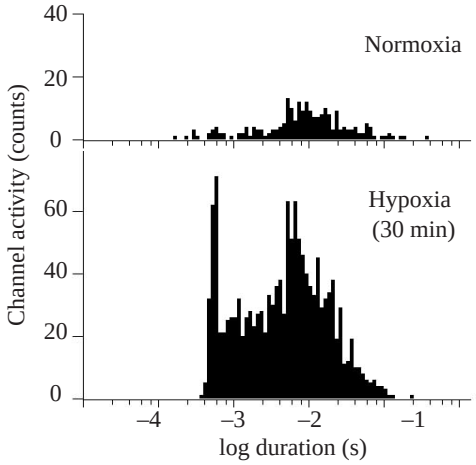

Fig. 4. Cardiac sarcolemmal K_{ATP} channel activity was increased during hypoxia (6.0±0.3 kPa) in cell-attached patches on isolated goldfish ventricular myocytes. In a representative experiment, duration histograms display the relative frequency of dwell times (s) between transitions from any one current level to another in a patch containing multiple K_{ATP} channels. While channel closed- and open-lifetime distribution was unchanged, overall activity was enhanced. Recordings were obtained within the first 10 s after application of a depolarizing holding potential (+80 mV).

Effects of BDM, ODQ and L-NAME on hypoxia-induced changes. To determine whether the hypoxia-induced enhancement of channel activity could be inhibited by the K_{ATP} channel antagonist BDM, this agent was made up in the N_2-gassed solution and administered simultaneously with hypoxic solution (N=4). At the concentration tested (60 μmol l^{-1}), BDM prevented any significant increase in channel activity during hypoxia. Channel activity at 10 min, 20 min and 30 min, as determined by the number of active channels per patch, by overall P_o (Fig. 5) and by channel closed- and open-lifetime distribution, was not significantly changed.

To further determine whether hypoxia-induced changes in K_{ATP} channel activity were mediated by a cGMP-dependent mechanism, the guanylate cyclase inhibitor ODQ was administered simultaneously with the onset of hypoxia (N=6). ODQ also prevented the significant increase in K_{ATP} activity observed with hypoxia alone (Fig. 5).

To clarify the role of NO in the hypoxia-induced elevation of K_{ATP} channel activity, the NO synthase inhibitor L-NAME was also administered simultaneously with the low oxygen solution (50 μmol l^{-1}; N=4). After 20 min, channel activity was significantly elevated relative to normoxia; however, the extent of the increase was much less than that noted with hypoxia alone (Fig. 5).

To assess the influence of these agents when given alone, additional patches were treated with either BDM, ODQ or L-NAME under normoxic conditions. In four experiments each, exposure to BDM induced a consistent but not significant decline in K_{ATP} channel activity. At the concentrations tested, neither ODQ nor L-NAME induced any significant change in K_{ATP} channel P_o (Fig. 5).

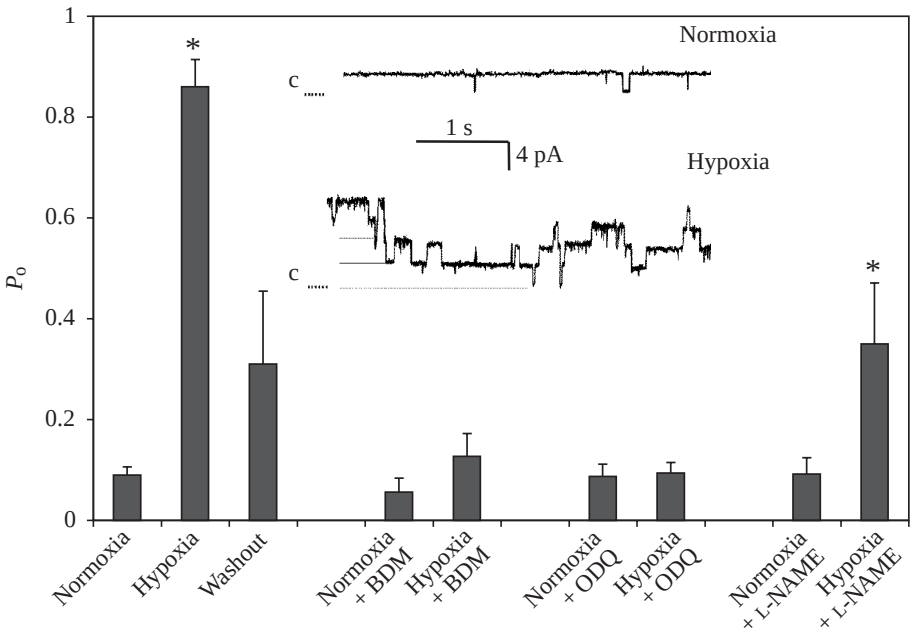

Fig. 5. Effects of 30 min hypoxia alone and in combination with other agents on the activity of sarcolemmal K_{ATP} channels in cell-attached patches. Channel open state probability (P_o) was significantly increased in response to hypoxia, a response that was maximal after 20 min (N=8; mean ± 1 S.E.M.) and reversible upon the return of normoxic solution (washout). The inset shows a representative recording of K_{ATP} channel activity from a single experiment; after only 10 min hypoxia, the number of active channels/patch was substantially increased. Channel openings are shown as upward deflections in response to depolarization by 80 mV; c, all channels closed. Simultaneous application of hypoxia and BDM (N=4), or hypoxia and an inhibitor of NO-sensitive guanylate cyclase (ODQ; 20 μmol l^{-1}; N=6), eliminated the hypoxia-induced increase in channel activity. Hypoxia plus an inhibitor of NO synthase (L-NAME; 50 μmol l^{-1}; N=4) reduced the magnitude of the increase in P_o seen with 20 min hypoxia alone. *****, significantly different from normoxia (P<0.05).

Cell-free patch-clamp experiments

Effects of NO. The inside-out patch clamp configuration was employed to determine the effects of the NO donor SNAP on sarcolemmal K_{ATP} channel activity. When applied directly to the intracellular surface of the membrane, SNAP significantly increased K_{ATP} channel activity, as measured by P_o (Fig. 6, left) and by the number of open channels per patch (N=6). The NO-induced augmentation of channel activity was usually observed within the first minute of exposure to SNAP and was maximal after 3 min.

Effects of 8-Br-cGMP. In four experiments, the effects on K_{ATP} channel activity of the stable cGMP analog 8-Br-cGMP were monitored. Under normoxic conditions, this agent induced a significant increase in overall P_o in all preparations (Fig. 6, right). When 8-Br-cGMP and BDM were applied simultaneously, however, there was no significant change in channel activity, suggesting that BDM blocks K_{ATP} activation by cGMP (Fig. 6; N=4).

Discussion

Data arising from this study indicate that sarcolemmal ATP-sensitive K^+ channels are present in the cardiac muscle of goldfish and that these channels may be activated under conditions of moderate environmental hypoxia. The present results further support the hypothesis that hypoxia-induced channel activation occurs as a result of the synthesis of nitric oxide within cardiac myocytes, with NO acting through a cGMP-dependent mechanism. Channel activation under conditions of metabolic stress may serve a cardioprotective function in this hypoxia-tolerant species.

K_{ATP} channels and hypoxia

Previous work in our laboratory has established that sarcolemmal K_{ATP} channels show reduced ATP sensitivity in animals acclimated at low temperatures, such that overall channel activity is enhanced (Ganim et al., 1998). In the present study, we observed a significant shortening of action potential duration during hypoxia in the isolated goldfish ventricle (Fig. 1), a response that was inhibited by the K_{ATP} channel antagonist BDM (Fig. 2). Hypoxia-induced APD shortening in cardiac muscle is well established in the mammalian heart and has been repeatedly ascribed to the activation of sarcolemmal K_{ATP} channels (Nakaya et al., 1991; Nichols and Cukras, 2001; Shigematsu and Arita, 1997). Moreover, we showed that single K_{ATP} channels are activated during hypoxia (Figs 4, 5), and that this response is also sensitive to inhibition by BDM (Fig. 5). A similar response by sarcolemmal K_{ATP} channels in trout heart might provide a mechanism for the protective effects of anoxic myocardial preconditioning reported for that species (Gamperl et al., 2001), a phenomenon widely attributed in mammals to K_{ATP} channel activation (Grover and Garlid, 2000; O'Rourke, 2000). Mitochondrial K_{ATP} channels in the heart may promote hypoxia tolerance in the yellowtail flounder (*Limanda*) by stabilizing intracellular Ca^{2+} concentration (MacCormack and Driedzic, 2002).

Activation of cardiac K_{ATP} channels during moderate hypoxia has not previously been demonstrated in ectotherms using single-channel recording techniques. During complete metabolic inhibition induced by sodium dithionate and/or iodoacetic acid, K_{ATP} channel activity has been induced in cardiac myocytes of the closely related crucian carp (*C. carassius*), even in the presence of 4 mmol l^{-1} ATP (Paajanen

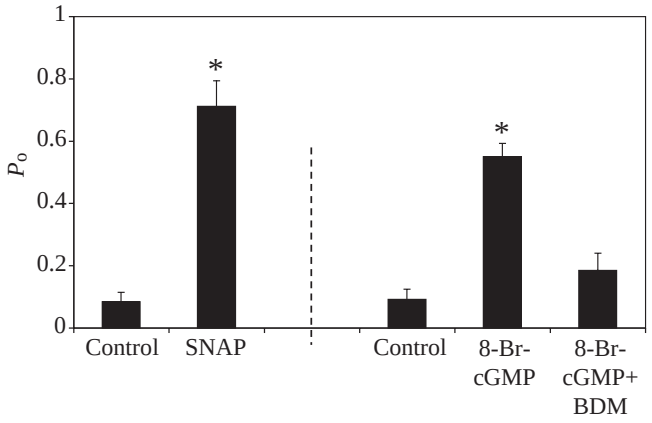

Fig. 6. The cell-free, inside-out patch clamp recording configuration was used to assess the effects on K_{ATP} channel open-state probability (P_o) of the NO donor SNAP (100 μmol l^{-1}; left), the stable cGMP analog 8-Br-cGMP (200 μmol l^{-1}) and 8-Br-cGMP in combination with the K_{ATP} antagonist BDM (60 μmol l^{-1}; right), all under normoxic conditions. Three min exposure to SNAP at the intracellular face of the membrane significantly increased P_o (N=6). The cGMP analog also raised P_o (N=4), an effect that was substantially eliminated by simultaneous administration of the K_{ATP} antagonist (N=4). Values are means ± 1 S.E.M. *****, significantly different from control (P<0.05).

and Vornanen, 2002). In this species, however, severe chemical anoxia using oxygen scavengers or cyanide (Vornanen and Tuomennoro, 1999) first induces a large inward current that prolongs APD and overwhelms the current through the K_{ATP} channel, reducing its cardioprotective influence (Paajanen and Vornanen, 2002). In mammals, the cell-attached patch-clamp configuration and a hypoxia regime similar to that employed in the present study have been used to demonstrate a potentially cardioprotective K_{ATP} current in guinea pig myocytes (Liu et al., 2001).

While myocardial ischemia may be a prominent component of the pathophysiology of heart disease in humans, ATP/ADP ratios would not ordinarily be expected to vary significantly in the healthy mammalian heart. Aquatic ectotherms, however, may regularly be exposed to acute or chronic depletion of environmental oxygen, and many are remarkably tolerant of complete anoxia. Hochachka (1986) and Hochachka et al. (1996) have proposed two principal strategies for extending tolerance of hypoxia in such species. One mechanism involves the matching of ATP requirements to reduced rates of ATP synthesis by simply arresting metabolism. Alternatively, an organism might remain active, compensating for reduced ATP-dependent ion pumping by increasing the energetic efficiency of metabolic processes. One way to manage this would be to decrease membrane permeability to Ca^{2+}; thus, despite decreased ATP turnover rates, ionic and electrical gradients across the cell membrane would not be lost and intracellular Ca^{2+} accumulation could be avoided. An adaptive strategy that increases tissue ATP levels or blocks uncontrolled Ca^{2+} influx might lessen the effects of 'leaky' membranes and promote hypoxia tolerance. Specific measures that could be expected to diminish Ca^{2+} entry would include the inhibition of sarcolemmal Ca^{2+} channels (Wasser, 1996) and the activation of outward potassium currents, hyperpolarizing the cell and blocking Ca^{2+} influx through voltage-gated channels (Nilsson, 2001; Pek-Scott and Lutz, 1998). It is possible that K_{ATP} channel activation in critical tissues such as heart or brain could, as one component in a suite of protective mechanisms (Hochachka and Lutz, 2001), extend tolerance of hypoxia in those species most capable of survival under such conditions (Cameron and Baghdady, 1994; Lutz and Prentice, 2002).

Nitric oxide

The role of endogenous NO generation in the regulation of myocardial energetics in mammals has been studied extensively in recent years (Kelly et al., 1996). Each of the three principal isoforms of nitric oxide synthase (NOS) has been localized within the heart and within individual cardiac myocytes (Hare and Stamler, 1999). Indeed, NOS has been shown to be upregulated in cardiac myocytes during ischemia, serving a cardioprotective function (Wang et al., 2002). Subcellular localization of two isoforms in particular, NOS1 (nNOS) and NOS3 (eNOS), allows for varied effects of NO on cardiac ion channels in different parts of the cell and even opposing effects on the heart with regard to increased *vs* decreased contractility (Barouch et al., 2002).

Many recent studies suggest a cardioprotective role for NO during ischemia in the mammalian heart (Dawn and Bolli, 2002; Nandagopal et al., 2001; Rakhit and Marber, 2001). A variety of mechanisms by which NO release could protect the heart during ischemia has been proposed; one possibility is that NO binds to a heme moiety at the active site on soluble guanylate cyclase, leading to an increase in the intracellular concentration of the second messenger cGMP. This molecule could in turn influence the activity of specific Ca^{2+} or K^+ channels, reducing myocardial contractility, oxygen consumption and energy demand (Rakhit and Marber, 2001). K_{ATP} channels have been proposed as a downstream effector in this NO-dependent cardioprotective pathway (Dawn and Bolli, 2002).

Relevant to the present study, NO has been reported to activate K_{ATP} channels in mammalian vascular smooth muscle (Miyoshi et al., 1994; Murphy and Brayden, 1995), as well as sarcolemmal (Moncada et al., 2000) and mitochondrial (Sasaki et al., 2000) channels in ventricular myocytes. The latter investigators used whole-cell recording techniques to show that SNAP activated mitochondrial but not sarcolemmal K_{ATP} channels in rabbit ventricular myocytes (Sasaki et al., 2000). This is in contrast to the present observations in goldfish (Figs 3, 6) and to those of others using a different NO donor and guinea pig myocytes (Moncada et al., 2000) or endogenous NO and intact hearts (Chen et al., 2000).

The cardioprotective effects of NO have been associated with the acute or chronic activation of mitochondrial K_{ATP} channels in mammals (Eells et al., 2000; Ockaili et al., 1999; Wang et al., 2001). There is evidence, however, that some

forms of SNAP-induced cardioprotection in mammals may indeed involve the mitochondria, yet remain entirely independent of K_{ATP} channel activation (Rakhit et al., 2000, 2001). Mitochondrial K_{ATP} channels have been shown to exist in the fish heart (MacCormack and Driedzic, 2002), and NO in turn has been found to play a prominent role in regulating peripheral vascular resistance and heart function in a variety of teleosts (Fritsche et al., 2000; Imbrogno et al., 2001; Pellegrino et al., 2002, 2003). Vascular NOS production is enhanced during hypoxia in elasmobranchs (Renshaw and Dyson, 1999), just as it is in mammals (Wang et al., 2002). The present results with the NOS inhibitor L-NAME (Fig. 5) indicate that a hypoxia-induced increase in NO production in goldfish heart could activate sarcolemmal K_{ATP} channels (Figs 3, 6), potentially enhancing tolerance of oxygen stress. The data also show that if this is the operative mechanism for K_{ATP} channel activation, NOS must be localized within individual myocytes, as endothelial cells were not present during patch-clamp experiments. Even more NOS would be available in the intact heart, in light of the fact that the endocardial endothelium in fish has a much greater surface area relative to the volume of the ventricle than does the equivalent tissue in mammals (Imbrogno et al., 2001).

In mammals, the hypoxia-induced activation of sarcolemmal K_{ATP} channels is thought to involve NO and to be a cGMP-dependent mechanism (Chen et al., 2000; Han et al., 2001). Prior experiments with teleost fish involving the stable and lipid soluble cGMP analog 8-Br-cGMP and the guanylate cyclase inhibitor ODQ indicate that an NO–cGMP-dependent pathway is also operative in the vasculature and the heart of the eel *Anguilla anguilla* (Imbrogno et al., 2001; Pellegrino et al., 2002). In the present study, Fig. 5 indicates that the hypoxia-induced activation of sarcolemmal K_{ATP} channels in goldfish can be blocked by ODQ. In addition, Fig. 6 illustrates that the effect of SNAP on these channels can be mimicked by 8-Br-cGMP and that this response is prevented by the K_{ATP} channel antagonist BDM. These observations are in accord with those in mammals, where 8-Br-cGMP has been shown to mirror the effects of NO on sarcolemmal K_{ATP} channels, decreasing APD by a mechanism that could be blocked by a K_{ATP} antagonist (Chen et al., 2000). On the other hand, 8-Br-cGMP appears to have little effect on mitochondrial K_{ATP} channels (Sasaki et al., 2000).

Tolerance of environmental hypoxia has evolved independently in a number of different teleost lineages (Hochachka and Lutz, 2001). Few species have been thoroughly investigated in this regard, and some observations suggest that there are broad interspecific differences in terms of the precise suites of defense mechanisms employed among various taxa. The evidence increasingly supports the possibility, however, that certain mechanisms arose early in the vertebrate line and serve to extend the hypoxia tolerance of specific tissues in a variety of living species. While a full evolutionary analysis of hypoxia adaptations in fishes must await further investigation, the present data support the hypothesis that K_{ATP} channels in goldfish play a cardioprotective role in hypoxia, just as they do in mammals (Grover and Garlid, 2000). These results further suggest that a hypoxia-induced enhancement of NO production in the heart of this species opens sarcolemmal K_{ATP} channels *via* a cGMP-dependent mechanism.

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