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A Comparison of the Effects of Cumene Hydroperoxide and Hydrogen Peroxide on Retzius Nerve Cells of the Leech *Haemopis sanguisuga*

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Abstract: Oxidative stress and the production of reactive oxygen species are known to play a major role in neuronal cell damage, but the exact mechanisms responsible for neuronal injury and death remain uncertain. In the present study, we examined the effects of oxidative stress on spontaneous spike activity and depolarizing outward potassium current by exposing the Retzius neurons of the leech to cumene hydroperoxide (CHP) and hydrogen peroxide (H_2O_2), the oxidants commonly used to examine oxidative mechanisms mediating cell death. We observed that relatively low concentrations of CHP (0.25, 1, and 1.5 mM) led to a marked prolongation of spontaneous repetitive activity. The prolonged action potentials showed an initial, spike-like depolarization followed by a plateau phase. In contrast, H_2O_2 at the same and much higher concentrations (0.25 to 5 mM) did not significantly change the duration of spontaneous spike potentials of leech Retzius nerve cells (LRNCs). In the voltage clamp experiments, calcium-activated outward potassium currents, needed for the repolarization of the action potential, were suppressed with CHP, but not with H_2O_2. The present findings indicate that CHP is a more potent oxidant and neurotoxin than H_2O_2 and that the effect of CHP on the electrophysiological properties of LRNCs may be due to the inhibition of the potassium channels.
Key words: cumene hydroperoxide, hydrogen peroxide, leech, oxidative stress, potassium currents

Introduction

Based on numerous results accumulated over the past decade, it is clear that oxidative stress and production of reactive oxygen species (ROS) are involved in neuronal damage, but the precise mechanisms responsible for neuronal injury and death are not understood completely. Electrophysiological studies have shown that oxidative stress may affect cell membrane. The cell membrane would seem of special interest because of its large surface area and because of the vulnerability of membrane lipids and proteins to oxidative damage. Cell membranes are either a source of neurotoxic lipid peroxidation products or the target of pathogenic processes that cause permeability changes or ion transport pathway modifications [3]. Experimental evidence indicates that the effects of reactive oxygen metabolites on membrane properties could include changes in membrane potential and current, ionic gradients, action potential duration and amplitude, spontaneous activity, and excitability [28]. Considering neuronal function, ROS can modify ion channels and transporters directly or indirectly via peroxidation of membrane lipids [6, 19] and by affecting associated signaling molecules [14].

Several previous studies indicate that a major form of ROS, hydrogen peroxide (H_2O_2), alters energy metabolism, ATP-sensitive K^+ currents, L-type Ca^{2+} currents [11, 24] and delayed rectifier K^+ currents [10]. The

H_2O_2-derived hydroxyl radical (HO•) has been proposed as the species initiating lipid peroxidation. In addition to initiating lipid peroxidation, HO• has been implicated in direct cellular damage. According to Zhu *et al.* (2005), H_2O_2 may mediate cell damage either through direct oxidation of lipids, proteins, and DNA or by acting as a signaling molecule to trigger intracellular pathways leading to cell death [36]. It is well known that the effects of H_2O_2 on neuronal membranes are not simply due to lipid peroxidation but also to alterations of cell membrane proteins and intracellular signaling pathways. Although H_2O_2 is generally considered to be a toxic by-product of respiration, recent evidence suggests that at low levels, H_2O_2 also functions as a signaling molecule, particularly in higher organisms [27]. H_2O_2 can also mediate subsecond signaling via activation and/or inhibition of the ion channels. H_2O_2 can regulate the function of calcium channels by directly altering the thiol redox state of proteins. The observations of Zou *et al.* (2011) suggest that increased levels of reduced glutathione (GSH) may be a major component of the protection observed by the overexpression of the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) and the efficiently protected t-BHP-induced mitochondrial membrane potential loss and apoptosis in cultured retinal pigment epithelial cells [37]. A recent paper reported that relatively low concentrations (100 μM) of cumene hydroperoxide (CHP) led to a significant decrease in the cellular content of ATP and GSH [33].

Electrophysiological analyses showed that a Ca^{2+}-activated K^+ current constituted the major outward current in Retzius nerve cells [26]. According to Frey *et al.* (1998), the Ca^{2+}-dependent K^+ channel may play a role in generating the resting membrane potential of leech Retzius neurons, as it shows maximum activity at the physiological intracellular Ca^{2+} concentration [9]. Ca^{2+}-activated K^+ channels have been shown to contribute to the repolarization phase of the action potential, the rate of firing, and the slow afterhyperpolarization responsible for spike frequency adaptation [20].

The purpose of the present study was to examine the effect of long lasting oxidants on a simple system such as an isolated leech nerve cell. Bearing in mind the importance of H_2O_2 and CHP in the pathophysiology of both invertebrates and vertebrates, we compared their electrophysiological effects on our model. The accessibility and simple organization of the nervous system of invertebrates has proven exceptionally useful for electrophysiological investigations.

Materials and Methods

All experiments were carried out at room temperature (22–25°C) on Retzius nerve cells of isolated abdominal segmental ganglia of the ventral nerve cord of the horse leech *Haemopis sanguisuga*. The method of dissection has been previously described [4] and complies with institutional research council guidelines. Dissected segments of 4 ganglia were immediately transferred to a 2.5 ml plastic chamber with a leech Ringer's solution (for the composition, see Solutions) and fixed by means of fine steel clips. The plastic chamber was then placed in a grounded Faraday cage mounted on a fixed table in a manner that prevents vibrations. Identification and penetration of the cells were performed in the cage under a stereomicroscope. The Retzius neurons were identified based on the size, characteristic position in the ganglion, and the bioelectrical properties of the cells. The Retzius cells, the largest neurons (40–60 μm in diameter) in the leech central nervous system, are situated on the ventral side of the ganglia, exhibit stable resting membrane potential and are nonbursting neurons with a low spontaneous firing rate. Prior to the experiments, the chamber was flushed with fresh leech Ringer's solution. A microelectrode was dipped into the solution and allowed 20–30 min for equilibration.

Electrical methods

The recordings were carried out by the use of conventional glass microelectrodes filled with 3 M KCl. The microelectrodes were manufactured with borosilicate glass capillaries (1.5 mm outside diameter, 0.6 mm inside diameter, Clark Electromedical Instruments, Edenbridge, UK) using a puller (Industrial Associates, Inc., Ridgewood, NY, USA) and then filled with a 3 M KCl-containing solution shortly after being pulled. Microelectrodes with a resistance ranging from 5 to 10 MΩ (when filled with 3 M KCl solution) and tip potentials less than 5 mV were selected for use. The recordings were amplified using a Bioelectric Instrument DS2C high input resistant amplifier. Microelectrodes were connected to the amplifier *via* an Ag-AgCl wire. The ground electrode was an Ag-AgCl wire in a separate chamber filled with Ringer solution connected to the experimental chamber by a 3 mM KCl 3% agar bridge. Activity K^+ channels

Table 1. The effects of the bath application of cumene hydroperoxide (CHP) on the action potential duration (APD) of the Retzius nerve cells of the leech

CHP (mM)	Control APD (ms) (Leech Ringer)	APD (ms) (CHP)	Δ (ms)	P	n
0.25	10.45 ± 0.98	14.40 ± 0.80	3.95 ± 1.18	>0.05	6
1	9.66 ± 0.52	35.33 ± 13.60	25.67 ± 7.92	≤0.01	6
1.5	9.66 ± 2.18	127.80 ± 15.95*	118.14 ± 12.04	≤0.01	11

Data shown as means ± SD. P: t-test significance level. n: number of cells. *: repetitive firing.

were studied in leech Retzius neurons by using the voltage-clamp technique. This was shown in voltage-clamped neurons by long depolarizing steps (to 300 ms) from the holding potential, which was more negative than −40 mV in a sodium-free leech Ringer solution, in order to induce fast and slow K^+ outward currents. Command pulses derived from a Tektronix 161 pulse generator. Voltage and current records were displaced on a Tektronix 564 oscilloscope. The signals were digitized by the use of an analog-to-digital converter (Digidata 1200; Axon Instruments, Jakarta, Indonesia) and saved in a computer for off-line analysis. The data were leak corrected by using hyperpolarizing pulses of equal magnitude and assuming a constant leak conductance.

Solutions

The normal leech saline solution (nutrient-free leech saline solution) contained (in mM) 115 NaCl, 4 KCl, 2 $CaCl_2$, 1.2 Na_2HPO_4, and 0.3 NaH_2PO_4 (pH 7.2). In the Na^+-free Ringer, 115 mM NaCl was completely replaced with an equal amount of Tris (hydroxymethyl) amino-methane-Cl (Tris Ringer), and Na_2HPO_4 and NaH_2PO_4 were omitted. Pharmacological agents were prepared and dissolved immediately before application in the physiological salt solution at the concentrations stated. H_2O_2 and CHP were added to the leech or Tris-Ringer. Solutions containing H_2O_2 (Zorka Pharma, Sabac, Serbia) were prepared freshly before their use from 30% H_2O_2 solution and added to the Ringer solution (or Tris-Ringer) at final concentrations of 0.25, 1, 1.5, and 5 mM. The CHP was obtained from Sigma (Sigma, St. Louis, MO, USA), dissolved in 0.01% dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO, USA) and added to the Ringer solution (or Tris-Ringer) in concentrations of 0.25, 1, and 1.5 mM. DMSO influenced neither the resting membrane potential nor the action potential shape of the Retzius neurons. For all drugs, administration was sufficient to reach a steady-state response (up to 30 min). The bath volume was 2 ml, and the solution changes were completed within 30 s.

Data analysis

Data are expressed as means ± SD. Comparisons between the mean values were made with the Student's t-test. P values <0.05 were considered significant.

Results

The effects of CHP and H_2O_2 on the action potential duration of leech Retzius nerve cells

In the first set of experiments, we investigated the time-dependent changes in action potential configuration in LRNCs. Table 1 summarizes the values of action potential duration (APD) in the leech Ringer solution and after adding different CHP concentrations. Exposure of LRNC to CHP (0.25, 1, and 1.5 mM) prolonged the duration of the action potentials in a concentration-dependent manner. The concentration of 0.25 mM did not significantly change, within 20 min, the APD of LRNCs, which decreased from 10.45 ± 0.98 to 3.95 ± 1.18 ms (P>0.05, n=6). During the 20 min of exposure to the leech Ringer solution containing 1 mM CHP, a significant change in APD, to 25.67 ± 7.92 ms (P≤0.01, n=6), and early afterdepolarization were recorded. A higher concentration of CHP (1.5 mM) caused an extreme change in the shape and action potential duration (from 9.66 ± 2.18 to 118.14 ± 12.04 ms; P≤0.01, n=11) in LRNCs. A cardiac-like action potential and early afterdepolarization were recorded. The time course of the CHP effect varied little, and a sustained plateau developed in all treated cells 20 min after their exposure to the CHP. The concentration of 1.5 mM led to the appearance of repetitive firing only a few minutes after the application of CHP, which was followed by loss of excitability of the leech Retzius nerve cells.

Figure 1 illustrates the representative record obtained after the exposure of an isolated ganglion to 1.5 mM CHP. Early afterdepolarization and a cardiac-like action potential with rapid depolarization followed by a sustained depolarization, or a plateau, which was termi-
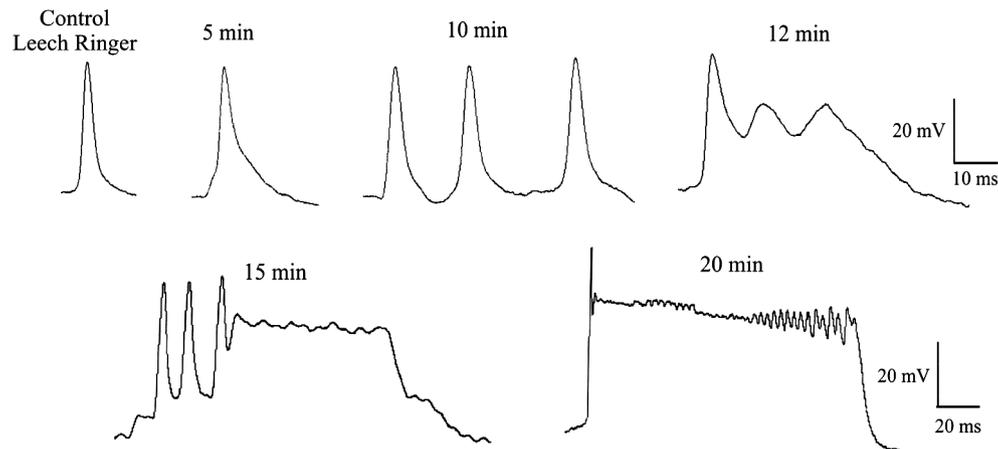
Fig. 1. Representative spontaneous action potentials recorded in leech Retzius nerve cells in leech Ringer solution and at 5, 10, 12, 15, and 20 min after exposure of the isolated ganglion to 1.5 mM CHP. Early afterdepolarization and repetitive firing were recorded.

Table 2. The effects of the bath application of hydrogen peroxide (H_2O_2) on the action potential duration (APD) of the Retzius nerve cells of the leech

H_2O_2 (mM)	Control APD (ms) (Leech Ringer)	APD (ms) (H_2O_2)	Δ (ms)	P	n
0.25	9.45 ± 1.12	10.14 ± 0.86	0.69 ± 0.18	>0.05	7
1	8.67 ± 0.72	9.96 ± 1.64	1.29 ± 0.98	>0.05	8
1.5	9.64 ± 1.48	10.72 ± 1.05	1.08 ± 0.34	>0.05	10
5	9.22 ± 1.14	10.45 ± 1.56	1.23 ± 0.85	>0.05	5

Data shown as means ± SD. *P*: *t*-test significance level. n: number of cells.

Table 3. Comparative effects of cumene hydroperoxide (CHP) and hydrogen peroxide (H_2O_2) on the action potential duration (APD) of the Retzius nerve cells of the leech

Conc. (mM)	Prolongation of APD (ms) in H_2O_2 solution	Prolongation of APD (ms) in CHP solution	P	n
0.25	0.69 ± 0.18	3.95 ± 1.18	≤0.01	5
1	1.29 ± 0.98	25.67 ± 7.92	≤0.01	5
1.5	2.80 ± 1.34	118.14 ± 12.04	≤0.01	5

Data shown as means ± SD. Conc.: applied concentration of H_2O_2 and CHP. *P*: *t*-test significance level. n: number of cells.

nated by rapid repolarization after 250 ms, were recorded.

The APDs of the leech RNC in different H_2O_2 concentrations are presented in Table 2. The results showed that treatment with an external solution containing 0.25, 1, 1.5, and 5 mM H_2O_2 did not significantly change (*P*>0.05), within 20 min, the APD of LRNCs. H_2O_2 is ineffective in generating either early afterdepolarization or cardiac-like action potential in LRNCs. Table 3 shows comparative effects of bath application of CHP and H_2O_2 on spontaneous spike activity of the Retzius nerve cells.

As can be seen in Table 3, H_2O_2 had a significantly smaller effect on the APD of Retzius neurons than CHP. The results obtained in the present study clearly demonstrate that with CHP extensive lengthening of the spike potential, or cardiac-like action potential, could be obtained with leech Retzius nerve cells. The effect of H_2O_2 on spontaneous spike activity was less pronounced.

*The effects of CHP and H_2O_2 on the Ca^{2+}-activated K^+ current of leech Retzius nerve cells*

Considering that the action potentials of LRNCs were

A   B

-30 mV

-20 mV

-7 mV

+3 mV

+10 mV

+17 mV

50 nA

50 ms
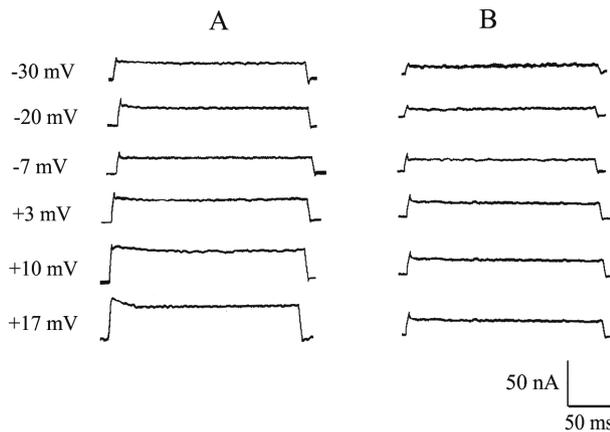
Fig. 2. Voltage clamp current records obtained from Retzius nerve cells in Tris Ringer and at 20 min after adding 1 mM CHP to the Tris Ringer during displacement of the membrane potential from a steady holding level of −73 mV to the potential given on each trace. A: records in Tris Ringer. B: records in CHP (1 mM).

elongated after exposure to CHP, the possibility of modification of membrane potassium currents was examined using the voltage clamp technique. It is well known that action potential duration depends on the activity of Ca^{2+}-activated K^+ channels. In leech Retzius nerve cells, three classes of K^+ channels (fast, slow calcium-activated, and late voltage-regulated) have been identified [4]. The effect on outward currents was studied; this was shown in voltage-clamped neurons by long depolarizing steps (to 300 ms) from the holding potential, which was less than −40 mV in sodium-free leech Ringer solution, in order to induce fast and slow potassium outward currents without any current artefact.

Thus, the prolonged effect of CHP on APD in leech RNC may be due to the inhibition of the Ca^{2+}-activated K^+ currents, which are needed for the repolarization of the action potential. To test this hypothesis, the effect of long lasting oxidants on Ca^{2+}-activated K^+ currents was studied. Figure 2 shows the representative current records obtained before (A) and 20 min after application of 1 mM CHP (B). The experimental procedure was as follows: first the fast and slow outward current was elicited by depolarizing steps from the steady holding level of −73 to +17 mV, and then 1 mM CHP was added and kept in the bath for 20 min. The cell was then washed out with Tris Ringer solution. In the corresponding current-voltage relationship (Fig. 3), a clear reduction in fast and slow components (without complete block) of K^+ outward current in the presence of CHP was ob-

○ Ikr
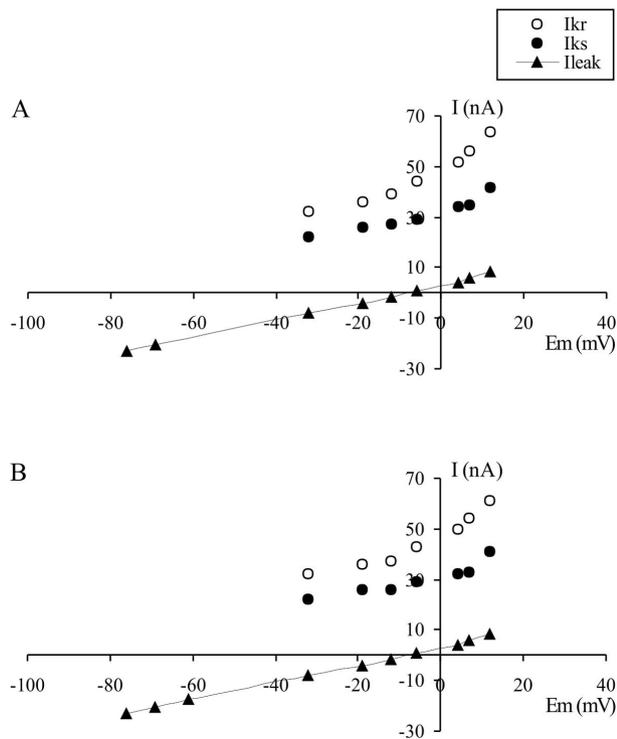● Iks
▲ Ileak

A   I (nA)

Em (mV)

B   I (nA)

Em (mV)
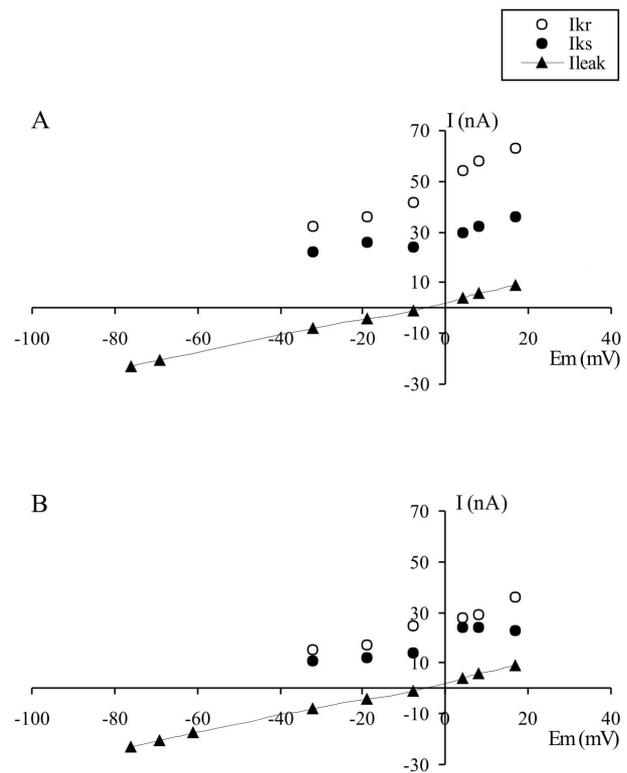
Fig. 3. The current-voltage relationship at the peak of the K^+ outward current (Ikr) and at the end of stimulation (Iks) in the absence (A) and presence of 1 mM CHP (B). Ikr–rapid Ca^{2+}-activated K^+ current; Iks–slow Ca^{2+}-activated K^+ current; Ileak–passive leak current.

tained. At the test potential of +17 mV, the fast and late steady parts of the K^+ outward current dropped from 63 to 36 nA (42.86%) and from 36 to 23 nA (36.12%).

Figure 4 illustrates the effect obtained with another long lasting oxidant, H_2O_2. In contrast to the effect of CHP, application of the H_2O_2 (1 mM) failed to inhibit fast and slow outward K^+ currents in the leech neuron. In the current-voltage relationship (Fig. 4), there were no significant changes in the early or late part of the K^+ outward current in the presence of H_2O_2. At the test potential of +12 mV from the holding potential of −77 mV, the fast and late steady parts of the K^+ outward current dropped by 4.69% (from 64 to 61 nA) and 2.39% (from 42 to 41 nA), respectively.

Discussion

Since long lasting oxidants play a significant role in the pathophysiology of the nervous system from invertebrates to mammalians, we have examined and com-

Fig. 4. The current-voltage relationship at the peak of the K^+ outward current (Ikr) and at the end of stimulation (Iks) in the absence (A) and presence of 1 mM H_2O_2 (B). Ikr–rapid Ca^{2+}-activated K^+ current; Iks–slow Ca^{2+}-activated K^+ current; Ileak–passive leak current.

pared the electrophysiological effects of CHP and H_2O_2. Although each of these oxidants has previously been examined individually in our laboratory and by other authors, comparison between them was either lacking or inconclusive. Our results showed that CHP treatment caused an extreme change in the duration of the action potential and suppression of Ca^{2+}-activated K^+ currents of LRNCs. In contrast, H_2O_2 did not significantly change the membrane properties of LRNCs. It could be concluded that CHP is a more potent oxidant in our model of oxidative stress than H_2O_2. There may exist various explanations for why H_2O_2 did not have a clear effect on the electrophysiological properties of Retzius neurons of the leech:

1. The leech Retzius nerve cells have a low concentration of polyunsaturated fatty acids, which are very sensitive to radical injury.
2. The leech ganglia have an efficient system against oxidative stress.
3. H_2O_2 might not have reached the Retzius neurons within the ganglion capsule.

The first of the above explanations is unlikely, since neuronal membranes are rich in lipids [34]. Another explanation could be that they have an efficient scavenging enzyme system that reacts rapidly with H_2O_2. Since changes in the membrane properties of LRNCs were not significant, it could be expected that upon exposure of LRNCs to external H_2O_2, the H_2O_2 was decomposed by antioxidant enzymes. According to Makino *et al.* (2004), in the nerve cells, antioxidant activity is not sufficiently high, and the membrane permeability for H_2O_2 is possibly an important factor in the antioxidant defense of the nervous system [21]. When cells are exposed to external H_2O_2, the intracellular consumption of H_2O_2 catalyzed by antioxidant enzymes inside the cells provides the driving force for setting up a gradient across the cellular membranes [8].

A more plausible explanation for weak responses of LRNCs to H_2O_2 is that the extracellular H_2O_2 application results in an intracellular concentration below that of the extracellular concentration. Contrary to what is widely assumed, recent studies have demonstrated that H_2O_2 does not freely diffuse across biological membranes. Indeed, in several cell types, such as Jurkat T-cells, a mammalian cell line [2], and *Saccharomyces cerevisiae* [5], it has been shown that H_2O_2 does not freely diffuse across cell membranes. In addition, H_2O_2 is relatively stable and poorly reactive in the absence of transition metals [12]. However, previous studies have shown that a 1 mM H_2O_2 treatment with 0.01 mM $FeCl_2$ did not significantly change the resting membrane potential of LRNCs [18]. Recent studies have suggested that limiting H_2O_2 diffusion into cells has a key role in the protection against extracellular H_2O_2. According to Branco *et al.* [5], the plasma membrane is important in the protection of cells against external H_2O_2, and an active regulation of biomembrane permeability characteristics is part of the cell response to oxidative stress. It is well known that concentration as well as time of exposure plays an important role in the response generated by ROS. The physiologically significant concentration of H_2O_2 may depend on the cell types. The range of $[H_2O_2]$ used by different authors varies from 0.1 to 50 mM [19]. The differences in the effect of the H_2O_2 when it is added to the intracellular or extracellular side imply different access to essential targets. In our experiments, bath application of H_2O_2 did not have a clear effect on the membrane properties of LRNCs. This is consistent with the studies of Soto *et al.* (2002) [25], who found that

H_2O_2 did not affect channel activity when added to the extracellular side, suggesting that the targets of H_2O_2 action are probably located on the intracellular side of K^+ channels.

How can we explain why CHP did have a clear effect on the membrane properties of LRNCs, in contrast to H_2O_2? A possible explanation is that organic hydroperoxides (e.g., CHP) and H_2O_2 generate distinct ROS. The organic hydroperoxide triggers the generation of the free radical intermediates peroxyl and alkoxyl radicals, which can cross cellular membranes and evoke the production of $HO^•$ [16]. In addition, CHP is more hydrophobic than H_2O_2. The present findings indicate that CHP could initiate lipid peroxidation as the mechanism of ROS-induced cell membrane injury. By contrast, H_2O_2 is a weaker oxidizing agent than other ROS, but the biological importance of H_2O_2 stems from its participation in the production of the extremely reactive $HO^•$. $HO^•$ generated by one-electron reduction of H_2O_2 damages adjacent molecules at diffusion-controlled rates. According to Soto *et al.* (2002), the H_2O_2 effect on the Ca^{2+}-activated K^+ channel activity is mediated by $HO^•$ [25]. However, $HO^•$ is effective only near the locus of production due to its extremely short half-life.

Previous studies indicate that large conductance Ca^{2+}-activated K^+ channels are subject to redox regulation. However, there are conflicting descriptions of the current changes induced by oxidants and an incomplete understanding of which is responsible for the observed changes in action potential duration. For example, Matsuura and Shattock (1991) demonstrated that oxidative stress induces a decrease in resting potassium conductance and an increase in Ca^{2+}-activated membrane conductance [22]. Both factors may underlie the depolarization of resting membrane potential, the prolongation of the action potential, and automaticity. Tarr and Valenzeno (1991) demonstrated that brief ROS exposure increased action potential duration, whereas longer exposure reduced action potential duration [29]. Tokube *et al.* (1996) reported biphasic changes in action potential duration, with initial lengthening of the action potential and subsequent shortening [31]. However, conflicting results exist concerning whether oxidation increases or decreases the channel activity. According to Tang *et al.* (2001), oxidizing agents such as H_2O_2 may induce upregulation or downregulation of large-conductance calcium-activated potassium channels [BK(Ca)], depending on experimental conditions [30]. According to

Huang *et al.* (2009), H_2O_2 may modulate open probability of BK (Ca) via the oxidation of cysteine residue [15]. It is thus suggested that the intracellular application of H_2O_2 decreases the open probability of BK(Ca) at a low concentration (<1 mM), and increases or decreases the open probability of BK(Ca) at a high concentration (5 mM). A recent paper reported that ROS donors (H_2O_2 and *t*-BHP) reduced the voltage-operated Ca^{2+} current but increased the amplitude of the delayed rectifier K^+ current in adult rat intracardiac ganglion neurons [7, 35].

Using whole-cell patch-clamp recording, Angelova and Muller (2006) demonstrated that H_2O_2 (80 μM) reduced both voltage-gated transient (I_A) and delayed rectifier K^+ in CA1 neurons in a hippocampal slice [1]. The results of Hasan *et al.* (2007) suggest that oxidative stress, which inhibits the delayed rectifier current, can alter neural activity [13]. Vega-Saenz de Miera and Rudy (1992) reported that H_2O_2 inhibited three cloned voltage-gated K^+ channels expressed in Xenopus oocytes [32]. In contrast to this, another study found that peroxide activates Ca^{2+}-dependent K^+ channels, leading to hyperpolarization and cessation of voltage-dependent Ca^{2+} influx [17]. According to Nani *et al.* (2010), hydrogen peroxide (1 mM; 30 min) induced an inward current or membrane depolarization accompanied by an increase in input resistance, enhanced firing, and depressed spontaneous synaptic events [23].

Taken together, the conclusion may be drawn that an alkyl hydroperoxide, CHP, is a more potent oxidant and neurotoxin than H_2O_2 in our model of oxidative stress. According to results described previously, we can assume that the prolongation of the action potential observed in our study is the result of the CHP effect on Ca^{2+}-activated K^+ channels. The oxidative mechanism of CHP and H_2O_2 action on electrophysiological properties of LRNCs proposed in this paper might have broader significance, pertaining not only to leeches but mammalian neurons as well.

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