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Abstract

Reactive oxygen species (ROS) are produced as by-products of oxidative metabolism and occur in the heart during ischemia and coronary artery reperfusion. The effects of ROS on the electrophysiological properties of intracardiac neurons were investigated in the intracardiac ganglion (ICG) plexus in situ and in dissociated neurons from neonatal and adult rat hearts using the whole-cell patch clamp recording configuration. Bath application of ROS donors, hydrogen peroxide (H_2O_2) and tert-butyl hydroperoxide (t-BHP) hyperpolarized, and increased the action potential duration of both neonatal and adult ICG neurons. This action was also recorded in ICG neurons in an adult in situ ganglion preparation. H_2O_2 and t-BHP also inhibited voltage-gated calcium channel (VGCC) currents and shifted the current-voltage (I-V) relationship to more hyperpolarized potentials. In contrast, H_2O_2 increased the amplitude of the delayed rectifier K+ current in neonatal ICG neurons. In neonatal ICG neurons, bath application of either superoxide dismutase (SOD) or catalase, scavengers of ROS, prior to H_2O_2 attenuated the hyperpolarizing shift but not the inhibition of VGCC by H_2O_2. In contrast, in adult ICG neurons, application of SOD alone had no effect upon either VGCC current amplitude or the I-V relationship, whereas application of SOD prior to H_2O_2 exposure abolished both the H_2O_2-mediated hyperpolarizing shift and inhibition. These data indicate that ROS alter depolarization-activated Ca2+ and K+ conductances which underlie neuronal excitability of ICG neurons. This affects action potential duration and therefore probably modifies autonomic control of the heart during ischemia/reperfusion.

Disciplines
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Reactive oxygen species modulate neuronal excitability in rat intrinsic cardiac ganglia

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ABSTRACT

Reactive oxygen species (ROS) are produced as by-products of oxidative metabolism and occur in the heart during ischemia and coronary artery reperfusion. The effects of ROS on the electrophysiological properties of intracardiac neurons were investigated in the intracardiac ganglion (ICG) plexus *in situ* and in dissociated neurons from neonatal and adult rat hearts using the whole-cell patch clamp recording configuration. Bath application of ROS donors, hydrogen peroxide (H_2O_2) and *tert*-butyl hydroperoxide (*t*-BHP) hyperpolarized, and increased the action potential duration of both neonatal and adult ICG neurons. This action was also recorded in ICG neurons in an adult *in situ* ganglion preparation. H_2O_2 and *t*-BHP also inhibited voltage-gated calcium channel (VGCC) currents and shifted the current–voltage (*I–V*) relationship to more hyperpolarized potentials. In contrast, H_2O_2 increased the amplitude of the delayed rectifier K^+ current in neonatal ICG neurons. In neonatal ICG neurons, bath application of either superoxide dismutase (SOD) or catalase, scavengers of ROS, prior to H_2O_2 attenuated the hyperpolarizing shift but not the inhibition of VGCC by H_2O_2. In contrast, in adult ICG neurons, application of SOD alone had no effect upon either VGCC current amplitude or the *I–V* relationship, whereas application of SOD prior to H_2O_2 exposure abolished both the H_2O_2-mediated hyperpolarizing shift and inhibition. These data indicate that ROS alter depolarization-activated Ca^{2+} and K^+ conductances which underlie neuronal excitability of ICG neurons. This affects action potential duration and therefore probably modifies autonomic control of the heart during ischemia/reperfusion.

1. Introduction

Mammalian intrinsic cardiac ganglia (ICG) are composed of clusters of neurons located throughout the atrial epicardium and interatrial septum, which receive both parasympathetic (vagal) and sympathetic input (Adams and Cuevas, 2004; Parsons, 2004). Given that electrical or chemical activation of mammalian intrinsic cardiac neurons are able to modify cardiac function (Butler et al., 1990a,b), it has been proposed that the intrinsic cardiac nervous system acts as the final common pathway for the control of regional cardiac function (Horackova and Armour, 1995; Arora et al., 2000).

The location of the mammalian ICG make them susceptible to the effects of myocardial ischemia and reperfusion associated with coronary heart disease (Horackova and Armour, 1995; Armour, 1999). Each ganglionated plexus in the human heart is perfused by two or more arterial branches arising from different major coronary arteries, and the activity of ICG neurons is modified by transient coronary occlusion

(Huang et al., 1993). Coronary occlusion results in lack of oxygen and metabolic substrates, as well as the accumulation of local ischemic products such as oxygen-derived free radicals. The resulting autonomic imbalance may contribute to the development of arrhythmias during acute myocardial ischemia. Disordered function of autonomic efferent neurons can also result in coronary vascular dysfunction, which in turn can lead to loss of cardiomyocyte contractile function and heart failure (Armour, 1999).

Oxygen-derived oxidants, $O_2^-\bullet$, H_2O_2 and $\bullet OH^-$, are produced as by-products of oxidative metabolism (for review see Kourie, 1998; Dröge, 2001). Reactive oxygen species (ROS) are produced extracellularly and intracellularly in the heart during ischemia and during coronary artery reperfusion and have been implicated in cardiac dysfunction (Huang et al., 1993; O'Neill et al., 1996; Armour, 1999). The proximity of ICG to the coronary blood supply make them susceptible to the effects of ROS. Studies carried out on canine cardiac ganglia *in situ* have revealed that H_2O_2 administered to the local blood supply modifies neuronal firing activity (Thompson et al., 1998). ROS produced by the myocardium during ischemia-reperfusion have been shown to alter the firing properties of cardiac sensory neurites associated with afferent axons in vagal and sympathetic nerves (Ustinova and Schultz, 1994; Huang et al., 1995). However, the effects of ROS on membrane currents and neuronal excitability on individual neurons in mammalian ICG have not been investigated either in intact ganglia or after dissociation.

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The major hypothesis of this study is that ROS, generated during myocardial ischemia and post-ischemic reperfusion, influence the electrical activity of ICG neurons via the modulation of voltage-dependent Ca^{2+} and K^+ conductances. Given the proposed importance of the intrinsic cardiac nervous system to the control of regional cardiac function, the response of ICG neurons to ROS donors may shed light on the pathological changes affecting the ability of these cells to stabilize cardiac function. Preliminary reports of some of these results have been presented in abstract form (Whyte et al., 2003; Dyavanapalli et al., 2008).

2. Materials and methods

2.1. Electrophysiological recordings from intracardiac ganglion preparations in situ

Young adult female Wistar rats (150–220 g) were killed by stun and cervical dislocation, in accordance with current UK Home Office guidelines. The hearts were quickly excised and atria isolated and placed in cold Krebs solution. The right atrial ganglion plexus and underlying myocardium were isolated from the dorsal surface of the atria. The preparation was pinned to the Sylgard (Dow-Corning, Midland, MI) covered base of a 35 mm tissue culture dish and superfused with a bicarbonate-buffered Krebs solution comprising (in mM): NaCl 118.4, NaHCO$_3$ 25.0, NaH$_2$PO$_4$ 1.13, CaCl$_2$ 1.8, KCl 4.7, MgCl$_2$ 1.3 and glucose 11.1, gassed with Carbogen (95% O$_2$:5% CO$_2$) to pH 7.4 (Rimmer and Harper, 2006). The recording chamber was continuously superfused (~2 ml/min) with Krebs solution at 35 °C. The temperature of the solution was controlled by Peltier thermoelectric elements (Medical systems PDMI-2 micro incubator, NY, USA) and monitored by an independent thermistor probe in the recording chamber (Yellow Springs Instruments, Yellow Springs, OH). Intracellular recordings were made using sharp glass microelectrodes (GC120F; Harvard Apparatus Ltd., Edenbridge, UK) with ~120 MΩ resistance when filled with 0.5 M KCl. Membrane voltage responses were recorded with a conventional bridge amplifier (Axoclamp 2A; Molecular Devices, Sunnyvale, CA). Signals were filtered at 20 kHz, digitized at 50 kHz and transferred to a Pentium 4 computer using an analogue-to-digital converter (Micro 1401 interface, CED, Cambridge, UK) and Spike 2 software (CED). Electrophysiological properties and responses of the neurons were analyzed using the same program. Pharmacological antagonists were bath applied at the concentrations indicated.

Brief intracellular depolarizing currents (≤3 ms in duration) were used to directly evoke single somatic action potentials. Action potential parameters measured were: the overshoot, rate of rise (d$rise$/dt), rate of fall (d$fall$/dt) and the afterhyperpolarization (AHP) following the action potential, characterized by its amplitude and decay time. Long (500 ms) hyperpolarizing and depolarizing pulses were applied to measure time constant, and evoked discharge characteristics. Time constant (τ) was measured at small hyperpolarizing current pulses (≤−0.1 nA) using Spike 2 software. Membrane resistance (R_m) was calculated from the time constant, $\tau = R_m \cdot C_m$, where C_m is the specific membrane capacitance, assumed to be 1 µF cm^{-2}.

2.2. Dissociated intracardiac ganglion neurons

Neurons from neonatal (3–10 day old) and adult (>6 weeks old) rat ICG were isolated and placed in tissue culture. The majority of neurons (>60%) in the rat intracardiac nerve plexus are unipolar with either none or few neurites (Pauza et al., 1997). The procedures for isolation of the ICG neurons have been described previously (Xu and Adams, 1992; Hogg et al., 2001). The protocols of our experiments were approved by the University of Queensland Animal Experimentation Committee, an independent review committee, and the investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). Briefly, Wistar rats were sacrificed by stunning and cervical dislocation

and the heart excised and placed in Hank's balanced salt solution (HBSS). Atria were isolated and the atrial ganglion plexus located around the pulmonary veins and superior vena cava on the dorsal surface of the atria (Batulevičius et al., 2003) were dissected and transferred to HBSS containing 1.2 mg/ml collagenase (Type 2, 300 U/ml; Worthington-Biochemical, Freehold, NJ). Following 1 h digestion, the ganglia were rinsed twice in HBSS and triturated in culture medium (Dulbecco's modified Eagle medium containing 10% (v/v) fetal calf serum and 1% (v/v) penicillin–streptomycin) (Gibco — Invitrogen, Melbourne, Australia) using a fine-bore Pasteur pipette. The dissociated neurons were plated on laminin-coated coverslips and incubated at 37 °C in a 95% O$_2$:5% CO$_2$ atmosphere for 12–24 h.

2.3. Electrophysiological recording

Membrane voltage and current were recorded in a whole-cell (dialyzed or perforated) patch-clamp configuration using an Axopatch 200A patch-clamp amplifier (Molecular Devices, Sunnyvale, CA). Voltage and current protocols were applied using pClamp software (Molecular Devices). Signals were filtered at 5 kHz, digitized at 20 kHz and transferred to a Pentium PC using a Digidata 1200 A/D interface (Molecular Devices).

In voltage-clamp (dialyzed whole-cell) experiments to record depolarization-activated calcium channel currents, Ba^{2+} was used as the charge carrier and the extracellular solution contained (in mM): 143 TEACl, 5 BaCl$_2$, 1 MgCl$_2$, 10 glucose, and 10 HEPES, adjusted to pH 7.4 with TEAOH. Pipettes were filled with solution containing (in mM): 135 CsCl, 1 MgCl$_2$, 14 triphosphocreatinine, 3.6 MgATP, 50 µl/ml creatinine phosphokinase and 10 HEPES, adjusted to pH 7.2 with CsOH. The osmolarity of the extra- and intracellular solutions was monitored by a vapor pressure osmometer (Wescor 5500, Logan, UT) and were in the range 295–310 mOsm/kg.

In experiments to record outward K$^+$ currents (voltage clamp) and action potential firing (current clamp), cells were recorded using the perforated-patch recording configuration. Cells were superfused with extracellular solution containing (in mM): 140 NaCl, 3 KCl, 2.5 CaCl$_2$, 1.2 MgCl$_2$, 7.7 glucose and 10 HEPES-NaOH, pH 7.2. Action potential duration (APD) at 0 mV was measured in the absence and presence of ROS donors bath applied at maximally effective concentrations. The concentrations tested (100–1000 µM) were similar to those shown previously to affect the excitability of canine intrinsic cardiac neurons *in situ* (Thompson et al., 1998). Total outward K$^+$ currents were recorded in the presence of 300 nM tetrodotoxin (TTX) to inhibit depolarization-activated Na$^+$ currents, and delayed rectifier K$^+$ currents were isolated by the addition of 100 µM CdCl$_2$ to the extracellular solution to inhibit voltage-dependent Ca^{2+} currents and Ca^{2+}-activated K$^+$ currents (Xu and Adams, 1992). Pipettes were filled with solution containing (in mM): 75 K$_2$SO$_4$, 55 KCl, 5 MgSO$_4$ and 10 HEPES-KOH, adjusted to pH 7.2 with N-methyl-d-glucamine. Amphotericin-B in dimethylsulfoxide was added at a final concentration of 240 µg/ml. The temperature of the superfusing solutions was controlled ±1 °C by a Peltier thermoelectric device and monitored by a thermistor probe in the recording chamber. Recordings were made at either 22 °C or 37 °C as indicated. Data are expressed as mean ± S.E.M. of the number of observations indicated, and were compared using ANOVA and paired t-tests (SigmaStat 3.1, Systat Software Inc., San Jose, CA) with statistical significance (P value) as indicated.

All chemicals were of analytical grade. These included amphotericin-B, hydrogen peroxide (H$_2$O$_2$;) *tert*-butyl hydroperoxide (*t*-BHP), catalase, glibenclamide, superoxide dismutase (SOD), tetraethylammonium chloride (TEA), and tetrodotoxin (all from Sigma Chemical Co.).

3. Results

Dissociated neonatal ICG neurons were smaller in size than those from adults, and had a mean cell capacitance of 18.8 ± 3.2 pF ($n = 45$)
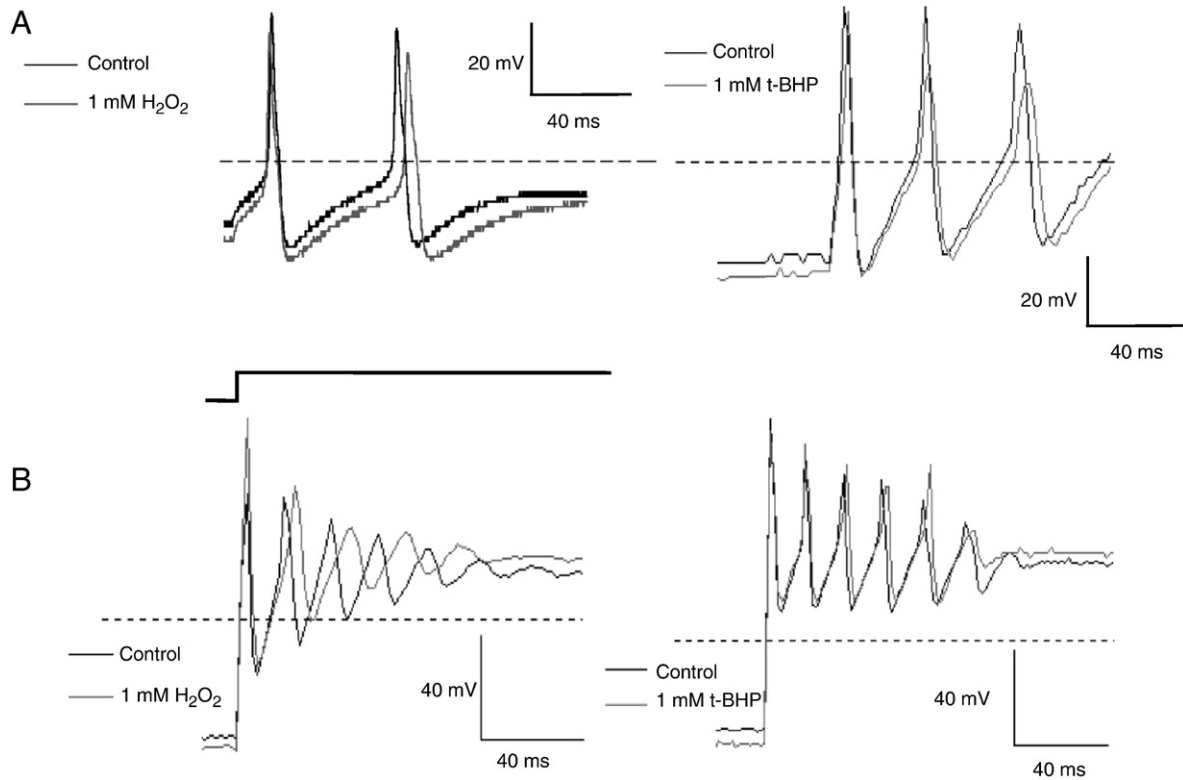
Fig. 1. Effect of ROS donors, H_2O_2 and t-BHP, on resting and action potentials in neonatal and adult rat ICG neurons. Superimposed traces of action potential discharge in neonatal (A) and adult (B) rat ICG neurons evoked by a $+200$ pA current pulse from a holding potential of -60 mV. Temperature 22 °C. The dashed line represents 0 mV. In the presence of either 1 mM H_2O_2 or t-BHP (light traces), ICG neurons were reversibly hyperpolarized by >5 mV and the action potential duration increased ($n \geq 4$).

compared with 52.4 ± 5.6 pF ($n = 39$) for adult neurons. Resting membrane potential was -53.0 ± 1.2 mV ($n = 45$) in neonatal rat ICG neurons, and -53.6 ± 1.6 ($n = 39$) in adult cells, which is in accordance with previously reported values (Hogg et al., 2001).

3.1. Effect of ROS donors on resting and action potentials

Under current clamp conditions, bath application of the ROS donors H_2O_2 or t-BHP reversibly hyperpolarized dissociated neonatal and adult ICG neurons, at both 22 °C and 37 °C (Fig. 1). At 22 °C, H_2O_2 and t-BHP, at a maximally effective concentration of 1 mM, hyperpolarized the neonatal and adult ICG neurons by approximately 4–10 mV (see Table 1). At 37 °C, 1 mM H_2O_2 and 1 mM t-BHP hyperpolarized the neonatal ICG neurons by 9 ± 1.4 mV ($n = 4$) and 4 ± 0.6 mV ($n = 3$), respectively, whereas 1 mM H_2O_2 hyperpolarized the adult cells by 13.2 ± 2.2 mV ($n = 3$). At both temperatures, membrane hyperpolarization caused by exposure to either H_2O_2 or t-BHP was not reversed by bath application of either 10 μM glibenclamide ($n = 4$) or Ba^{2+} ($n = 3$), inhibitors of K_{ATP} channels present in adult ICG neurons (Hogg and Adams, 2001).

Table 1
Effect of ROS donors on resting membrane potential (RMP) and action potential duration (APD) in rat ICG neurons.

Preparation		1 mM H_2O_2 (n)		1 mM t-BHP (n)	
		Δ RMP (mV)	APD (% increase)	Δ RMP (mV)	APD (% increase)
Neonate ICG	22 °C	-7.9 ± 2.0 (4)	16.2 ± 2.6 (4)**	-5.6 ± 1.6 (4)	11.2 ± 2.8 (4)*
	37 °C	-9.0 ± 1.4 (4)	–	-4.0 ± 0.6 (3)	–
Adult ICG	22 °C	-5.7 ± 0.9 (9)	24.9 ± 2.8 (9)**	-8.0 ± 1.2 (3)	24.1 ± 1.8 (9)**
	37 °C	-13.2 ± 2.2 (3)	–	–	–

Δ RMP, change in resting membrane potential; –, not determined; n, number of cells; *$P<0.05$, ** $P<0.01$.

The action of the ROS donors H_2O_2 and t-BHP on the resting membrane potential of adult ICG neurons in the *in situ* preparation was in good agreement with that on dissociated ICG neurons. In the *in situ* ICG preparation, at 35 °C, both H_2O_2 and t-BHP evoked a membrane hyperpolarization. Superfusion of H_2O_2 (1 mM) hyperpolarized the resting membrane potential by 16.7 ± 0.4 mV ($n = 9$, from -47.5 ± 2.4 mV to -64.2 ± 2.9 mV, $P<0.001$, paired t-test.) A lower concentration of H_2O_2 (0.5 mM) also caused a modest hyperpolarization of 6.7 ± 1.3 mV ($n = 4$). Similarly, t-BHP (1 mM) produced a hyperpolarizing shift in membrane potential of -5.5 ± 0.2 mV ($n = 8$, from -51.5 ± 3.0 mV to -57.0 ± 2.8 mV). The hyperpolarization produced by H_2O_2 was accompanied by a decrease in membrane resistance from 5.1 ± 0.8 kΩ cm² to 4.0 ± 0.6 kΩ cm² ($n = 7, P<0.05$) and the membrane resistance was reduced to 0.57 ± 0.14 of control values ($n = 5, P<0.05$) upon superfusion of t-BHP (1 mM) indicating that an increase in K^+ conductance was most likely responsible. To test for this, tetraethylammonium (TEA, 10 mM), an inhibitor of voltage- and Ca^{2+}-dependent K^+ channels and Ba^2 (1 mM), a wide-spectrum K^+ channel inhibitor, were applied to preparations in which the H_2O_2-induced hyperpolarization had fully developed. The hyperpolarizing shift in membrane potential was partially reversed by TEA, but fully reversed by Ba^{2+} (see Fig. 2A). In a group of 5 neurons, the resting membrane potential was -48.1 ± 3.3 mV in control conditions, -64.6 ± 3.6 mV in the presence of H_2O_2 and -45.6 ± 2.4 mV following superfusion of Ba^{2+} (see Fig. 2B). Bath application of apamin (100 nM), an SK_{Ca} channel inhibitor, iberiotoxin (100 nM), a BK_{Ca} channel inhibitor, and glibenclamide (10 μM) did not reverse the effects of H_2O_2 (data not shown).

Bath application of either 1 mM H_2O_2 or 1 mM t-BHP increased action potential duration by 11–16% in dissociated neonatal ICG neurons ($n = 8; P<0.05$) and by ~25% in adult cells compared to control ($n = 18; P<0.01$). Action potential duration was measured as neither H_2O_2 nor t-BHP appreciably changed the frequency of action potential firing in response to depolarizing current pulses in dissociated ICG neurons
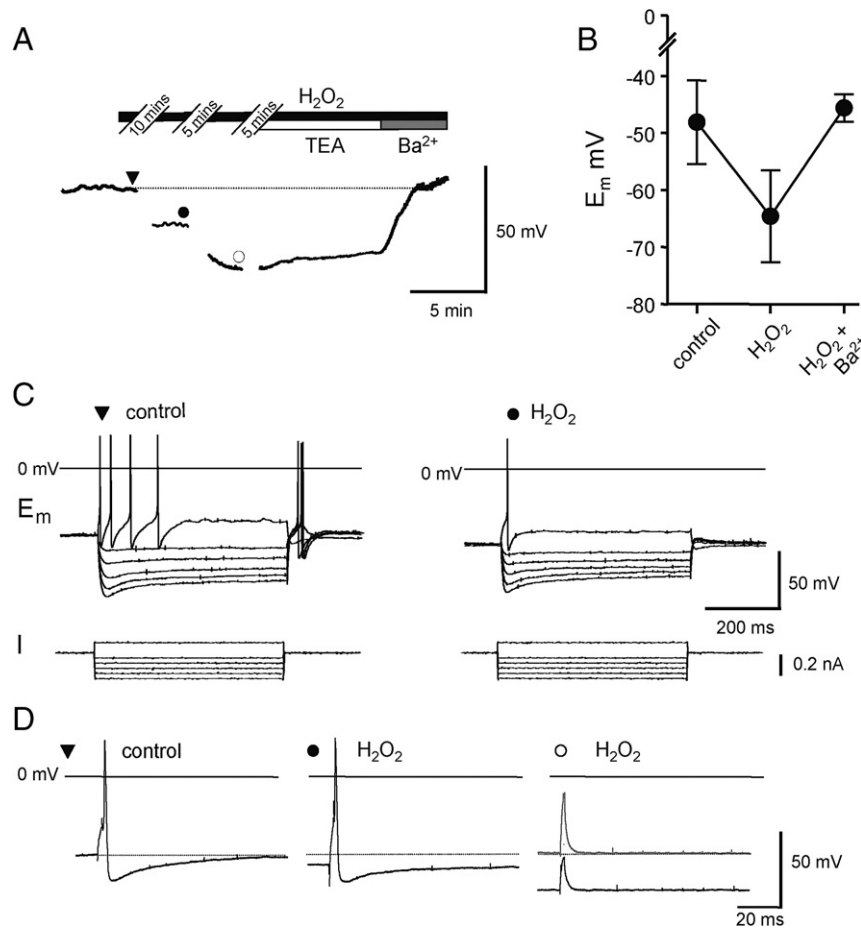
Fig. 2. Effect of H_2O_2 on resting and action potentials in adult rat ICG neurons *in situ*. (A) Representative membrane potential responses of an adult ICG neuron *in situ* to superfusion of H_2O_2 (1 mM) and subsequently co application of 10 mM TEA and 1 mM Ba^{2+}. Temperature 35 °C. (B) Ba^{2+} (1 mM) reverses the membrane hyperpolarization observed in the presence of H_2O_2. The membrane potential, taken for a group of 5 neurons, is presented for control conditions, in the presence of H_2O_2 (1 mM) and following superfusion of Ba^{2+}. (C) Voltage responses (upper traces) obtained in response to depolarizing and hyperpolarizing current pulses ($+0.2$, -0.1 to -0.5 nA, lower traces) are shown for control conditions and after 10 min exposure to 1 mM H_2O_2. (D) Representative somatic action potential traces elicited by brief depolarizing current pulses (2 ms) showing the time-dependent decrease in excitability in the presence of H_2O_2. The symbols denote the times at which the responses were taken in A. The membrane potential was reset to control values (grey trace) in the recording taken at 20 min to test if inexcitability was secondary to membrane hyperpolarization. Stimulus current was $+0.4$ nA (▼), $+0.6$ nA (●), and $+0.6$ nA (○) in the absence and presence of a $+0.25$ nA holding current (grey trace).

(Fig. 1A,B). In contrast, in the *in situ* preparation, the evoked response to long depolarizing current pulses was switched from predominantly multiple adapting to phasic or unresponsive by H_2O_2 and *t*-BHP (see Fig. 2C). Furthermore, *t*-BHP (1 mM) reduced the action potential AHP amplitude from 16.5 ± 5.1 mV (control) to 9.9 ± 4.4 mV (*t*-BHP; $n = 10$, $P<0.001$) but had no significant effect on either action potential overshoot or AHP duration, as gauged by time to 50% recovery. However, the absolute amplitude of AHP (measured from 0 mV) was unchanged in the presence of *t*-BHP indicating the change was secondary to the membrane hyperpolarization produced by *t*-BHP (Fig. 2D). *t*-BHP reduced the maximum rate of rise and fall of the action potential to $74 \pm 14\%$ and $72 \pm 6\%$, respectively, of its value under control conditions ($n = 6$; $P<0.05$ max d*rise*/d*t* and $P<0.005$ d*fall*/d*t* paired *t*-test). This resulted in an increase in action potential duration (measured at 0 mV) of approximately 55%.

## 3.2. Inhibition of voltage-gated Ca^{2+} channel currents by ROS donors

Under voltage clamp conditions, bath application of either 1 mM H_2O_2 or 1 mM *t*-BHP significantly inhibited the depolarization-activated Ca^{2+} channel current in both neonatal and adult ICG neurons (Fig. 3). In the presence of 300 nM TTX and using Ba^{2+} as the charge carrier, voltage ramps from -70 mV to $+40$ mV evoked an inward Ba^{2+} current which

was inhibited by 14–18% and 10–16% in neonatal and adult ICG neurons, respectively, in the presence of the ROS donors (see Table 2). The whole-cell current–voltage (*I–V*) relationship obtained in both neonatal and adult ICG neurons was shifted to a more hyperpolarized membrane potential (Fig. 3A,B) which was reversible upon washout of H_2O_2 and *t*-BHP.

3.3. Effect of pretreatment with ROS scavengers

In neonatal ICG neurons, both SOD and catalase (100 U/ml) reduced non-significantly the voltage-gated Ca^{2+} channel (VGCC) current amplitude (Table 2) and shifted the *I–V* relationship to more a depolarized potential as shown in Fig. 4A. Bath application of SOD or catalase prior to exposure to either H_2O_2 or *t*-BHP attenuated the hyperpolarizing shift, but did not reverse the inhibition of the inward Ba^{2+} current amplitude by H_2O_2 and *t*-BHP (data not shown).

In contrast, application of SOD (100 U/ml) alone had no significant effect upon either inward Ba^{2+} current amplitude (Table 2) or the *I–V* relationship in adult ICG neurons, but application of 100 U/ml SOD prior to exposure to either H_2O_2 or *t*-BHP abolished the hyperpolarizing shift of the *I–V* curve, as well as inhibition of the Ba^{2+} current by both ROS donors (Fig. 4B).
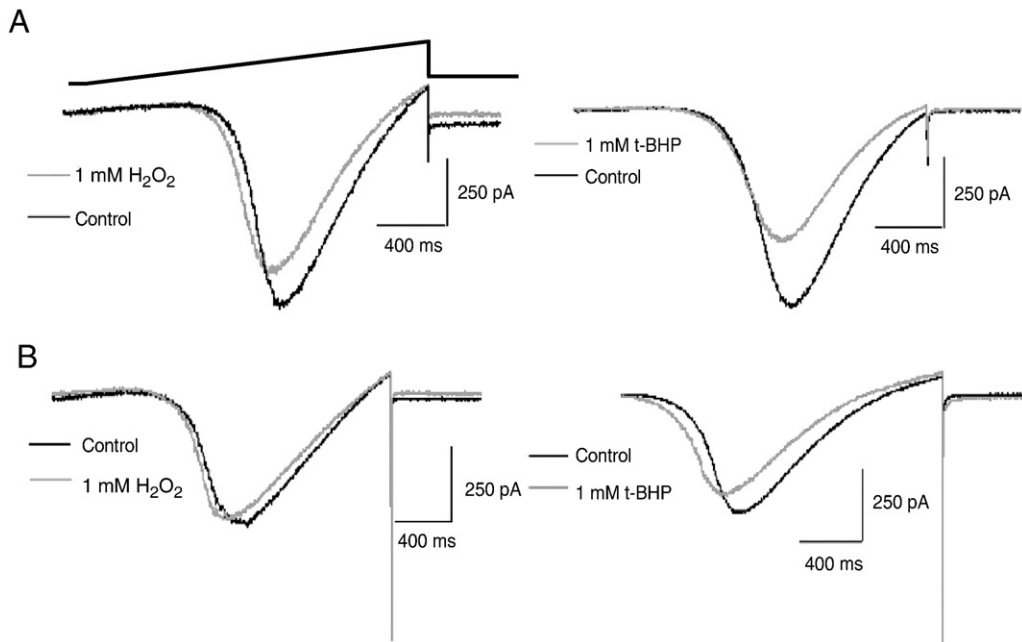
Fig. 3. Effect of ROS donors on Ca^{2+} channel currents in neonatal and adult ICG neurons. Superimposed inward Ba^{2+} currents evoked in response to voltage ramps from -70 mV to $+40$ mV in the absence (control) and presence of 1 mM H_2O_2 and t-BHP (light traces) in neonatal (A) and adult (B) rat ICG neurons ($n=3-6$). Temperature 22 °C.

Considering the *in situ* ICG preparation, superfusion of either SOD (100 U/ml) or catalase (100 U/ml) had no significant action on membrane potential or directly evoked somatic action potential characteristics. Superfusion of SOD (100 U/ml) and H_2O_2 (1 mM) attenuated the membrane hyperpolarization of H_2O_2 in the majority of neurons studied (3/5). Similarly, the action of H_2O_2 was abrogated by catalase (100 U/ml) changing by -1.8 ± 0.9 mV ($n=4$) upon co-application of H_2O_2 and catalase (data not shown).

3.4. Potentiation of delayed rectifier K^+ currents by ROS donors

The total outward K^+ current recorded in rat ICG neurons consists of both Ca^{2+}-dependent and Ca^{2+}-independent, delayed rectifier K^+ currents, with the Ca^{2+}-activated K^+ current accounting for approximately one-third of the total outward K^+ current (Xu and Adams, 1992; Xi-Moy and Dun, 1995). In the presence of either H_2O_2 or t-BHP (1 mM), the total outward K^+ current was reduced by $12.8\pm2.3\%$ and $13.9\pm1.3\%$ compared to control ($n=4$; $P<0.05$) in neonatal and adult ICG neurons, respectively. The reduction in the amplitude of the total outward K^+ current is largely a consequence of the inhibition of the VGCC-mediated Ca^{2+} influx by ROS donors. In contrast, bath application of either 1 mM H_2O_2 or 1 mM t-BHP increased the amplitude of the Ca^{2+}-independent, delayed rectifier K^+ current evoked by depolarizing voltage steps from -50 to $+50$ mV (Fig. 5A,B). H_2O_2 (1 mM) increased the delayed rectifier K^+ current amplitude at $+50$ mV by $15.5\pm0.4\%$ and $11.5\pm1.0\%$ compared to control ($n=3$; $P<0.05$) in neonatal and adult ICG neurons, respectively. Similarly, t-BHP increased the delayed rectifier K^+ current amplitude at $+50$ mV by $18.3\pm2.1\%$ ($n=3$; $P<0.01$) in neonatal ICG neurons and $12.9\pm4.5\%$ ($n=3$) in adult ICG neurons. The delayed

rectifier K^+ current recorded in the presence of H_2O_2 and t-BHP was reversibly inhibited by extracellular TEA (10 mM) (data not shown).

4. Discussion

This is the first study to directly examine the effects of ROS donors on the electrophysiological properties of mammalian intrinsic cardiac neurons. The ROS donors H_2O_2 and t-BHP reversibly hyperpolarized and increased the action potential duration in dissociated neurons of both neonatal and adult rat ICG. The action of these agents on neurons was also investigated in the *in situ* adult ICG preparation. Dissociated neurons do not have surrounding satellite glial cells which encapsulate ICG neuronal somata (Baluk, 1995) and may release and/or take up substances which buffer or exacerbate the effects of ROS donors. Furthermore, there is accruing evidence that the expression and distribution of membrane receptors and ion channels in dissociated autonomic ganglion neurons are not necessarily the same as that in the intact ganglion (e.g. Martínez-Pinna et al., 2002; Rimmer and Harper, 2006). Therefore, in order to relate the effects of ROS on the electrophysiological properties of dissociated neurons to physiological function, it is important to complement these with studies in intact ganglion preparations. The action of H_2O_2 and t-BHP on the passive and active electrical properties of neurons in the intact adult ICG are in good agreement with those for dissociated adult ICG neurons. The small depolarization recorded with co application of H_2O_2 and Ba^{2+} compared with control values is consistent with the contribution of the M-current to the resting membrane potential in these neurons (Cuevas et al., 1997; Rimmer and Harper, 2004).

H_2O_2 and t-BHP also inhibited VGCC currents and shifted the *I–V* relationship to more hyperpolarized potentials. The ROS scavengers SOD and catalase inhibited the VGCC currents in neonatal ICG cells but shifted the *I–V* relationship to more depolarized potentials. Application of SOD or catalase to neonatal ICG cells prior to H_2O_2 or t-BHP exposure attenuated the hyperpolarizing shift, but not the inhibition by these ROS donors. In contrast, in adult cells, application of SOD alone had no effect upon either VGCC current amplitude or the *I–V* relationship. Furthermore, application of SOD prior to H_2O_2 treatment abolished the hyperpolarizing shift and inhibition of VGCC currents by H_2O_2.

Table 2
Effect of ROS donors and pretreatment with SOD and catalase on voltage-gated calcium channel (VGCC) currents from neonatal and adult rat ICG neurons (22 °C).

Preparation	% inhibition of VGCC current amplitude (n)			
	1 mM H_2O_2	1 mM t-BHP	100 U/ml SOD	100 U/ml catalase
Neonate ICG	13.9 ± 2.6 (8) **	17.9 ± 1.9 (5) **	12.5 ± 4.8 (4)	13.7 ± 3.7 (4)
Adult ICG	9.8 ± 1.4 (6) *	16.5 ± 1.6 (3) **	0 (3)	ND

ND, not determined; n, number of cells; *$P<0.05$, **$P<0.01$.
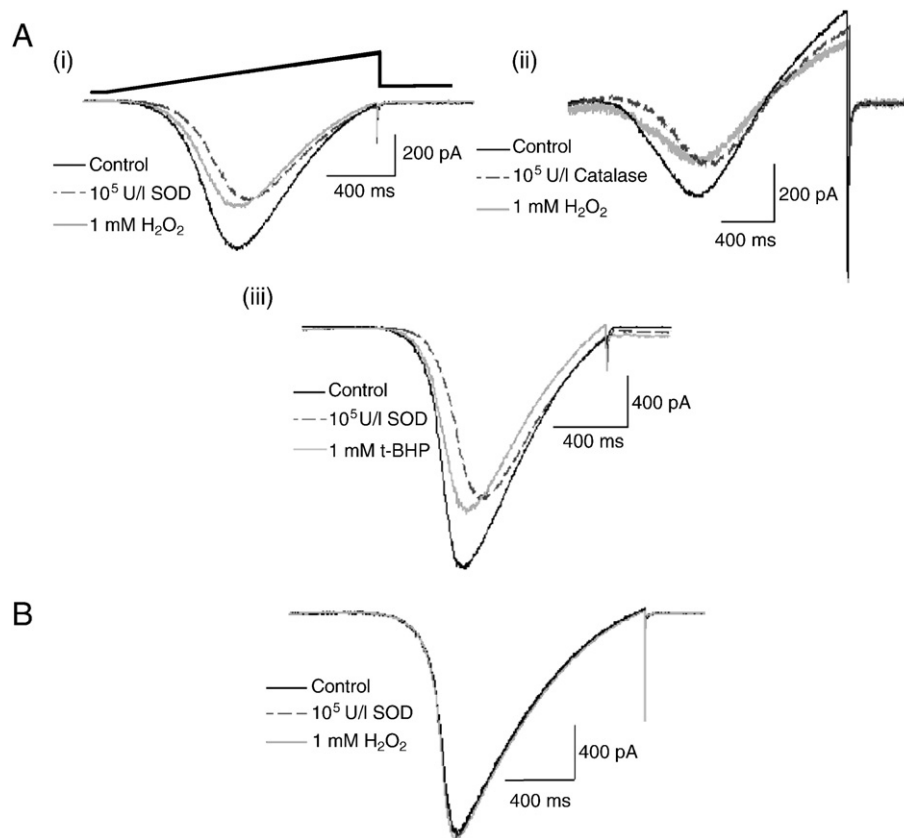
Fig. 4. Effect of pre-application of ROS scavengers on the effects of H_2O_2 and t-BHP on Ca^{2+} channel currents in neonatal and adult rat ICG neurons. A: Superimposed inward Ba^{2+} currents recorded at 22 °C in response to voltage ramps from -70 mV to $+40$ mV in neonatal rat ICG neurons. Representative currents obtained in the absence (control) and presence of either 100 U/ml SOD (i) or catalase (ii) applied before and following exposure to 1 mM H_2O_2 (light traces). (iii) Bath application of SOD prior to exposure to 1 mM t-BHP attenuated the hyperpolarizing shift of the I–V relationship but not the inhibition of the inward Ba^{2+} current by t-BHP ($n=11$). B: Superimposed inward Ba^{2+} currents obtained in the absence (control) and presence of 100 U/ml SOD before and after application of 1 mM H_2O_2 in adult ICG neurons ($n \geq 3$). Inward currents were evoked by a 110 mV voltage ramp as illustrated in A(i).
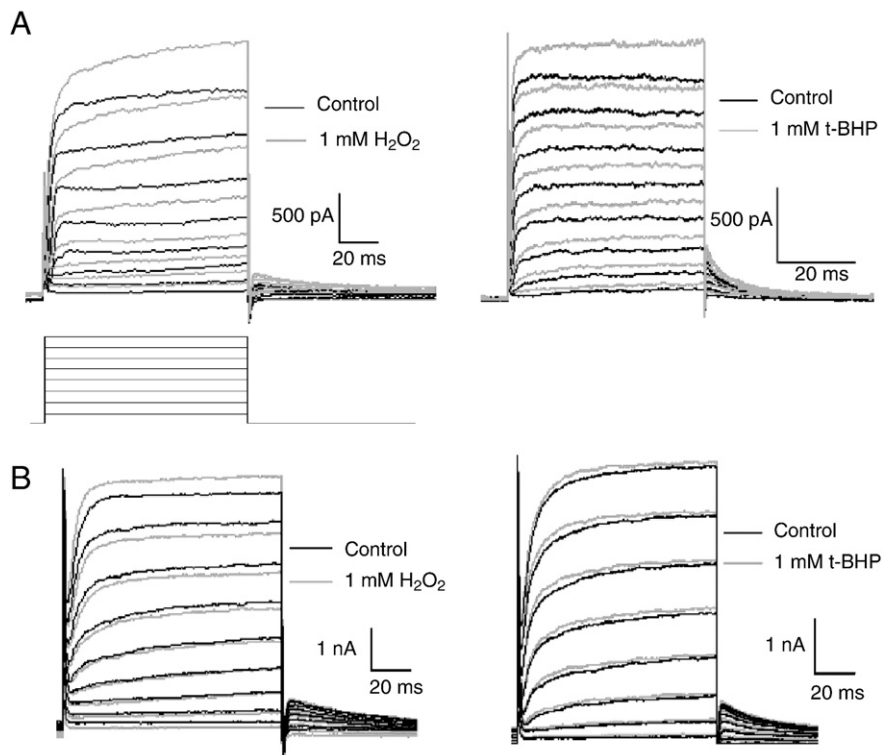
Fig. 5. Effect of ROS donors, H_2O_2 and t-BHP, on the delayed rectifier K^+ current in neonatal and adult rat ICG neurons. Superimposed outward K^+ currents obtained in the absence (control) and presence of 1 mM H_2O_2 and 1 mM t-BHP bath applied (light traces) in neonatal (A) and adult (B) rat ICG neurons ($n=5$). Outward K^+ currents recorded at 22 °C in the presence of 300 nM TTX and 100 μM Cd^{2+} extracellularly, were evoked by depolarizing voltage steps in 10 mV increments from a holding potential of -50 mV.

Outward repolarizing K^+ currents are important determinants of action potential duration and neuronal firing properties. The action potential duration in both neonatal and adult ICG neurons was increased by approximately 11–25% in the presence of either H_2O_2 or t-BHP but the frequency of action potential firing in response to depolarizing current pulses was unchanged. In the presence of either H_2O_2 or t-BHP, the total outward K^+ current was reduced by ~13% as a consequence of the reduced Ca^{2+} influx through open VGCCs. However, in contrast, H_2O_2 and t-BHP increased the amplitude of the Ca^{2+}-independent, delayed rectifier K^+ currents by approximately 12–18% in both neonatal and adult ICG neurons. Given that both delayed rectifier K^+ currents and Ca^{2+}-dependent K^+ currents contribute to the repolarization of the action potential in rat ICG neurons (Adams and Cuevas, 2004), the action potential duration is likely to reflect the balance between the inhibition of the Ca^{2+}-dependent K^+ current and the potentiation of the delayed rectifier K^+ current. We also observed the reversible prolongation of action potential duration by ROS donors in adult rat ICG neurons of the whole-mount right atrial ganglion plexus preparation using sharp microelectrode recording techniques. The prolongation of action potential duration is likely to affect transmitter release from postganglionic nerve terminals in the heart, consistent with the actions of H_2O_2 on quantal release and presynaptic potentiation during high frequency stimulation observed at the neuromuscular junction (Giniatullin and Giniatullin, 2003).

Although the actions of ROS on membrane conductances have not been investigated previously in mammalian intracardiac neurons, the effects of ROS on neuronal excitability have been studied in myenteric neurons in guinea-pig distal colon *in situ* (Wada-Takahashi and Tamura, 2000) and neurons isolated from guinea-pig intestine and grown in primary culture (Vogalis and Harvey, 2003). The majority of distal colon myenteric neurons exhibited a transient membrane hyperpolarization followed by a long-lasting depolarization in response to hypoxanthine and xanthine oxidase which was attributed to suppression and augmentation of the conductance of Ca^{2+}-dependent K^+ channels, respectively (Wada-Takahashi and Tamura, 2000). In contrast, application of sub-millimolar H_2O_2 to cultured intestinal neurons induced membrane hyperpolarization, and a concomitant suppression of the slow after-hyperpolarization which was attenuated by internal perfusion of catalase and glutathione (Vogalis and Harvey, 2003). Under voltage clamp conditions, the acute oxidative stress produced by H_2O_2 was shown to be due to the suppression of a hyperpolarization-activated inward current (I_h) and the activation of an inwardly rectifying outward current blocked by glibenclamide and TEA.

Electrophysiological studies have also shown varied effects of ROS donors on action potential amplitude and duration in guinea pig ventricular myocytes (Hayashi et al., 1989; Cerbai et al., 1991). In this system, the prolongation of action potential duration by dihydroxyfumarate-generated ROS was accompanied by a decrease in VGCC current and time-dependent outward K^+ current amplitude (Cerbai et al., 1991). Similarly, H_2O_2 inhibited the L-type Ca^{2+} current but either activated an outward membrane current consistent with the ATP-sensitive K^+ current (Goldhaber and Liu, 1994) or enhanced the delayed rectifier K^+ current (Satoh and Matsui, 1997). However, in these experiments, no attempt was made to differentiate between the effects of ROS on the I_{Kr} (rapidly activating/deactivating) and I_{Ks} (slowly activating/deactivating) components of the delayed rectifier K^+ current and the inward rectifier K^+ current was unaffected (Sanguinetti and Jurkiewicz, 1990). Similarly, investigation of the effects of H_2O_2 (200 μM) on action potentials and underlying ionic currents in single myocytes from the ventricles of adult rat hearts using the amphotericin-perforated patch clamp method showed that H_2O_2 caused a marked prolongation of the action potential but no significant changes in either the Ca^{2+}-independent transient outward K^+ current (I_{to}) or the inwardly rectifying K^+ current (I_{K1}) (Ward and Giles, 1997). The most prominent effect of H_2O_2 on the ionic currents, which underlie the action potential, was a slowing of inactivation of the TTX-sensitive Na^+ current. ROS produced by exposure

to either H_2O_2 or $FeSO_4$ and ascorbic acid has also been shown to inhibit several cloned K^+ channels expressed in *Xenopus* oocytes, including HERG ($K_V11.1$), HukII ($K_V1.4$), KShIIID ($K_V3.3$), and KShIIIC ($K_V4.4$) but not rDRK1 ($K_V2.1$), bEAG ($K_V10.1$) or mIRK1 ($K_{ir}2.1$) (Vega-Saenz de Miera and Rudy, 1992; Taglialatela et al., 1997). Likewise, the activity of three Shaker K^+ channels ($K_V1.3$, $K_V1.4$, and $K_V1.5$), one Shaw channel ($K_V3.4$) and one inward rectifier K^+ channel (IRK3) expressed in *Xenopus* oocytes was inhibited by photoactivation of rose bengal, a generator of ROS, whereas other K^+ channel types ($K_V1.2$, $K_V2.1$, $K_V2.2$, $K_V4.1$, inward rectifiers IRK1 and ROMK1 and hIsK) were completely resistant to this treatment (Duprat et al., 1995). A recent study of the effect of H_2O_2 on the K_V7 family demonstrated a strong enhancement of $K_V7.2$, 7.4 and 7.5 mediated K^+ currents by H_2O_2 (0.3 mM) due to the oxidative modification of cysteines in a triple cysteine pocket within the cytosolic S2–S3 linker (Gamper et al., 2006). Oxidative-induced enhancement of M-current induced a hyperpolarization and a reduction of action potential firing frequency in rat sympathetic neurons (Gamper et al., 2006) consistent with our observations in rat ICG neurons *in situ*.

In conclusion, there is considerable evidence demonstrating that cardiac autonomic regulation may be modulated by ROS, the behavior of which is dictated by the oxidative stress of the microenvironment (for review see Danson and Paterson, 2006). Our results suggest that free radicals generated during ischemia and reperfusion may contribute to electrophysiologic abnormalities in neonatal and adult rat ICG neurons by modifying resting and depolarization-activated Ca^{2+} and K^+ conductances which underlie neuronal excitability. Changes to these membrane conductances account for the prolongation of action potential duration by ROS donors and therefore probably modify autonomic control of the mammalian heart.

Acknowledgements

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