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Toxicity Induced by Cumene Hydroperoxide in Leech Retzius Nerve Cells: the Protective Role of Glutathione

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In the present study, we studied the ability of glutathione (GSH) to detoxify exogenously applied cumene hydroperoxide (CHP). Exposure of leech Retzius nerve cells to CHP (1.5 mM) induced a marked prolongation of the spontaneous spike potential of these cells. Early after depolarization, and a cardiac-like action potential with a rapid depolarization followed by a sustained depolarization or plateau, which is terminated by a rapid repolarization were recorded. GSH (0.2 mM) significantly inhibited the effects of CHP on the duration of the action potential and suppressed CHP-induced spontaneous repetitive activity. Voltage-clamp recordings showed that CHP (1.5 mM) caused significant changes in the outward potassium currents. The fast and slow steady part of the potassium outward current was reduced by 46% and 39%, respectively. GSH applied in a concentration of 0.2 mM partially blocked the effect of CHP on the calcium-activated potassium currents. The fast and slow calcium-activated potassium currents were suppressed by about 20% and 15%, respectively. These results suggest that the neurotoxic effect of CHP on spontaneous spike electrogenesis and calcium-activated potassium currents of leech Retzius nerve cells was reduced in the presence of GSH.

Key words: leech, cumene hydroperoxide, glutathione, action potential.

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In comparison with other organs of the body, the brain may, for a number of biochemical, physiological and anatomical reasons, be especially vulnerable to oxidative stress and reactive oxygen species (ROS)-mediated injury. The brain has a high level of metabolic activity, requiring a continuous supply of oxygen. On the other hand, the brain is poor in catalytic activity and has a low level of protective antioxidant enzymes, namely catalase and glutathione peroxidase. Therefore, defense against the neurotoxic effects of ROS is an essential task within the brain. An important component of the cellular detoxification of ROS is antioxidant reduced glutathione (GSH) (DRINGEN & GUTTERER 2002; DRINGEN & HIRRLINGER 2003). The glutathione system exerts a key function in the detoxification of hydrogen peroxide (H_2O_2) by neurons (HASKEW *et al*. 2010; LIMON-PACHECO & GONSEBATT 2010). A growing body of evidence suggests that glutathione plays an important role in the detoxification of ROS in the brain and that glutathione is the main antioxidant molecule in neurons (AOYAMA *et al*. 2008; ESCARTIN *et al*. 2011). The glutathione system of peroxide detoxification in neurons is less efficient than in astroglial cells. Because H_2O_2 is the peroxide generated in the highest quantity in the brain, astrocyte -mediated protection of neurons against the toxicity caused by H_2O_2 appears to be particularly important (DESANGHER *et al*. 1996; LANGEVELD *et al*. 1995). These data support the hypothesis that brain neurons are more vulnerable to oxidative stress than astrocytes due to an insufficient detoxification of ROS via their glutathione system (DRINGEN *et al*. 1999; MARTIN & TEISMANN 2009). Because neurons have limited antioxidant capacity, they rely heavily on their metabolic coupling with astrocytes to combat oxidative stress. In the brain, the nuclear E2-related factor 2 (Nrf2) is the main pathway responsible for cell defense against oxidative stress and maintaining the cellular glutathione level and redox balance (STEPKOWSKI & KRUSZEWSKI 2011; CORREA *et al*. 2011). However, the concentration of glutathione is relatively lower in the brain as compared to other organs of the body (SKAPER *et al*. 1999). In a mouse brain the specific activity of glutathione reductase is 32% and 65% that of the kidney or liver, respectively (HO *et al*.

1997). While H_2O_2 serves as a signaling molecule at physiological levels (FORMAN *et al*. 2010), an excessive amount of these molecules leads to oxidative modification and, therefore, dysfunction of proteins, nucleic acids, and lipids.

One class of compounds has proved to be very useful in studying oxidative damage resulting from lipid hydroperoxide: organic hydroperoxides (DRINGEN *et al*. 1998; CHEN & MASON 2002). Organic hydroperoxides, such as cumene hydroperoxide, are relatively stable but toxic molecules. Their toxicity is partly due to their ability to generate free organic radicals which can react with a variety of biomolecules. Organic hydroperoxide triggers the generation of the free radical intermediates, peroxyl and alkoxyl radicals, which can cross cellular membranes and evoke the production of the hydroxyl radical ($HO^{\bullet}$) (HWANG *et al*. 2009). Cumene hydroperoxide has been employed as a prototypic inducer of oxidative stress in a variety of *in vitro* and *in vivo* systems. According to JOVANOVIC & BELESLIN (1997), cumene hydroperoxide is a more efficient neurotoxin and oxidant than hydrogen peroxide. It was found that a mixture of 1 mM H_2O_2 and 0.02 mM $FeCl_2$ did not significantly change the resting membrane potential of leech Retzius nerve cells (LRNC).

The mechanism of ROS-induced modifications in ion transport pathways involves oxidation of sulfhydryl (SH) groups on the ion transport proteins, lipid peroxidation, and alterations of Ca^{2+} homeostasis. Considering neuronal function, ROS can attack ion channels and transporters either directly, or indirectly by causing lipid peroxidation (CARMELIET 1999) and affecting associated signaling molecules (HOOL 2006). Peroxidation of membrane lipids has been demonstrated to affect various transmembrane processes, such as receptor activation, formation of intracellular second messengers and Ca^{2+} homeostasis. ROS can also react with proteins directly and in this case seem to have a prevalence for SH groups or disulfide bridges (VAN DER VLIET & BAST 1992). Ion channels and transporters are susceptible to oxidative stress. For example, voltage-dependent Na^+, K^+, and Ca^{2+} channels, Ca^{2+}-activated K^+ channels, and K_{ATP} channels have all been identified as targets for ROS (HOOL 2006). Several previous studies indicate that H_2O_2 alters energy metabolism, ATP-sensitive K^+currents, L-type Ca^{2+}currents (GOLDHABER & LIU 1994; RACAY *et al*. 1997), as well as delayed rectifier K^+ currents (GOLDHABER *et al*. 1989).

The aim of the present study was to explore whether glutathione could protect LRNC from toxicity induced by cumene hydroperoxide. Although invertebrates may represent "simple systems", in the sense that their nervous systems contain fewer neurons than a vertebrate brain, at the cellular and molecular level, the individual neurons are as complex as neurons in any vertebrate (BURRELL & SAHLEY 2001; BIER & MCGINNIS 2004). The invertebrate nervous system has proven exceptionally useful in electrophysiological neuroscience for the understanding of some processes of the central nervous system. The main advantages of studying nerve cells in leech brain are the large sizes of Retzius nerve cells and their easy accessibility for electrophysiological recordings.

Material and Methods

Experimental animals

All experiments were carried out at room temperature (22-25°C) on the Retzius nerve cells of isolated abdominal segmental ganglia of the ventral nerve cord of the horse leech, *Haemopis saguisuga.* The dissection procedure, the recording method and point voltage clamp technique were employed as described previously (BELESLIN *et al*. 1987). The leeches were first anaesthetized in 10% ethanol. Then, the ventral nerve cord was removed from the animal in short segments of four ganglia via a ventral longitudinal incision. Dissected segments were immediately transferred to a 2.5 ml plastic chamber with a leech Ringer and fixed by means of fine steel clips. The plastic chamber was then placed in a grounded Faraday's cage mounted on a fixed table in a manner that prevents vibrations. Identification and penetration of the cells was performed in the cage under a stereomicroscope. Retzius cells are the largest neurons (40-60 μm in diameter) situated on the ventral side of the ganglia.To change the solution, the chamber was flushed continuously with a volume of fluid at least five times that of the chamber volume. The perfusion rate was kept low so that implanted microelectrodes remained inside the cells during the perfusion.

Electrical measurements

Spontaneous spike activity was recorded with a conventional 3 M KCl microelectrode. Membrane voltage and current were recorded using voltage-clamp techniques. This was shown in voltage-clamped neurons by long depolarizing steps (to 500 ms) from the holding potential which was more negative than -40 mV in a sodium free leech Ringer, in order to induce fast and slow K^+ outward currents. The recording electrodes were prepared from 1.5 mm borosilicate capillaries (Clark Electromedical Instruments, UK) and filled with a 3 M KCl-containing solution. The pipette resistance ranged from 5 to 10 MΩ. Usually the microelectrode was connected through a Ringer bridge

and Ag-AgCl electrode via a negative capacitance high input resistant amplifier Bioelectric Instrument DS2C to a computer. Command pulses were derived from a Tektronix 161 pulse generator. The signals were digitized by the use of an analog-to-digital converter (Digidata 1200; Axon Instruments) and saved in a computer for off-line analysis.

Solutions

The Ringer solution used in these experiments had the following composition (in mM): NaCl,115; KCl, 4; $CaCl_2$, 2; Na_2HPO_4, 1.2; NaH_2PO_4, 0.3 (pH 7.2). The sodium-free Ringer (Tris-Ringer) contained 115 mM Tris-Cl instead of NaCl without a phosphate buffer. CHP was obtained from Sigma (St. Louis, U.S.A.), dissolved in 0.01 % dimethyl sulfoxide (Sigma, St. Louis, U.S.A.) and added to the Ringer solution (or Tris-Ringer) in a concentration of 1.5 mM.

GSH (Sigma, St. Louis, U.S.A.) was added to the Ringer solution to produce a final concentration of 0.2 mM.

Data analysis

The data are presented as original recordings and expressed as means ± SD (n = number of observations). Comparisons between the mean values were made with Student's t-analysis. P values <0.05 were considered significant.

Results

Modification of the action potential in LRNC by cumene hydroperoxide

In the first set of experiments we tested the effect of CHP on the action potential of the Retzius nerve cells of the leech. CHP was used as an oxidant agent. Superfusion of leech abdominal ganglia with CHP (1.5 mM) caused an extreme change to the shape and action potential duration (APD) in leech Retzius nerve cells (Fig. 1). Early depolarization during the 20 min of exposure with the leech Ringer containing 1.5 mM CHP was also recorded. A higher concentration of CHP 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 LRNC. Data were normally taken in controlled conditions, and following 30 min superfusion of LRNC to the ROS donor, CHP. Bath application of CHP produced a marked prolongation of the action potential (from 9.66±2.18 ms, before the application of CHP to 127.80±15.95 ms, 30 min after adding CHP to the Leech Ringer solution) (Table 1).

The protective effects of glutathione against cumene hydroperoxide-induced modulation of the action potential in LRNC

Because it has been shown that CHP affects the lasting action potentials of Retzius nerve cells, the possibility of recovering changes by the effect of antioxidant, reduced glutathione was also examined. The application of 0.2 mM GSH solution significantly decreased the bursting frequency, duration and amplitude of depolarization plateaus, and the number of spikes per plateau. First, Retzius cells were exposed to the effect of CHP (1.5 mM) then GSH was added in a concentration of 0.2 mM to the Leech Ringer solution. GSH largely inhibited the effects of CHP on the duration of the action potential. In the presence of GSH, the APD was extended by 9.22±1.14 ms, in controlled conditions, to 12.45±1.56 ms, 30 min after adding CHP and GSH to the Leech Ringer solution, which did not have any significant statistical result (Table 1). In the presence of glutathione, repetitive firings were

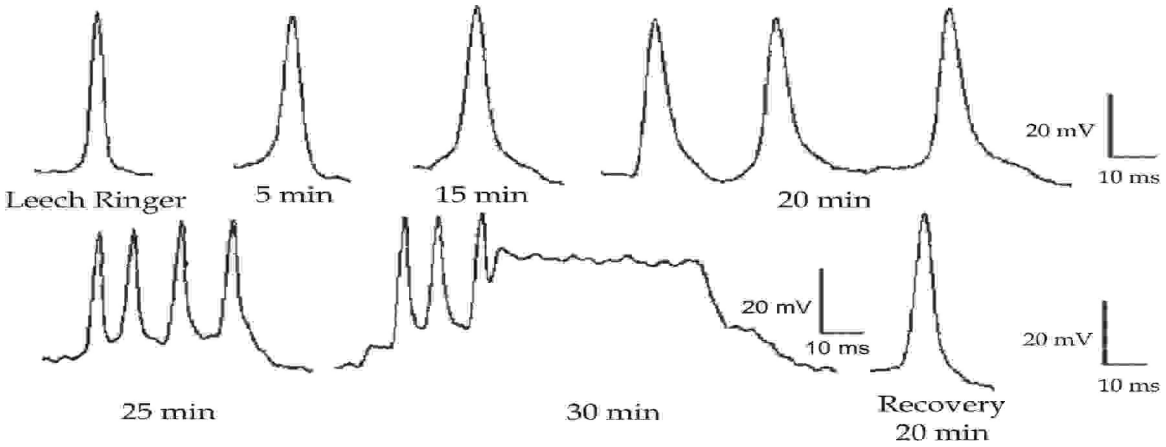

Fig. 1. Effects of CHP on the action potential duration of LRNC. Representative action potential recordings under control conditions (Leech Ringer), after exposure to 1.5 mM CHP and after washout of CHP. Early after-depolarization and cardiac-like action potential recorded in LRNC after exposure of isolated ganglion to CHP.

Table 1

Effects of reduced glutathione on cumene hydroperoxide-induced prolongation of the action potential duration in leech Retzius nerve cells. Data are expressed as mean ± SD; P – level of significance; CHP – cumene hydroperoxide; GSH – reduced glutathione; n=number of cells, * – repetitive firing

Action potential duration (ms) in leech Retzius nerve cells						
	Leech Ringer	5 min	15 min	20 min	30 min	Recovery 20 min
1.5 mM CHP n= 11	9.66±2.18	16.09±3.15	41.64±8.27*	68.72±13.40*	127.80±15.95* P≤0.01	23.43±4.61
1.5 mM CHP + 0.2 mM GSH n=10	9.22±1.14	8.87±2.34	9.67±1.44	10.76±2.32	12.45±1.56 P>0.05	10.34±1.21

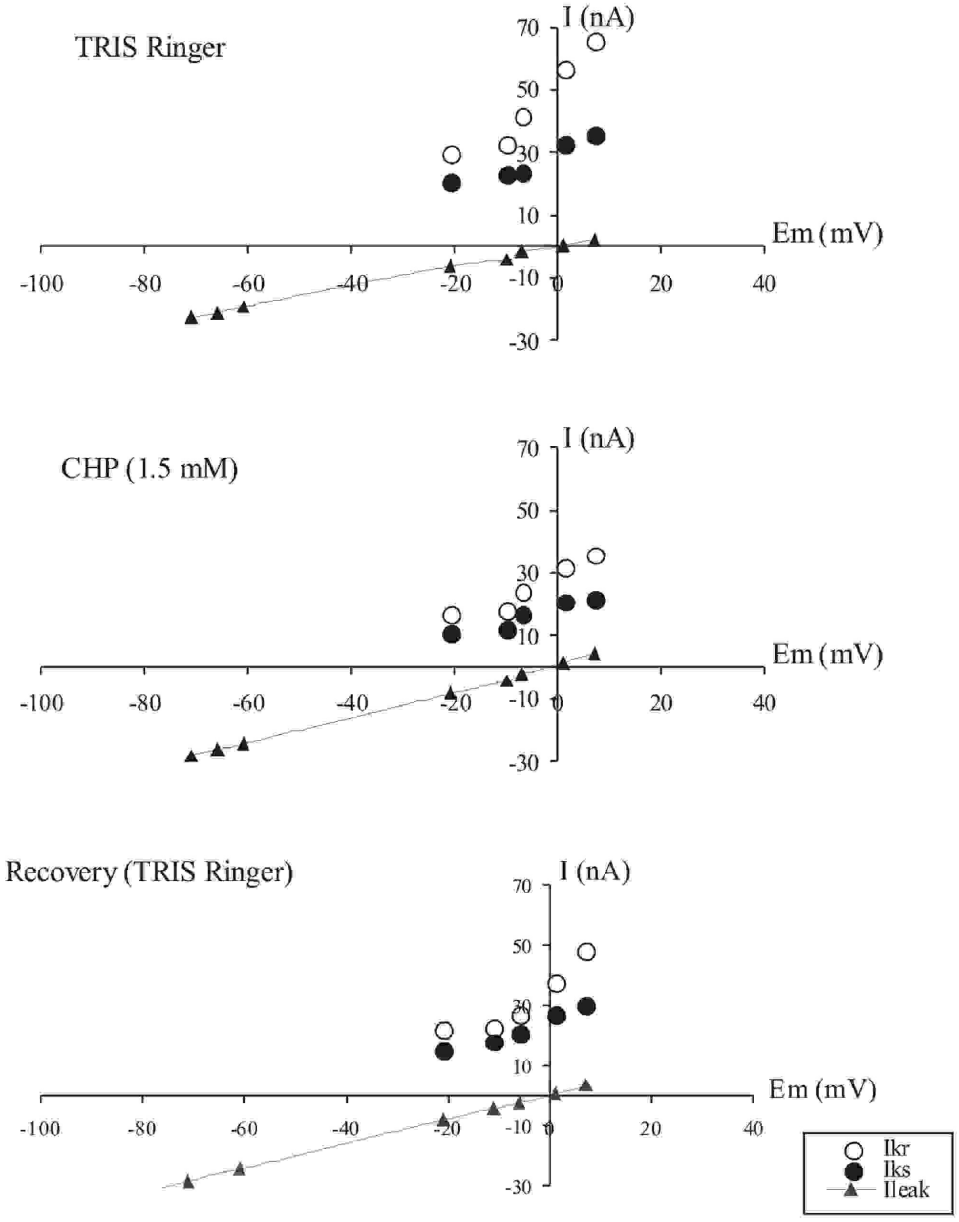

Fig. 2. The current-voltage relationship for the same cell, measured at the peak of the potassium outward current (open circles) and at the end of stimulation (solid circles) in the Tris-Ringer, 25 min after adding 1.5 mM CHP and during the recovery. Ikr-rapid Ca^{2+} activated K^+ current; Iks-slow Ca^{2+} activated K^+ current; Ileak-passive leak current.

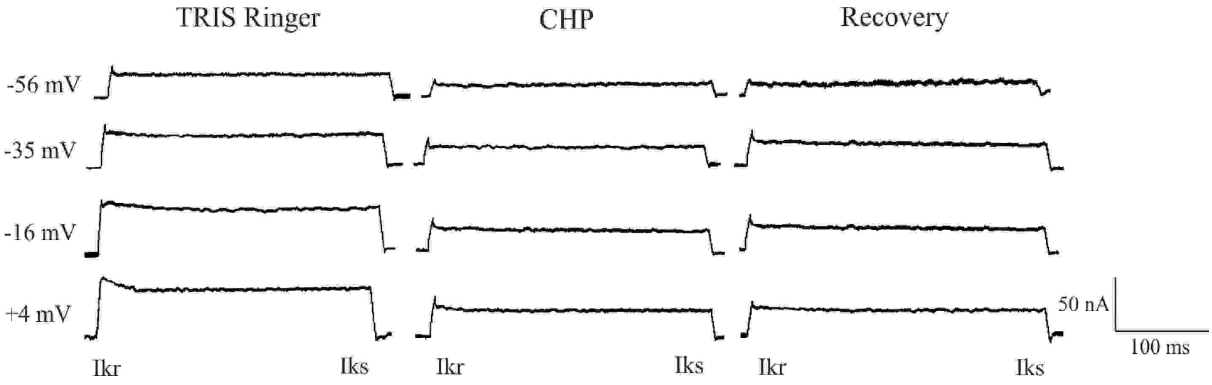

Fig. 3. Recordings of Ca^{2+} activated K^+ currents of LRNC under control conditions (TRIS Ringer), in the presence of 1.5 mM CHP and after washout of CHP (recovery), during the voltage step from -56 mV to +4 mV.

not registered in any examined cells. The application of GSH, a free radical scavenger, to a bathing solution reverses the CHP effects. These observations emphasize the significance of glutathione in the protection of sulfhydryl groups of membrane proteins as well as lipids in oxidative stress caused by CHP.

Inhibition of Ca^{2+}-activated outward K^+ currents in LRNC by cumene hydroperoxide

The elongation of action potentials by CHP suggested that CHP decreased the magnitude of ion currents needed for the repolarization phase of action potentials. The action potentials of LRNC elongated after the exposure to 1.5 mM of CHP, suggested that CHP modified the outward K^+ currents that form action potentials together with the Na^+ current. In order to explore the ionic mechanism by which CHP prolongs spike potential, we examined its effects on membrane K^+ currents. The K^+ channels, key regulators of neuronal excitability, are targets of ROS. Application of CHP caused suppression of fast and slow Ca^{2+}-activated outward K^+ currents. Figure 2 shows the current-voltage relationship, separately, for the peak and established a steady level of depolarizing potassium outward current. At the test potential of +4 mV, the fast and late steady part of the K^+ outward current dropped from 68 to 37 nA (46%) and from 36 to 22 nA (39%). These results demonstrate the marked electrophysiological effects of CHP in LRNC.

Figure 3 shows representative current records obtained 20 min after application of the CHP.

The protective effects of glutathione against cumene hydroperoxide-induced modulation of Ca^{2+}-activated outward K^+ currents in LRNC

GSH applied in a concentration of 0.2 mM partially blocked the effect of CHP on Ca^{2+}-activated K^+ currents. In the current-voltage relationship (Fig. 4) there were no significant changes on the early or late part of the K^+ outward current in the presence of 1.5 mM CHP and 0.2 mM GSH. At the test potential of +17 mV, the fast and late steady part of the K^+ outward current dropped from 65 to 52 nA (20%) and from 46 to 39 nA (15%). Figure 5 recorded from LRNC during control (TRIS Ringer) and after addition of 1.5 mM CHP and 0.2 mM GSH during a voltage step from -57 mV to +17 mV.

Discussion

A growing body of evidence suggests that many neuronal ion channels and receptors can be modulated by oxidative modifications. The literature describing the effects of ROS on membrane currents is sometimes contradictory. The electrophysiological effects of ROS generally consist of an initial action potential prolongation, followed by development of after-depolarizations, and finally marked depolarization and inexcitability (TARR & VALENZENO 1989; SATOH & MATSUI 1997), although, only a reduction (HAYASHI *et al*. 1989; COETZEE *et al*. 1990) or an increase in action potential duration (BARRINGTON1994) have also been reported. According to BARRINGTON (1994) the effects of H_2O_2 on action potentials are strongly dependent upon recording conditions. The effect of ROS generated by light and the photosensitizer Rose Bengal on ionic currents in single frog atrial cells was studied by TARR & VALENZENO (1991). They reported that the excitatory inward sodium and calcium currents were both suppressed by ROS as was the outward, delayed rectifier potassium current. A recent report by TAKEUCHI & YOSHII (2008) demonstrated that ROS produced by exposure to light, prolongs the duration of action potentials, and increases the

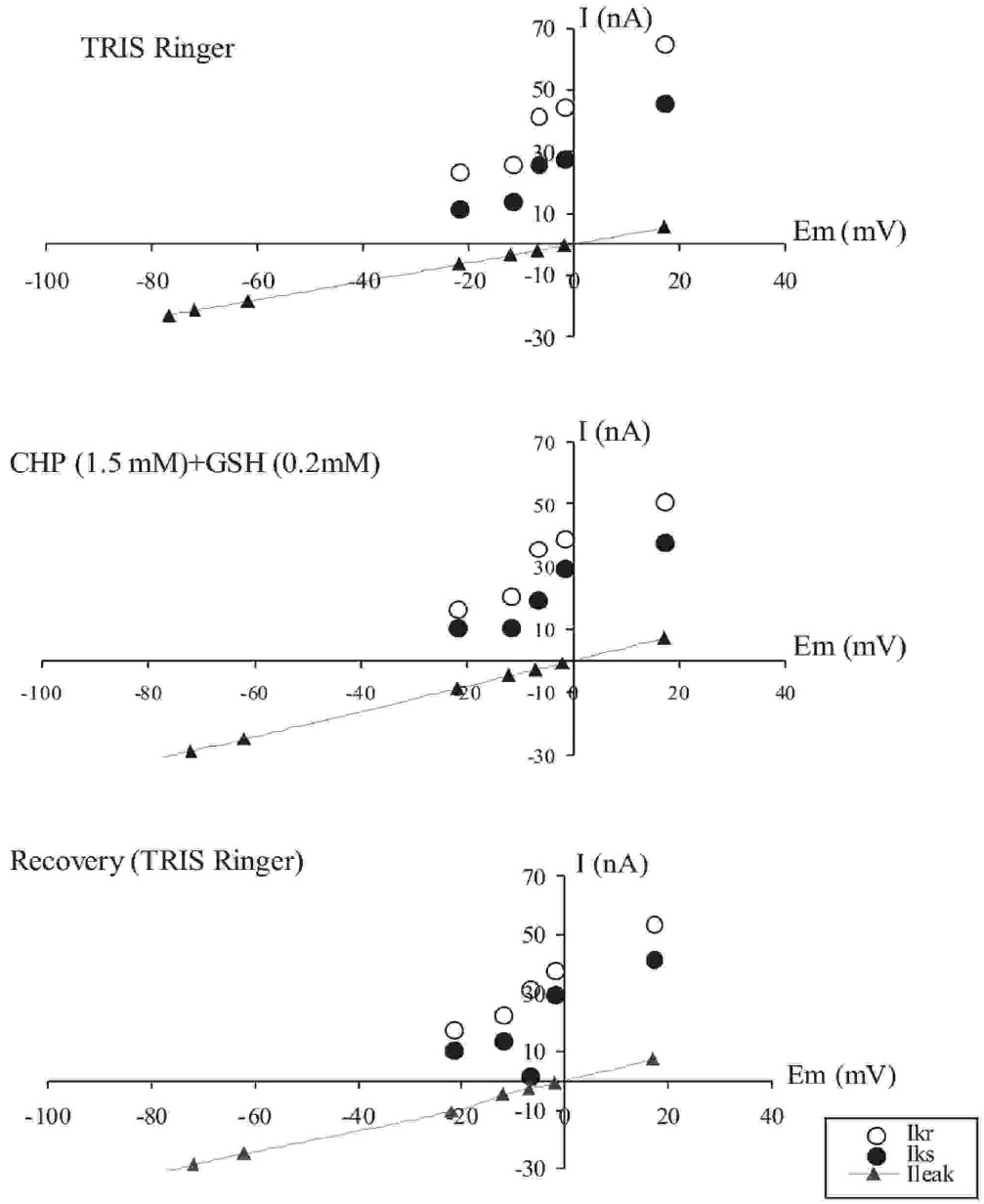

Fig. 4. The current-voltage relationship for the same cell measured at the peak of the potassium outward current (open circles) and at the end of stimulation (solid circles) in the Tris-Ringer, 25 min after adding 1.5 mM CHP and 0.2 mM GSH and during the recovery. Ikr-rapid Ca^{2+} activated K^+ current; Iks-slow Ca^{2+} activated K^+ current; Ileak-passive leak current.

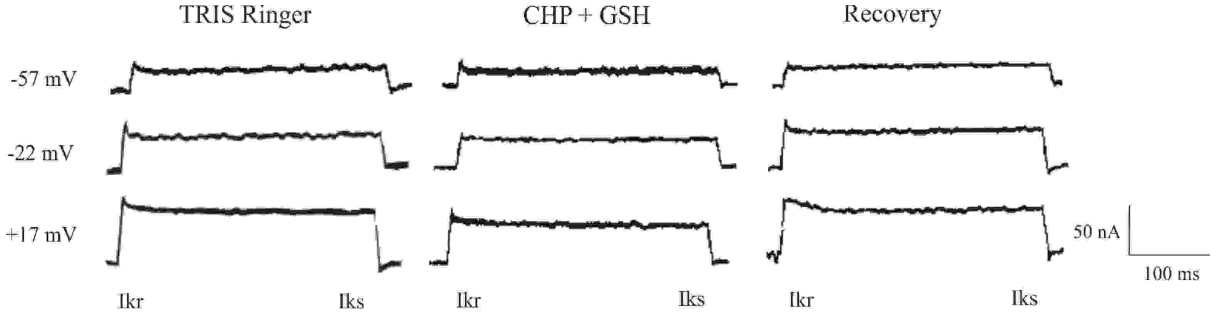

Fig. 5. Effect of glutathione on cumene hydroperoxide-induced suppression of the Ca^{2+} activated K^+ current of LRNC. Representative traces recorded from LRNC in Na-free fluid, after adding 1.5 mM CHP and 0.2 mM GSH, and in TRIS Ringer (recovery), during displacement of the holding potential from -57 mV to +17 mV.

magnitude of outward rectifier K^+ currents and inward rectifier K^+ currents in cultured mouse hippocampal neurons. In guinea pig ventricular myocytes, dihydroxyfumarate-generated ROS reduced I_K (CERBAI *et al*. 1991), while the direct application of 100 μM H_2O_2 appeared to slightly increase I_K at highly (+70 mV) depolarizing pulses (SATOH & MATSUI 1997). However, in these experiments, no attempt was made to differentiate between the effects of ROS on Ikr (rapid) and Iks (slow), the two components of the delayed rectifier K^+ current (SANGUINETTI & JURKIEWICZ 1990). VEGA-SAEZ DE MIERA & RUDY (1992) reported that H_2O_2 inhibited three cloned voltage-gated K+ channels expressed in *Xenopus* oocytes. A report by DUPRAT *et al*. (1995) demonstrated that photoactivation of Rose Bengal induced inhibition of the cloned K^+ channel activity. 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 (DYAVANA--PALLI *et al*. 2010; WHYTE *et al*. 2009). These findings indicate that ROS effects depend on the ROS--generating system as well as on the type of channel protein under investigation. It is likely that this variability arises from both species differences and variations in recording techniques and conditions.

The most important finding of the present study is that CHP modulates Ca^{2+} activated K^+ channels in leech Retzius nerve cells. In the voltage clamp experiments, fast and slow Ca^{2+} activated outward K^+ currents were suppressed with CHP. Outward currents play a principal but not exclusive role in repolarization in many types of excitable cells. In leech Retzius nerve cells three classes of K^+ channels (fast, slow calcium-activated and late voltage--regulated) have been identified (BELESLIN *et al*. 1987). Ikr (rapid) and Iks (slow) are important determinants of APD and play a dominant role in the repolarization of the action potential. The present results support the view that CHP stimulates lipid peroxidation as a mechanism of ROS-induced cell membrane injury. Organic hydroperoxide triggers the generation of free radical intermediates peroxyl and alkoxyl radicals, which can cross cellular membranes and evoke the production of $HO^{\bullet}$ (HWANG *et al*. 2009). It was well known that $HO^{\bullet}$ could cause peroxidation of lipids that inactivates membrane-associated protein, increasing membrane permeability. A recent paper reported that relatively low concentrations of cumene hydroperoxide (100 μM) led to a significant decrease in the cellular content of ATP and reduced glutathione, as well as decreased mitochondrial potential (VIMARD *et al*. 2011).

The results of our study demonstrate that a SH reducing agent, GSH, incompletely inhibited the effect of CHP on calcium-activated potassium channels in LRNC. SH groups are known to be important for the function of potassium channels (LEE *et al*. 1994; HAN *et al*. 1996). There are several explanations for the incomplete recovery of calcium-activated potassium channels. The simplest could be that CHP treatment must modify other amino acid residues (e.g., as methionine or tryptophan, besides cysteine). In addition, part of the changes in channel properties depend on cysteine residues located on the cytoplasmic domains of the calcium-activated potassium channels in LRNC. Of course, it is possible that the oxidant agent affects other components associated to the membrane or channel (β-subunit or some membrane-bound enzyme able to promote channel phosphorylation).

In conclusion, it was found that cumene hydroperoxide increases the excitability of leech Retzius neurones by an inhibition of outward potassium currents, responsible for the repolarization of action potentials. The protective effects of glutathione against cumene hydroperoxide -induced neurotoxicity may be due, at least in part, to its ability to scavenge ROS and to protect sulfhydryl groups on the ion transport proteins. It has been concluded that leech ganglia are good models for studying protective mechanisms against ROS.

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