Altered Excitability of Intestinal Neurons in Primary Culture Caused by Acute Oxidative Stress

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Vogalis, Fivos and John R. Harvey. Altered excitability of intestinal neurons in primary culture caused by acute oxidative stress. J Neurophysiol 89: 3039–3050, 2003; 10.1152/jn.01005.2002. Neurons were isolated from the intestine of guinea pigs and grown in primary culture for ≤15 days. Using conventional whole cell recording techniques, we demonstrated that the majority of neurons express a prolonged poststimulus afterhyperpolarization (slow AHP). These neurons also had large-amplitude (∼100 mV), broad-duration (∼2 ms) action potentials and generated a hyperpolarization-activated inward current (Ih). Application of H2O2 (0.22–8.8 mM) hyperpolarized these neurons but not those lacking slow AHPs. The H2O2-induced hyperpolarization was followed by irreversible depolarization at higher concentrations (more than ∼1 mM) of H2O2 while it was maintained after washout of submillimolar H2O2. The ionic mechanisms underlying the hyperpolarization included the suppression of Ih and the activation of an inwardly rectifying outward current, which was blocked by glibenclamide (25–50 μM) and TEA (30 mM). In addition, H2O2 suppressed the slow AHP and its underlying current. Internal perfusion of catalase and glutathione opposed the H2O2-mediated decrease in Ih. Our results indicate that acute oxidative stress has neuron- and conductance-specific actions in intestinal neurons that may underlie pathophysiological conditions.

INTRODUCTION

Superoxide is produced endogenously in all cells by components of the mitochondrial electron transport chain (ETC) (Halliwell and Gutteridge 1985; McCord 2000). Normally, generation of superoxide accounts for <5% of the oxygen consumed, but on reoxygenation, after hypoxia, superoxide production is elevated above normal due to an increased leakage of electrons to oxygen, resulting from the downregulation of cytochrome oxidase and from a decrease in the activity of Mn-dependent superoxide dismutase (MnSOD) (Li and Jackson 2002). The poor lipid solubility of superoxide limits its actions to mitochondrial components thus affecting ATP production. However hydrogen peroxide (H2O2), which is generated from superoxide by MnSOD, is lipid soluble and diffuses out into the cytoplasm (Halliwell 1992). Although relatively inert, on contact with ferrous-containing low-molecular-weight proteins or with free cytoplasmic Fe2+ (Halliwell 1992), H2O2 is a source of hydroxyl radical that is one of the most potent of known oxidants that oxidizes proteins and lipids and breaks apart DNA strands (Stadtman and Levine 2000). Under normoxic conditions, production of hydroxyl radicals is checked by enzymes such as catalase and glutathione peroxidase that convert H2O2 to water and molecular oxygen, and to water respectively (Halliwell 1992). However, during oxidative stress, apart from rundown of ATP-dependent processes, there is an overproduction of oxygen radicals and reactive oxygen intermediates (ROIs) that may directly affect neuronal excitability.

Hypoxia is also a trigger for the conversion of cytoplasmic xanthine dehydrogenase (XD) to xanthine oxidase (XO). Under normoxic conditions, XD catalyzes the conversion of hypoxanthines to xanithines (derived from the breakdown of ATP and AMP) and then to uric acid, using NAD+ as the electron acceptor (McCord 1985) but XO, which accumulates during hypoxia, uses molecular oxygen as the electron acceptor to break down hypoxanthines, thereby generating superoxide. On reoxygenation, the increased availability of oxygen leads to increased production of superoxide, H2O2, and destructive hydroxyl radical (McCord 1985). Although the role of neuronal XD/XO in ischemia/reperfusion injury is unclear, these enzymes are implicated in the ROI-mediated damage to epithelial cells of the gastrointestinal tract and in the vascular endothelium (Li and Jackson 2002; McCord 1985). Endothelial cells also contain NAD(P)H-oxidase, which actively synthesizes superoxide and which becomes superactivated after hypoxia (Droge 2002). A phagocytic isoform of this enzyme plays a central role in the destruction of pathogens by activated macrophages and neutrophils (Droge 2002) and extravasation of activated neutrophils to sites of infection is accompanied by the increased production of ROIs and with inflammation. In the gastrointestinal tract, activated macrophages increase the level of H2O2 in the muscle layers (Gonzalez and Sarna 2001) and ROIs may participate in inflammatory bowel diseases (Fiocchi 1998). A potential target of ROIs are likely to be the intrinsic neurons of the intestine that control secretion and absorption at the mucosa and motility in the muscle (Kunze and Furness 1999).

In the present study, we investigated the actions of H2O2 on the electrical properties of intestinal neurons isolated and grown in primary culture that minimized contamination from muscle-derived factors. Specifically we investigated the actions of H2O2 on the excitability of neurons that generate slow afterhyperpolarizing potentials (slow AHPs; AH neurons) because membrane ion channels and transporters are key targets of ROIs (Kourie 1998). A recent study on intact AH/type 2 neurons reported that superoxide generated by the extracellular

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application of hypoxanthine and XO had strong actions on AH/type 2 neurons in the ileum, including a transient hyperpolarization followed by a depolarization, and a sustained hyperpolarization after washout of the substrate-enzyme combination (Wada-Takahashi and Tamura 2000). These actions were thought to be mediated by hydroxyl radicals and were closely mimicked by H₂O₂. In the present study, we investigated the actions of H₂O₂ on the excitability of AH neurons to determine its scope of actions.

METHODS

Primary culture of myenteric neurons from the duodenum of the guinea pig

A segment of duodenum (3–4 cm in length) was removed from a guinea pig of either sex, killed by cervical dislocation and exsanguination, in compliance with the requirements of the Animal Ethics Committee at the University of Melbourne. The duodenal segment was cleaned of its contents by flushing physiological saline through the lumen and then cut longitudinally and pinned out flat, mucosa surface uppermost, in a petri dish lined with silicone elastomer (Sylgard, Dow Corning). The dish was filled with preoxygenated Ca²⁺-free Hanks solution at room temperature that was replaced regularly during the dissection. The mucosa and the underlying circular muscle layer were removed using fine forceps and scissors under a binocular microscope to reveal the myenteric plexus that remained adhered to the longitudinal muscle. This longitudinal muscle-myenteric plexus preparation was then cut into small pieces (~2 mm²) and transferred to a test-tube containing 0.2% collagenase Type 1A (Sigma) dissolved in Ca²⁺-free Hanks solution (composition given below). The tissue was then incubated in this medium for 15 min at 37°C after which it was then incubated in this medium for 15 min at 37°C and aliquots of this solution were added to 35-mm culture dishes (NALG NUNC) that contained cell culture medium consisting of Dulbecco’s modified Eagle’s medium (DMEM; JRH Biosciences) with 10% heat-inactivated fetal calf serum (CSL Australia). This culture medium was supplemented with 1% glutamine and 2% antibiotics (penicillin, 10,000 units/ml; streptomycin, 10 mg/ml) to inhibit microbial contamination. Each dish also contained 50 ng of mouse nerve growth factor (Sigma). After 2–3 days in culture, the culture medium was exchanged with fresh medium to which were added 0.075% fluorodeoxyuridine (FDU) and 0.175% uridine to inhibit mitotic activity of nonneural cells. Thereafter, antimitotic drugs were added to the culture medium, which was exchanged every 3 days, until the day of use, ~7–15 days postplating. The Ca²⁺-free Hanks solution contained (in mM) 137.9 NaCl, 5.5 glucose, 5.4 KCl, 0.44 K₂HPO₄, and 0.4 Na₂HPO₄; 0.35 g/l of NaHCO₃ was added to this solution on the day of use, and it was then filtered through a 0.22-µm filter before use.

Electrophysiological recordings from cultured neurons

In preparation for patch-clamp recording, a dish containing cultured neurons was placed on the stage of an inverted microscope (Olympus, CK40) and was perfused continuously (1–2 ml/min) with preoxygenated Krebs solution (bubbled with carbogen, 95% O₂–5% CO₂), heated to 33–35°C when the temperature was measured in the dish. Neurons were allowed to equilibrate in Krebs solution for 5–10 min. The composition of the Krebs solution was (in mM) 118 NaCl, 4.8 KCl, 25 NaHCO₃, 1.0 NaH₂PO₄, 1.2 MgSO₄, 11.1 glucose, and 2.5 CaCl₂. After bubbling with carbogen, the pH of this solution was 7.35. Neurons with larger cell bodies were selected for recordings because such neurons were more likely to generate slow afterhyperpolarizing potentials (slow AHPs) (Vogalis et al. 2000). We also recorded from a small number of cells that had a bipolar morphology (Fig. 1Bi) but found that these cells were unable to generate regenerative responses and had low resting potentials (Fig. 1Bi).

Patch electrodes were pulled from borosilicate glass capillary tubing (GC150F-10, Harvard Apparatus) to have resistances of 4–8 MΩ when filled with the standard intracellular pipette-filling solution, the composition of which was (in mM) 130 KCl, 1 MgCl₂, 0.45 CaCl₂, 10 HEPES, 1 EGTA, and 2 ATP_K₃. pH was adjusted to 7.2 using 4 M KOH, adding ~11 mO K⁺. The concentration of Ca²⁺ in the internal solution was estimated to be 70 nM using Maxchelator (http://www.stanford.edu/~cattone/maxc.html). Internal solution containing 10 mM EGTA contained no added Ca²⁺ but was otherwise identical to the standard pipette solution. Patch electrodes were connected to the head-stage of an Axopatch 200B amplifier (Axon) and positioned using a fine micromanipulator (Sutter). Membrane currents and voltage were digitized through a Digidata 1200A (Axon) and analog stimulus pulses were applied via the same instrument, using pClamp 8.2 (Axon) acquisition software. We also used a current stimulator (Master-8, AMPI) to inject constant current pulses into cells. To minimize liquid junction potential artifacts, we used a KCl (3 M) agar-bridge as a ground electrode in the dish. The active signal was low-pass filtered (8-pole Bessel filter, Krohn-Hite; 1–2 kHz for current in voltage-clamp or at 5 kHz for voltage in current-clamp mode) before digitization. Recordings were stored on the hard drive of a PC computer (Dell) and analyzed off-line using Clampfit (Axon) and Igor 4 (WaveMetrics). Under voltage clamp, series resistance compensation was employed to compensate for the voltage drop across the electrode, up to the point before ringing occurred. Currents generated by discharge of the cell capacitance were minimized using the cancellation dials on the patch-clamp amplifier.

Drugs were added directly to the Krebs solution and perfused through the dish, unless otherwise stated. The standard protocol for testing the action of hydrogen peroxide (H₂O₂) was to perfuse 10–15 ml of the solution through the dish and record the effects on mem-

FIG. 1. Appearance of intestinal neurons and glial cells in culture and corresponding electrical activity. A, i: phase contrast image of typical cluster of neurons 10 days postplating. The neuron about to be patched is indicated (↓). ii: electrical activity of the patched neuron recorded in current-clamp mode (bottom) in response to injection of 150-ms current steps (top). Note hyperpolarization-activated depolarizing sag in the electrotonic potential. B, i: typical bipolar cells usually found among neuronal clusters. ↑, a cell from which the recording was obtained: ii: note absence of regenerative activity and low resting potential.
brane potential and on ionic currents for 5–10 min during wash-in and then 5–10 min during the washout of H$_2$O$_2$. The following agents were used: H$_2$O$_2$ (30% analytical grade, Ajax Chemicals), which was made up fresh for each trial from stock solution; glybenclamide (Tocris); tetraethylammonium chloride (TEA, Sigma); clotrimazole (Sigma); forskolin (Sigma); caffeine (Sigma); catalase and reduced glutathione (GSH; Sigma). Glybenclamide was made up as a 0.1 M stock solution dissolved in DMSO as was forskolin. Clotrimazole was also dissolved in DMSO at a stock concentration of 0.05M and stored at 0–4°C until use.

Stimulus and recording protocols and analysis of data

The capacitance of neurons was estimated by fitting a single exponential function to the electrotonic potential, elicited by injection of a 20- to 60-pA, 150-ms hyperpolarizing current step at the resting membrane potential (RMP). The time course of the electrotonic potential was fitted with a single exponential function whose time constant was used to derive the cell capacitance ($C_{cell}$) by dividing the time constant by the input resistance ($R_{in}$). $R_{in}$ was obtained from the ratio of the voltage deflection of the electrotonic potential divided by the amplitude of the injected current step. To determine whether neurons were able to generate slow AHPs, the standard stimulus protocol consisted of three 10-ms intracellular depolarizing current pulses, 250–400 pA in amplitude, delivered at 50 Hz, with (triple-pulse stimulation). The value of the RMP from which the peak of the ensuing slow AHP was determined, was taken as the averaged voltage over 1–2 s of recording prior to the stimulus pulse. In general, slow AHPs could be distinguished from other faster AHPs because they were >5 s in duration and had an apparent delay in their onsets.

To record the current underlying the slow AHP, the standard voltage-clamp protocol consisted of holding the membrane potential of the cell at −65 mV, stepping the membrane potential to +50 mV for 100 ms, and then repolarizing the cell to −55 mV for 20 s to record the peak and the deactivation phase of the slow AHP current ($I_{sAHP}$). The magnitude of the $I_{sAHP}$ was taken as the difference between the peak current at −55 mV, and the current at the end of the step. The deactivation phase of the $I_{sAHP}$ was well-fitted with a single exponential function to obtain the deactivation time constant ($\tau_{deact}$).

In voltage-clamp mode, we also applied voltage ramps to determine the I-V relationships of the membrane currents affected by the various treatments. These consisted of ramp depolarizations from −100 to −30 mV, applied over 10 s, at a rate of 7 mV/s.

The data were tabulated with Excel (Microsoft) to calculate means ± SE and n refers to the number of cells. Statistical significance between means was determined using two-tailed paired Student’s t-test, unless otherwise stated and statistical significance was set for $P < 0.05$.

RESULTS

Properties of myenteric neurons in primary culture

Neurons that were patch clamped usually had multiple processes emanating from their cell bodies (Fig. 1Ai). Whole cell current-clamp recording revealed that the majority of them possessed many of the electrophysiological features of AH neurons in intact myenteric ganglia, including broad-duration action potentials and a distinct hyperpolarization-activated depolarizing “sag” (Fig. 1Aii). In contrast, cells with a bipolar appearance (Fig. 1Bi) were assumed to be glial cells as they lacked regenerative activity and had low RMPs (−20 to −30 mV; Fig. 1Bii).

From a total of >250 neurons whose activity was recorded, >60% were found to generate a slow afterhyperpolarization (slow AH), defined as a hyperpolarization of the membrane potential that lasted >5 s and that followed a triggered volley or burst (200–1,000 ms) of action potentials. The slow AHP that was evoked by triple-pulse stimulation (3 10-ms depolarizing pulses, 250–400 pA, at 50 Hz) hyperpolarized AH neurons by 5.3 ± 0.4 mV ($n = 86$; Fig. 2Ai). The amplitude of slow AHPs evoked by 200-ms depolarizing pulses were larger (7.1 ± 0.5 mV, $n = 12$), in keeping with the greater number of action potentials (~8) triggered per burst, at a frequency of 40 ± 4.4 Hz ($n = 12$) (Hillsley et al. 2000). The $C_{cell}$ of AH neurons averaged 50.9 ± 2.3 pF ($n = 117$) and differed significantly ($P < 0.05$, unpaired t-test) from the $C_{cell}$ of neurons that lacked slow AHPs (non-AH neurons: 36.4 ± 2.1 pF, $n = 83$). As shown in Fig. 2B, there was a large overlap between the two distributions of $C_{cell}$ of the two groups of neuron. A comparison of the electrical properties of the two groups of neurons is given in Table 1. In general, AH neurons had a more negative RMP and a lower $R_{in}$ than non-AH neurons and were not spontaneously active. The action potentials of AH neurons displayed a prominent “hump” on the repolarizing phase which was due to the opening of N-type Ca$^{2+}$ channels as it was blocked by omega-conotoxin GVIA (0.5 μM).

Under voltage-clamp, AH neurons generated a postdepolarization outward current ($I_{sAHP}$) that was responsible for the

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**FIG. 2.** Electrical properties of slow afterhyperpolarization (AHP)-generating (AH) neurons. A: i: current-clamp recording from an AH neuron on a slow time base showing action potentials evoked by triple-pulse stimulation (C) and the ensuing slow AHP. ii: the current underlying the slow AHP ($I_{sAHP}$) recorded under voltage clamp with protocol shown (top). B: histogram distribution of cell capacitance measurements ($C_{cell}$) of AH and non-AH neurons. Bin width was set to 5 pF. AH neurons had a larger mean $C_{cell}$ than non-AH neurons. C: i: positive correlation ($r = 0.745$) between $I_{sAHP}$ amplitude and $C_{cell}$. ii: approximately inverse relationship between input resistance ($R_{in}$) and $C_{cell}$. Data points represent means ± SE.
hydroperoxide (H$_2$O$_2$) on the activity of an AH neuron. A: $i$: continuous current-clamp recording showing bursts of action potentials evoked by current injection (200-ms pulses, 300 pA, filled squares), followed by slow AHPs. Wash in of H$_2$O$_2$ (8.8 mM, filled bar) inhibited the slow AHPs as the resting potential (RMP; dotted line) hyperpolarized. $ii$: action potential evoked during current pulses before (1), and at 2 time points during H$_2$O$_2$ application (2 and 3). Note that despite the increase in the threshold depolarization required to trigger an action potential at the peak of the H$_2$O$_2$-induced hyperpolarization, the action potential waveform is similar to the control spikes. B: responses of the AH neuron to injection of 150-ms current pulses before ($i$) and after wash-in of H$_2$O$_2$ ($ii$). $iii$: peak voltage deflection plotted as a function of injected current (V-I relationship) under the two conditions. The slopes of the fitted regression lines indicate that the input resistance was decreased from 245 to 174 MΩ by H$_2$O$_2$.
developed large nonspecific resting potentials toward 0 mV. Such neurons, the RMP before application of \( \text{H}_2\text{O}_2 \) averaged 449 ± 110 mV (\( n = 36 \)) for \( \text{H}_2\text{O}_2 \) indicated that on average AH neurons were hyperpolarized from –55.4 ± 1.5 mV (\( n = 22 \)) to a peak of –63.4 ± 2.9 mV (\( n = 22 \)) within 5 min of wash-in of \( \text{H}_2\text{O}_2 \). At the same time, their \( R_{\text{in}} \) was halved, from an average of 449 ± 66 MΩ (\( n = 22 \)) to 219 ± 36 MΩ (\( n = 18 \)) (\( P < 0.05 \)). In addition, the slow AHP elicited by triple-pulse stimulation in these neurons was decreased from 3.5 ± 0.7 mV (\( n = 19 \)) to 0.1 ± 0.1 mV (\( n = 19 \); \( P < 0.05 \); Fig. 3Ai) and similarly under voltage clamp, the \( I_{\text{AHP}} \) was decreased from 107 ± 45 pA (\( n = 8 \)) to 22 ± 12 (\( n = 8 \); \( P < 0.05 \)).

The transient hyperpolarization elicited by supra-millimolar \( \text{H}_2\text{O}_2 \) was superseded by an irreversible depolarization. In five neurons examined, the RMP had depolarized to –50 ± 5.7 mV (\( n = 5 \)) within 5–10 min of washout of \( \text{H}_2\text{O}_2 \), and the \( R_{\text{in}} \) decreased to 95 ± 32 MΩ (\( n = 5 \)). Thereafter, neurons developed large nonspecific leak conductances that drove their resting potentials toward 0 mV.

Application of supra-millimolar \( \text{H}_2\text{O}_2 \) to non-AH neurons failed to significantly hyperpolarize them (Fig. 4A1). In seven such neurons, the RMP before application of \( \text{H}_2\text{O}_2 \) averaged –51.9 ± 4.2 mV (\( n = 7 \)) and –52.0 ± 3.3 mV (\( n = 7 \)) 5–10 min after wash-in of \( \text{H}_2\text{O}_2 \) (1.7 ± 0.5 mM), and their \( R_{\text{in}} \) was decreased by ~20% from 523 ± 106 MΩ (\( n = 7 \)) to 393 ± 72 MΩ (\( n = 7 \); \( P = 0.073 \); Fig. 4Bi). The peak amplitude of the action potentials, however, was significantly decreased (Fig. 4Bii) from 91.1 ± 6.3 mV (\( n = 7 \)) to 77.9 ± 6.5 mV (\( n = 7 \); \( P < 0.05 \)).

Effects of sub-millimolar \( \text{H}_2\text{O}_2 \) on AH neurons

To circumvent the deleterious actions of supra-millimolar \( \text{H}_2\text{O}_2 \) on the integrity of neuronal membranes, we tested the actions of lower concentrations of \( \text{H}_2\text{O}_2 \) on the excitability of AH neurons. We settled on a test concentration of 0.88 mM (sub-millimolar \( \text{H}_2\text{O}_2 \)) because this concentration elicited reproducible results within the time period of a typical recording. Overall we found that within 5–10 min of wash-in of sub-millimolar \( \text{H}_2\text{O}_2 \), the RMP of AH neurons had hyperpolarized by 5–10 mV (Fig. 5A), and this was associated with the abolition of the slow AHP, while under voltage clamp, there was a large decrease (>50%) in the amplitude of the \( I_{\text{AHP}} \) (Fig. 5B; \( i \) and \( ii \); Table 1). The abolition of the slow AHP and the hyperpolarization persisted after washout of \( \text{H}_2\text{O}_2 \). Ramp depolarizations (~100 to ~30 mV) applied under voltage clamp, in the absence and in the presence of \( \text{H}_2\text{O}_2 \), revealed...
that several ionic conductances were affected by H₂O₂ treatment. The high membrane resistance near the resting potential (−50 to −60 mV; Fig. 5Ci, control) decreased after wash-out of H₂O₂ as did the inward rectification at potentials negative of the RMP (Fig. 5Ci, H₂O₂). In the presence of H₂O₂, the ramp current was increased in slope and became quasi-linear while its reversal potential was shifted negative (Fig. 5Ci, H₂O₂). On average, the peak outward ramp current at −30 mV was increased from 121 ± 26 to 234 ± 56 pA (n = 15; P < 0.05) while the peak inward current at potentials between −100 and −90 mV was decreased from −347 ± 63 to −269 ± 51 pA (n = 15; P < 0.05).

To investigate the possibility that the slow AHP was decreased because H₂O₂ activated the same conductance that was responsible for the slow AHP (Iₘ,AH), in three AH neurons we tested the action of clotrimazole (10 μM), a fungicide that has been shown to inhibit the Iₘ,AH (Shah et al. 2001; Vogalis et al. 2002) (see Fig. 6B). As illustrated in Fig. 5A, in an AH neuron hyperpolarized by H₂O₂, clotrimazole repolarized the membrane potential toward its pre-H₂O₂ level while partially restoring the slope of the ramp current (Fig. 5Ci, clotrimazole). As shown in Fig. 5Ci, the current that was blocked by clotrimazole reversed at −86 mV and showed weak inward rectification, confirming that it was carried by K⁺.

Treatment with H₂O₂ also activated a sustained outward ramp current of variable magnitude that persisted after washout of H₂O₂ (Fig. 6Aii). This current was insensitive to clotrimazole at a concentration (10 μM) that was sufficient to block the residual Iₘ,AH (Fig. 6B). The clotrimazole-insensitive H₂O₂-induced current was blocked, however, by a high concentration of TEA (30 mM; Fig. 6Aiii). In three neurons pretreated with H₂O₂, TEA repolarized their membrane potential from −71 ± 6.3 mV back to −54.3 ± 3.6 mV, while their Rₚ was increased from 197 ± 97 to 598 ± 68 MΩ, indicating that the clotrimazole-insensitive current contributes to the hyperpolarization elicited by H₂O₂. The ramp I-V relationship of the TEA (30 mM)-sensitive current displayed prominent inward rectification and reversed near Eₗ (Fig. 6C).

In three AH neurons, in which a sufficiently large-amplitude outward current persisted after washout of H₂O₂, we tested the effect of glybenclamide (25–50 μM). Using ramp depolarizations, we found that after activation of the outward current by H₂O₂, application of glybenclamide reduced the current (at −30 mV) to 37 ± 11% (Fig. 7C). In addition, in three AH neurons that were pretreated with glybenclamide (50 μM), H₂O₂ failed to elicit a significant hyperpolarization (RMP in glybenclamide alone, −59.7 ± 6.3 mV; glybenclamide and H₂O₂ −59.7 ± 4.0 mV), nor did it alter their Rₚ (305 ± 56 vs. 267 ± 94 MΩ). A 10-fold lower concentration of glybenclamide (1–2 μM), when applied after H₂O₂ exposure, failed to restore the RMP of the hyperpolarized neurons; RMP after H₂O₂ treatment averaged −62.8 ± 2.5 mV, and this hyperpolarized further to −71.3 ± 3.2 mV after application of 1–2 μM glybenclamide. At the higher concentrations, glybenclamide (25–50 μM) alone hyperpolarized AH neurons from an average of −53.4 ± 1.3 to −61.6 ± 3.5 mV (n = 5), but this was not associated with any change in Rₚ (control, 257 ± 30 MΩ; glybenclamide, 262 ± 43 MΩ, n = 5) nor did glybenclamide (25–50 μM) affect the Iₘ,AH (control, 46 ± 16 pA; glybenclamide, 52 ± 15 pA, n = 5).

The actions of glybenclamide applied after H₂O₂ treatment are illustrated in an AH neuron that was filled with catalase (25 mg/ml; see following section) in Fig. 7. Application of sub-millimolar H₂O₂ hyperpolarized this neuron and decreased the amplitude of evoked slow AHPs (Fig. 7A). The hyperpolarization was associated with a decrease in Rₚ (Fig. 7B, i and ii), which was decreased further after washout of H₂O₂ (Fig. 7Biii) as the neuron hyperpolarized further (Fig. 7A). Subsequent application of glybenclamide restored the RMP and the Iₘ,AH (Fig. 7Biv). Digital subtraction of the ramp currents recorded after washout of H₂O₂ and afterwash-in of glybenclamide (Fig. 7C) revealed that the glybenclamide-sensitive current had a reversal potential near Eₗ and showed inward rectification (Fig. 7Cii).

Suppression of a hyperpolarization-activated inward current (Iₖp) by H₂O₂

Hydrogen peroxide consistently decreased the magnitude of an inwardly rectifying current at potentials negative of −55 mV.

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**FIG. 6.** Sub-millimolar H₂O₂ activates a clotrimazole-insensitive outward current. A, i: ramp currents (bottom) evoked by ramp depolarizations (top) in an AH neuron, under control conditions, and after treatment with sub-millimolar H₂O₂. Note suppression of the peak inward ramp current and the activation of a small outward current near the resting potential. ii: application of clotrimazole inhibited the inward current at negative potentials but failed to suppress the outward current. Additional application of a high concentration of TEA (30 mM) nearly maximally inhibited the outward current near the RMP. B: corresponding I-MAPs recorded under the three conditions. Note inhibition of I-MAP by H₂O₂ and then block by clotrimazole. C: ramp I-V relationship of the TEA (30 mM)-sensitive outward current that displayed significant inward rectification at potentials positive to −50 mV.
neurons in which H₂O₂ activated only small-amplitude out-

Effects of internal EGTA, Ca²⁺-free bathing solution and forskolin and caffeine

To determine whether Ca²⁺ was required for H₂O₂ to activate the gₛAHP, we performed two series of experiments in which Ca²⁺ entry and Ca²⁺ release were compromised. First patch pipettes were filled with an internal solution containing 10 mM EGTA, which eliminated the slow AHP while having little effect on the RMP or Rₘ (Table 2). Under these conditions, H₂O₂ hyperpolarized the RMP in three of the six neurons tested from −60.6 to −66.7 mV, but overall neither the RMP nor the Rₘ were altered significantly by H₂O₂ (Table 2). Second, the slow AHP was eliminated by perfusing neurons with Ca²⁺-free Krebs solution. This slightly depolarized neurons and made them fire spontaneously (Fig. 9A; Table 2). As expected, the Ca²⁺-dependent shoulder on the repolarizing phase the action potential was abolished (Fig. 9Ai; Table 2). Application of H₂O₂ (0.88 mM) significantly hyperpolarized all five of these neurons, and this was associated with a significant increase in Rₘ (Fig. 9B, i and ii; Table 2). This increase in Rₘ was largely due to the inhibition of Iᵥ, which was decreased from −402.1 ± 108.5 to −319.9 ± 99.9 pA (P < 0.05), as measured at ramp potentials between −100 and −90 mV (Fig. 9C).

To demonstrate that H₂O₂ can activate a K⁺ conductance that is distinct from gₛAHP, we blocked the slow AHP with forskolin (1 µM), which had no significant effect on either the RMP or Rₘ of AH neurons (Table 2). Application of H₂O₂ (0.88 mM), however, hyperpolarized these neurons by −10 mV and decreased their Rₘ by 30% (Table 2). This hyperpolarization was maintained after washout of H₂O₂ and was associated with the activation of a net outward current at −55 mV. The residual slow AHP was also significantly decreased by H₂O₂ in the presence of forskolin (Table 2).

In three other AH neurons, we also tested the effect of H₂O₂ after suppression of the slow AHP with caffeine (4 mM). Caffeine did not affect the RMP (control, 62.3 ± 6.0 mV; caffeine, −58.3 ± 3.3 mV, n = 3) but increased Rₘ from 287 ± 64 to 477 ± 95 MΩ (n = 3). Application of H₂O₂ (0.88 mM) hyperpolarized these neurons by an average of 6 mV and significantly decreased Rₘ in all three cells to 415 ± 82 MΩ (P < 0.05), which was associated with activation of a sustained outward current.

Effects of enhancement of intracellular anti-oxidant capacity on H₂O₂-mediated actions

We added exogenous anti-oxidants to the internal pipette solution to determine whether the actions of H₂O₂ were sensitive to the capacity of cells to tolerate oxidative stress. First we tested the effect of adding catalase to the internal pipette solution (25–50 mg/ml). Under these conditions, the electrical properties of AH neurons were largely unchanged (Table 3). Application of H₂O₂ (0.88 mM) hyperpolarized the RMP of AH neurons (Fig. 10A; see also Fig. 8A), and this was associated with a substantial decrease in Rₘ (Table 3). In three of the
catalase-filled AH neurons, the peak inward ramp current (attributed to \( I_h \)) was decreased by 14% from −223 ± 114 to −191 ± 22 pA after application of \( H_2 O_2 \). Both the slow AHP and \( I_{sAHP} \) were decreased in the continued presence of \( H_2 O_2 \) (Table 3), but this outward current was decreased lesser (by 39%) than it was decreased in neurons that were not perfused internally with catalase (62%; Table 1). We also noted that during wash-in of \( H_2 O_2 \), the \( I_{sAHP} \) increased transiently (Fig. 10B, i and ii), and this was associated with an increase in the duration of the slow AHP (Fig. 10B, iii and iv). This transient increase in the \( I_{sAHP} \) was generally not seen in neurons that had not been filled with catalase. Washout of \( H_2 O_2 \) led to a modest recovery of the RMP and \( R_{in} \) (Table 3).

We also examined the effect of dialyzing into AH neurons exogenous GSH (5 mM), which is utilized by glutathione reductase to break down \( H_2 O_2 \) to water. As with catalase, internal GSH alone had no effect on the basal electrical firing properties of AH neurons (Table 3). Addition of \( H_2 O_2 \) hyperpolarized neurons by <5 mV, which partially repolarized in the continued presence of \( H_2 O_2 \). But unlike in catalase-filled neurons, there was no detectable change in \( R_{in} \) (Table 3). The peak amplitude of the \( I_{sAHP} \) was also decreased by application of \( H_2 O_2 \) by 54%, and the peak inward ramp current (at potentials between −100 and −90 mV) revealed that \( I_h \) was also decreased from an average of −281 ± 43 to −159 ± 26 pA (n = 6; \( P < 0.05 \)) after 5–10 min of exposure to \( H_2 O_2 \). These results indicate that the weak hyperpolarization elicited by \( H_2 O_2 \) in GSH-filled neurons was attributable largely to the inhibition of \( I_h \). Interestingly, washout of \( H_2 O_2 \) in four of these neurons was associated with a second hyperpolarizing phase and an associated increased in the outward ramp current.

**DISCUSSION**

**Properties of AH neurons in primary culture**

Myenteric neurons comprise a varied population of neurons, ranging from tonically firing neurons to ones that fire phasically at the onset of a stimulus pulse. The latter population includes AH neurons characterized by having broad-duration action potentials with a prominent Ca\(^{2+}\)-dependent “hump” and for generating prolonged afterhyperpolarizations (slow AHPs). These events last for 5–30 s and are responsible for conﬁrming on these neurons a pronounced adaptation in firing of GABA and acetylcholine, which may be important for the coordination of smooth muscle contractions.
AHPs and the corresponding physiological properties of AH neurons in intact ganglia is the comparatively smaller amplitude of slow AHPs recorded from neurons in intact ganglia (Vogalis et al. 2001) despite the fact that the cell capacitances of the two populations were similar. This suggests that trophic factors in intact ganglia may be important in regulating the density of slow AHP channels. Just over 60% of neurons that we patched in our culture system showed no AHPs. The remaining neurons with larger cell bodies. Cells with a characteristic bipolar appearance lacked any regenerative activity and were assumed to be glial cells. The strong representation of AH neurons in our cultures was probably lower because we had an AH neuronal phenotype, but the actual proportion of AH neurons in our cultures was probably lower because we deliberately selected neurons with larger cell bodies. Cells with a characteristic bipolar appearance lacked any regenerative activity and were assumed to be glial cells. The strong representation of AH neurons in our culture system indicates that it can be used in the future to explore the mechanisms affecting expression of AH neuron-specific conductances.

**Multi-targeted actions of H₂O₂ on AH neurons**

Hydrogen peroxide is generated within all cells that undergo oxidative phosphorylation, and although it is relatively inert, H₂O₂ freely diffuses across cell membranes (Halliwell 1992). Its potential as a source of destructive ROIs is minimized by intracellular enzymes such as catalase and glutathione reductase which convert H₂O₂ to nonreactive molecular oxygen and water, thereby keeping its radius of reactivity short. In the present study, we tested the actions of exogenous H₂O₂ by adding it to the bathing solution, thus mimicking the actions of ROIs produced by activated resident macrophages and extravasated neutrophils (Droge 2002) and also the effects of intracellularly generated H₂O₂ during reoxygenation. A consistent finding in the present study was that H₂O₂ decreased the amplitude of action potentials in both AH and in non-AH neurons by ~10 mV. This effect persisted after washout of the H₂O₂ and suggests that H₂O₂ has a direct effect on voltage-gated rectifying current at negative potentials (i.e., when the cell is hyperpolarized).

**Table 3. Effect of H₂O₂ on AH neurons’ electrical properties perfused internally with two anti-oxidants**

<table>
<thead>
<tr>
<th></th>
<th>Catalase (50 mg/ml)</th>
<th>GSH (5 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>H₂O₂</td>
</tr>
<tr>
<td>RMP, mV</td>
<td>−58.3 ± 1.2</td>
<td>−64.8 ± 2.2*</td>
</tr>
<tr>
<td>Rm, MΩ</td>
<td>226 ± 39</td>
<td>181 ± 32</td>
</tr>
<tr>
<td>AP_{amp}, mV</td>
<td>83.9 ± 4.5</td>
<td>78.8 ± 5.7</td>
</tr>
<tr>
<td>AP_{damp}, ms</td>
<td>2.05 ± 0.17</td>
<td>1.98 ± 0.14</td>
</tr>
<tr>
<td>sAHP, mV</td>
<td>5.6 ± 0.9</td>
<td>3.1 ± 0.7*</td>
</tr>
<tr>
<td>I_{AHP,−55 mV}, pA</td>
<td>113 ± 15</td>
<td>69 ± 13*</td>
</tr>
<tr>
<td>τ_{damp}, s</td>
<td>8.1 ± 1.5</td>
<td>30.5 ± 5.6*</td>
</tr>
</tbody>
</table>

Catalase (25–50 mg/ml) and glutathione (GSH; 5 mM) were the anti-oxidants used. Values are means ± SE; number of cells are in parentheses. *, significantly different (P < 0.05; paired t-test) from corresponding control mean.
depolarization, which was associated with a further decrease in $R_{in}$, indicated that H$_2$O$_2$ or H$_2$O$_2$-derived ROIs may activate an additional conductance, possibly permeable to cations (Herson et al. 1999; Jeulin et al. 2002). It is also likely, however, that the delayed depolarization may have resulted from breakdown of the cell membrane initiated by lipid peroxidation (Halliwell 1992), leading to the loss of selective permeability (Lim et al. 2002). This is supported by the granular appearance of the surface membrane of neurons.

Membrane hyperpolarization associated with suppression of $I_h$

A characteristic feature of AH neurons is a prominent hyperpolarization-activated depolarizing “sag” that is due to the activation of a hyperpolarization-activated cation current ($I_h$) (Galligan et al. 1997). This current activates at potentials negative to about −50 mV and opposes the slow AHP from hyperpolarizing the membrane potential to $E_K$. Using both ramp depolarizations and voltage steps, we have demonstrated that $I_h$ is strongly and irreversibly inhibited by H$_2$O$_2$, and this effect is due to a decrease in the maximal available conductance by ~30–40%. An analogous current in cardiac myocytes ($I_h$) is also inhibited by H$_2$O$_2$ (Ward and Giles 1997).

Inhibition of the $I_h$ alone would be expected to hyperpolarize AH neurons and to increase $R_{in}$ because in ganglionic AH neurons (Rugiero et al. 2002) as in other sensory neurons (Doan and Kunze 1999), this current is partially activated at the rest. Under normal conditions, however, H$_2$O$_2$ (0.88 mM) decreased $R_{in}$ by 31% as it hyperpolarized AH neurons. This was because the inhibition of $I_h$ was accompanied by the activation of at least two outward K$^+$ currents. Although it is possible that activation of these currents in the soma may have led to the apparent suppression of $I_h$ as a result of reduced space clamp of distant regions of the cell where $I_h$ channels are expressed, we found that in a minority of AH neurons in which H$_2$O$_2$ elicited relatively small outward currents, $I_h$ was decreased to a similar extent, ~20–30%. This suggests that H$_2$O$_2$ decreases either the unitary conductance of $I_h$ channels or decreases the number of channels that can open.

At present it is unclear whether H$_2$O$_2$ directly acts on $I_h$ channels or another ROI derivative is involved in the suppression of $I_h$. We found that internal perfusion of catalase inhibited the H$_2$O$_2$-mediated suppression of $I_h$, suggesting that the inhibitory action of H$_2$O$_2$ is mediated in part by H$_2$O$_2$-derived radicals, probably including hydroxyl radical formed by the reaction of H$_2$O$_2$ with cytoplasmic Fe$^{2+}$. This conclusion is supported by our finding that in AH neurons filled with EGTA (10 mM), which reduces the generation of hydroxyl radicals by chelating divalent metal ions (Britigan et al. 1998), there was no net change in either the RMP nor $R_{in}$ in response to H$_2$O$_2$.

Suppression of the slow AHP by H$_2$O$_2$

Although the hyperpolarization elicited by H$_2$O$_2$ occurred simultaneously with suppression of the slow AHP, the latter effect was unlikely to be due to inhibition of Ca$^{2+}$ entry because the Ca$^{2+}$-dependent “hump” of the action potentials remained intact. Neither was it attributable to a reduction in driving force occasioned by the hyperpolarization because under voltage-clamp, the current underlying the slow AHP,
$I_{\text{sAHP}}$ was also strongly suppressed by H$_2$O$_2$. It is possible that H$_2$O$_2$ blocks the release of Ca$^{2+}$ from internal stores triggered by Ca$^{2+}$ entry (CICR), which is critical for generation of the slow AHP and the $I_{\text{sAHP}}$ (Vogalis et al. 2001). Both H$_2$O$_2$ and superoxide, however, have been shown to activate Ca$^{2+}$-release channels in cardiac muscle (Boraso and Williams 1994; Kawakami and Okabe 1998), although it is possible that the sustained opening of oxidized Ca$^{2+}$ release channels may compromise the ability of stores to sequester Ca$^{2+}$, leading to the abolition of CICR, and the loss of the slow AHP.

In AH neurons perfused internally with catalase, the suppression of the slow AHP was preceded by a transient enhancement of the $I_{\text{sAHP}}$ and by an increase in the duration of the slow AHP, an effect that lasted for several minutes. This action is similar to the effect of HX/XO on AH neurons in situ (Wada-Takahashi and Tamura 2000), suggesting that the AH neurons perfused internally with catalase mimic more closely the responses of intact neurons to oxidative stress and highlight the importance of endogenous anti-oxidants.

Catalase, which breaks down H$_2$O$_2$ into water and molecular oxygen (Fridovich 1995), may lead to the transient overproduction of superoxide from oxygen by the ETC. This in turn may enhance CICR and thereby prolong slow AHPs. This possibility is supported by the threefold increase in the time constant of deactivation of the $I_{\text{sAHP}}$ during H$_2$O$_2$ application (Table 3), suggesting that the release of Ca$^{2+}$ from stores is prolonged. On the other hand, the eventual suppression of the $I_{\text{sAHP}}$ may be the result of the oxidation of the $I_{\text{sAHP}}$ channels by H$_2$O$_2$ and hydroxyl radicals, as demonstrated for large-conductance Ca$^{2+}$-activated K$^+$ (BK) channels (DiChiara and Reinhart 1997). This is consistent with our finding that in AH neurons perfused internally with catalase and GSH, both of which would blunt the generation of hydroxyl radicals, the $I_{\text{sAHP}}$ was inhibited to a lesser degree by H$_2$O$_2$ than in unfilled neurons.

We found that clotrimazole at concentrations that blocked the $I_{\text{sAHP}}$ reversed a large proportion of the hyperpolarization elicited by H$_2$O$_2$. This suggests that a component of the hyperpolarization was mediated by the opening of $I_{\text{sAHP}}$ channels. This was especially true at the onset of H$_2$O$_2$ application, which is consistent with H$_2$O$_2$ or H$_2$O$_2$-derived ROIs enhancing CICR, thereby prolonging the duration of the intracellular Ca$^{2+}$ transient. Although at present the exact mechanism by which the $I_{\text{sAHP}}$ channels are activated by Ca$^{2+}$ is not known, it is possible that H$_2$O$_2$ may also act through enzymatic mediators that control channel gating. However, the fact that the transient enhancement of the $I_{\text{sAHP}}$ was not seen in AH neurons in which the slow AHP was suppressed, either with internal EGTA or Ca$^{2+}$-free bathing solution, suggests intracellular Ca$^{2+}$ is necessary for activation of this conductance.

**Nature of the sustained outward currents activated by H$_2$O$_2$**

Glybenclamide, a blocker of $K_{\text{ATP}}$ channels (Ashcroft and Gribble 1998), had little or no effect on the $I_{\text{sAHP}}$ but inhibited the sustained component of the H$_2$O$_2$-induced outward current that was resistant to clotrimazole. In addition, this sustained outward current was blocked by a high concentration (30 mM) of external TEA. These features indicate that the sustained outward current induced by H$_2$O$_2$ is mediated by the opening of $K_{\text{ATP}}$ channels, and activation of these channels may underlie the hyperpolarization elicited under conditions where the $g_{\text{sAHP}}$ was eliminated with forskolin and caffeine. Although $K_{\text{ATP}}$ channel currents have not been recorded from AH neurons, tolbutamide (500 µM), another blocker of $K_{\text{ATP}}$ channels, inhibited the hyperpolarization elicited by reduction of the external glucose concentration in AH neurons in situ (Liu et al. 1997). Moreover these neurons were immunoreactive for the sulfonylurea receptor (SUR1) and for $K_{\text{c,6.2}}$ channel subunits (Liu et al. 1997). However, given the relatively high concentration of glybenclamide (25–50 µM) required to inhibit the H$_2$O$_2$-induced sustained outward current, it is likely that the $K_{\text{ATP}}$ channels expressed in AH neurons may also be composed of the less-sensitive SUR2 subunits (Bryan and Aguilar-Bryan 1999).

ROIs such as H$_2$O$_2$ are known to activate $K_{\text{ATP}}$ channels in cardiac myocytes (Goldhaber et al. 1989; Tokube et al. 1998) and in pancreatic β cells where H$_2$O$_2$ (>30 µM) inhibits insulin secretion (Nakazaki et al. 2000). In cardiac myocytes, H$_2$O$_2$ (1 mM) was shown to directly and irreversibly activate $K_{\text{ATP}}$ channels in excised patches, by decreasing their sensitivity to inhibition by ATP (Ichinari et al. 1996). In the present study, we obtained conventional whole cell patch recordings using pipettes that had resistances of between 5 and 8 MΩ and that were filled with internal solution containing 2 mM ATP. Under these internal conditions, it is likely that activation of $K_{\text{ATP}}$ channels by H$_2$O$_2$ occurred by a direct action of H$_2$O$_2$ or one of its derivatives on the $K_{\text{ATP}}$ channels. However, the rebound hyperpolarization that occurred in AH neurons perfused internally with GSH after washout of H$_2$O$_2$ is suggestive of possible inhibition of ATP synthesis (Krippert-Drews et al. 1997).

**Functional significance of decrease in excitability of AH neurons by H$_2$O$_2$**

Extravasated phagocytic neutrophils and activated macrophages release H$_2$O$_2$, whose concentration may approach ≥100 µM in their immediate vicinity (Droge 2002). This suggests that the effects that we have described using sub-millimolar H$_2$O$_2$ may reflect severe pathophysiological conditions within intestinal ganglia as well as the effects of intraneuronally generated H$_2$O$_2$ after reoxygenation. Infiltration of neutrophils into the musculature and the production of ROIs is known to occur after intestinal transplantation (Turler et al. 2002). Studies on innervated ileal muscle strips have shown that exogenous sub-millimolar H$_2$O$_2$ has an initial potentiating action on nerve-mediated contractions and then inhibits them (Moummi et al. 1991). Our own results suggest that H$_2$O$_2$ and other ROIs will have marked actions on reflexly initiated contractile activity that involves AH neurons. Our results also highlight the importance of the intracellular anti-oxidants in resisting the effects of oxidative stress, and the irreversible nature of these effects suggests that recovery from a bout of acute oxidative stress may require renewed protein synthesis in the surviving neurons.

In summary, H$_2$O$_2$ has potent actions on the electrical activity of AH intestinal neurons grown in primary culture by affecting several different membrane conductances. Chief among these actions is inhibition of an $I_h$ current and a concomitant suppression of the slow AHP and activation of a $K_{\text{ATP}}$-channel-mediated outward current. Higher concentra-
tions of H₂O₂ also activate after a delay an inward current that irreversibly depolarizes neurons and is associated with necrosis. Future studies will focus on the actions of specific ROIs on the specific ionic conductances. We will also utilize the HX/XO reaction in the absence or presence of SOD and/or catalase to determine the identity of the offending ROI with the aim of developing anti-oxidant therapies.

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F. Vogalis is a CR Roper Fellow, University of Melbourne.

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