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Effects of Oxidative Stress on the Electrophysiological Function of Neuronal Membranes

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1. Introduction

Numerous experimental and clinical observations suggest that reactive oxygen species (ROS) play a significant role in several pathological conditions of the central nervous system where they directly injure tissue and where their formation may also be a consequence of tissue injury. Reactive oxygen metabolites are particularly active in the brain and neuronal tissue, and they are involved in numerous cellular functions, including cell death and survival. 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 ROS mediated injury. A high metabolic rate (the brain consumes approximately 20% of total-body oxygen) and an abundant supply of transition metals, make the brain an ideal target for free radical attack (Facchinetti et al., 1998; Gutowicz, 2011). In addition, the brain has high susceptibility to oxidative stress due to high polyunsaturated fatty acid content and relatively lower regenerative capacity in comparison with other tissues. On the other hand, the brain is poor in catalytic activity and has moderate amounts of glutathione peroxidase and superoxide dismutase. Of all the cell types in the body, neuronal cells may be among the most vulnerable to oxidative stress. These cells are continuously exposed to ROS. Accumulating evidence demonstrating that the defense of nerve cells against ROS-mediated oxidative damage is essential for maintaining functionality of nerve cells. Because hydrogen peroxide (H_2O_2) is the peroxide generated in the highest quantity in the brain, the defense against the oxidative stress appears to be particularly important. When production exceeds antioxidant protection, oxidative stress leads to molecular damage. An important component of the cellular detoxification of ROS is the antioxidant glutathione (GSH) (Dringen & Gutterer, 2002; Dringen & Hirrlinger, 2003). Because neurons have limited antioxidant capacity, they rely heavily on their metabolic coupling with astrocytes to combat oxidative stress. Evidence is growing that glutathione plays an important role in the detoxification of H_2O_2 and organic hydroperoxides in the brain and that glutathione is the main antioxidant molecule in neurons (Aoyama et al., 2008; Haskew-Layton et al., 2010; Limon-Pacheco & Gonsebat, 2010). Ongoing studies have shown that neuron-glia compartmentalization of antioxidants is critical for neuronal signaling by H_2O_2 , as well as neuronal protection. The 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). But, the concentration of glutathione is in relatively lesser quantities in the brain in comparison to the other organs of the body (Skaper et al., 1999). In contrast to other ROS, H_2O_2 is neither a free radical nor an ion, which limits its reactivity (Cohen, 1994). However, in the presence of transition metals such as iron or copper, H_2O_2 can give rise to the indiscriminately reactive and toxic hydroxyl radical (HO^\bullet) by Fenton chemistry. H_2O_2 is able to diffuse across biological membranes, and therefore can diffuse freely from a site of generation (Bienert et al., 2007; Makino et al., 2004) so that it is well-suited as a diffusible messenger. Increasing evidence indicates that H_2O_2 is a particularly intriguing candidate as an intracellular and intercellular signaling molecule because it is neutral and membrane-permeable (Nistico et al., 2008; Forman et al., 2010). Recent research into mechanisms of ROS-induced modifications in ion transport pathways involves: oxidation of sulfhydryl (SH) groups on the ion transport proteins, lipid peroxidation, and alterations of calcium (Ca^{2+}) homeostasis, a major second messenger system (Kourie, 1998). Increases in Ca^{2+} initiate inappropriate activation of several enzyme systems e.g., nitric oxide synthase and phospholipase A_2 . Overactivation of these enzymes results in the breakdown of proteins and phospholipids and initiates several cascades that damage cells (Lee et al., 1999). It has been described that elevation in cytoplasmic Ca^{2+} levels activates the mitogen-activated protein kinase (MAPK) cascade (Liu & Templeton, 2008; Son et al., 2011) and the phosphatidylinositol 3'-kinase (PI3K)-Akt pathway (Cheng et al., 2003). ROS produce cell damage through multiple mechanisms, including excitotoxicity, metabolic dysfunction and disturbance of intracellular homeostasis of Ca^{2+} (Halliwell & Gutteridge, 1984; Del Maestro et al., 1980; Bracci, 1992). Activation of glutamate ionotropic receptors promptly triggers membrane depolarization and Ca^{2+} influx, resulting in the activation of several different protein kinases (Ca^{2+} -calmodulin-dependent kinase, protein kinase C and MAPK) and transcription factors, such as cyclic AMP response element binding protein (CREB). Neurons efficiently repair glutamate-induced oxidative DNA damage by a process involving CREB-mediated up-regulation of apurinic endonuclease 1 (APE1) (Yang et al., 2011).

Studies have demonstrated that ROS can induce or mediate the activation of the MAPK pathways (McCubrey et al., 2006). The mechanisms by which ROS can activate MAPK pathways are unclear. Because ROS can alter protein structure and function by modifying critical amino acid residues of proteins (Thannickal & Fanburg, 2000), the oxidative modification of signaling proteins by ROS may be one of the plausible mechanisms for the activation of MAPK pathways. However, the precise molecular target(s) of ROS is unknown. The prevention of oxidative stress by antioxidants blocks MAPK activation after cell stimulation with cellular stimuli indicating the involvement of ROS in activation of MAPK pathways. The recent observations provide a strong argument for activation of MAPK pathways by direct exposure of cells to exogenous H_2O_2 (Ruffels et al., 2004; Son et al., 2011).

The cell membrane would seem of special interest because of its large surface area and because of the susceptibility of membrane unsaturated fatty acids and proteins containing oxidizable amino acids (such as cysteine and methionine) to oxidant attack. Oxidative stress affects cellular membrane lipids and proteins. Cell membranes are either a source of neurotoxic lipid oxidation products or the target of pathogenic processes that cause permeability changes or ion channel formation (Axelsen, 2011). Reactive oxygen metabolites modify ion transport mechanisms either directly via ion transport pathway proteins and

regulatory proteins or indirectly via peroxidation of membrane lipids. The nature and sequence of events that lead to the disruptions of these ion transport pathways are not fully understood. Early studies revealed that the effects of ROS on membrane properties could be deduced from electrophysiological parameters of the membrane. These include changes in membrane potential and current, ionic gradients, action potential duration and amplitude, spontaneous activity and excitability (Tarr et al., 1995; Tarr & Valenzeno, 1989; Beleslin et al., 1998). Oxygen-derived free radicals are thought to induce alterations in nervous electrical activity, however, the underlying membrane ionic currents affected by ROS and the mechanisms by which ROS induce their effects on ion channels in the nerve cells are not well defined. Considering neuronal function, ROS can attack ion channels and transporters directly, or indirectly by causing lipid peroxidation (Kourie, 1998; Carmeliet, 1999) and affecting associated signaling molecules (Hool, 2006). The mechanism of initiation of ROS peroxidation is not understood completely. The hydroxyl radical ($\text{HO}\cdot$), a highly reactive oxidant, has been proposed as the initiating species. The ability of the $\text{HO}\cdot$ to initiate lipid peroxidation has been questioned by some investigators. In addition to initiating lipid peroxidation, the $\text{HO}\cdot$ has been implicated in direct cellular damage. Peroxidation of membrane phospholipids has been demonstrated to affect various transmembrane processes, such as receptor activation and formation of intracellular second messengers and Ca^{2+} homeostasis. Ca^{2+} ions also play a central role in the control of neuronal excitability. ROS oxidatively modify numerous membrane-bound proteins including ion channels. ROS can also react with proteins directly and in this case seem to have a prevalence for SH groups or disulfide bridges on the ion transport proteins (Van der Vliet & Bast, 1992). Oxidative sensitivity of ion channels is often conferred by amino acids containing sulfur atoms (Su et al., 2007). The mechanism of ROS-induced modifications in ion transport pathways involves the inhibition of membrane-bound regulatory enzymes and modification of the oxidative phosphorylation and ATP levels.

Studies have demonstrated that oxidative stress, perturbations in the cellular thiol level and redox balance, affects many cellular functions, including signaling pathways. In the CNS, cells respond to oxidative stress by initiating endogenous protective cascades often regulated at the transcriptional level. The transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2) plays an integral role in astrocyte-mediated protection of neurons from oxidative stress. Previous studies have reported that MAPKs may play a role in the induction and regulation of the Nrf2 system in the brain (Clark & Simon, 2009). When cells are exposed to oxidative stress, the Nrf2 binds to the antioxidant responsive element (ARE). The Nrf2-ARE pathway elicits transcriptional activation of antioxidant genes and detoxifying genes that protect cells and organisms from oxidative stress. Activation of this pathway protects cells from oxidative stress-induced cell death (Hur and Gray, 2011). The NRF2/KEAP1 signaling pathway is the main pathway responsible for cell defense against oxidative stress and maintaining the cellular redox state (Stepkowski & Kruszewski, 2011). The Nrf2-mediated GSH biosynthesis and release from astrocytes protects neurons from oxidative stress (Shih et al., 2003). Increased levels of GSH may be a major component of the protection observed by Nrf2 activation. In the CNS, Nrf2 plays an integral role in astrocyte-mediated protection of neurons from oxidative stress. Neuronal viability is enhanced significantly by an increased supply of GSH precursors from Nrf2-overexpressing glia. Thus Nrf2-dependent enhancement of glial GSH release appears to be necessary and sufficient for

neuronal protection. The observations of Correa et al. (2011) suggest that activated microglia can stimulate or reduce the GSH-related anti-oxidant defense in cultured astrocytes. Recently, Zou et al. (2011) reported that overexpression of Nrf2 increased GSH content and efficiently protected t-BHP-induced mitochondrial membrane potential loss and apoptosis in cultured human retinal pigment epithelial cells.

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). However in literature the data concerning the effect of ROS on potassium current are controversial. For example, Cerbai et al. (1991) and Ward & Giles (1997) did not observe any effect, in contrast to Tarr & Valenzeno (1989) who obtained a decrease in the outward, delayed rectifier potassium current. The results of Hasan et al. (2007) suggest that oxidative stress, which inhibits the delayed-rectifier current, can alter neural activity. Ward and Giles (1997) studied the effects of H_2O_2 on action potentials and underlying ionic currents in isolated rat ventricular myocytes. They showed that H_2O_2 caused no significant changes in either the Ca^{2+} -independent transient outward K^+ current (Ito) or the inwardly rectifying K^+ current (I_{K1}). The most prominent effect of H_2O_2 on the ionic currents which underlie the action potential is a slowing of inactivation of the I_{Na} . Potassium channels constitute a highly diverse class of ion channel and thus participate in multiple modulatory functions. Although altered potassium dynamics play a major role in this type of neuronal activity (Dudek et al., 1998) the role of K^+ channels is still incompletely understood. The voltage-activated K^+ channels are responsible for the establishment of the resting membrane potential, repolarization during action potentials and regulation of action potential frequency (Vacher et al., 2008). Three principal K^+ currents were identified in LRNC (Stewart et al., 1989). These differed in their time courses of activation and inactivation and in their responses to Ca^{2+} channel blockers. K^+ currents of the A-type (I_{A}) with rapid activation and inactivation kinetics, were not affected by Ca^{2+} channel blockers. The A-type K^+ currents were a minor component of the outward current in LRNC. A Ca^{2+} activated K^+ current (I_{Ca}), that inactivated more slowly and was reduced by Ca^{2+} channel blockers, constituted the major outward current in LRNC. The third K^+ current resembled the delayed rectifier currents (I_{K1} and I_{K2}) of squid axons, activated and inactivated slowly. Modifications of K^+ channel activity by ROS in the brain would lead to drastic changes in the electrical excitability of neuronal cells and could easily explain a tendency to brain hyperexcitability, or even neuronal death.

2. Materials and methods

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 *Haemopsis saguisuga*. The dissection procedure, the recording method and point voltage clamp technique were employed as described previously (Beleslin et al., 1988). Dissected segments of 4 ganglia 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 neurons were identified based on the position within the ganglion, the size and the bioelectrical properties of the cells. The Retzius cells, are the largest neurons (40-60 μm in diameter) situated on the ventral side of the ganglia. It is well known that the resting potential of Retzius nerve cells of medical and horse leeches are lower than in other neurons (Hagiwara & Morita, 1962; Beleslin, 1985). Theoretically, this can be due to the low resting potassium permeability or the high membrane permeability to sodium. The Retzius cell is spontaneously active and responds to depolarization with an increased firing rate proportional to the depolarization (Lent, 1977). In leech Retzius nerve cells three classes of K^+ channels (fast, slow calcium-activated and late voltage-regulated) have been identified (Beleslin et al., 1982; Beleslin, 1985). 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. The bath volume was 2 ml and the solution changes were completed within 30 sec.

2.1 Electrical methods

In this study, we investigated the time-dependent changes in action potential configuration and changes in steady-state membrane currents in leech Retzius nerve cells. The 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 $\text{M}\Omega$ (when filled with 3M KCl solution). Microelectrodes with a tip potential less than 5 mV in an artificial solution, were selected for use. 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.

2.2 Solutions

Pharmacological agents were prepared and dissolved immediately before application in the physiological salt solution at the concentrations stated. H_2O_2 , cumene hydroperoxide (CHP) and reduced glutathione (GSH), were added to the leech or Tris-Ringer. All drugs were administered 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 sec. The ganglia were bathed in a leech Ringer containing (mM): 115 NaCl, 4 KCl, 2 CaCl_2 , 1.2 Na_2HPO_4 , 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 (hydroxy-methyl)aminomethane-Cl (Tris Ringer) and Na_2HPO_4 and NaH_2PO_4 were omitted.

Solutions containing H_2O_2 were prepared freshly before their use from 30% H_2O_2 solution (Zorka Pharma, Sabac) and added to the Ringer solution (or Tris-Ringer) at final concentrations of 1, 5 and 10 mM. CHP and GSH were added to the normal or Tris-Ringer solution. The 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 concentrations of 0.25, 1 and 1.5 mM. A GSH (Sigma, St. Louis, U.S.A.) was added to the Ringer solution to produce a final concentration of 0.2 mM.

2.3 Data analysis

Data are expressed as mean \pm SEM. Comparisons between the mean values were made with a Student's t-analysis. P values <0.05 were considered significant.

3. Results

3.1 Effects of cumene hydroperoxide on the spontaneous spike potential of leech Retzius nerve cells

In our work we used CHP to stimulate lipid peroxidation as the mechanism of free radical-induced cell membrane damage. We investigated the time-dependent changes in action potential configuration and changes in steady-state membrane currents in LRNC. Superfusion of leech abdominal ganglia with CHP (0.25, 1 and 1.5 mM) caused an extreme change to the shape and action potential duration (APD) in LRNC. Exposure of LRNC to CHP prolonged the duration of the action potentials of the LRNC in a concentration-dependent manner. Figure 1 illustrates the representative record obtained 15, 20, 25 and 30 min after the exposure of an isolated ganglia to 1 mM of CHP. A cardiac-like action potential with a rapid depolarization, followed by a sustained depolarization or plateau and fast repolarization was recorded. During the 20 min of exposure with leech Ringer containing 1 mM CHP, early after depolarization was recorded. Higher concentration of CHP led to appearance of repetitive firing only a few minutes after application of CHP, which was followed by loss of excitability of leech Retzius nerve cells.

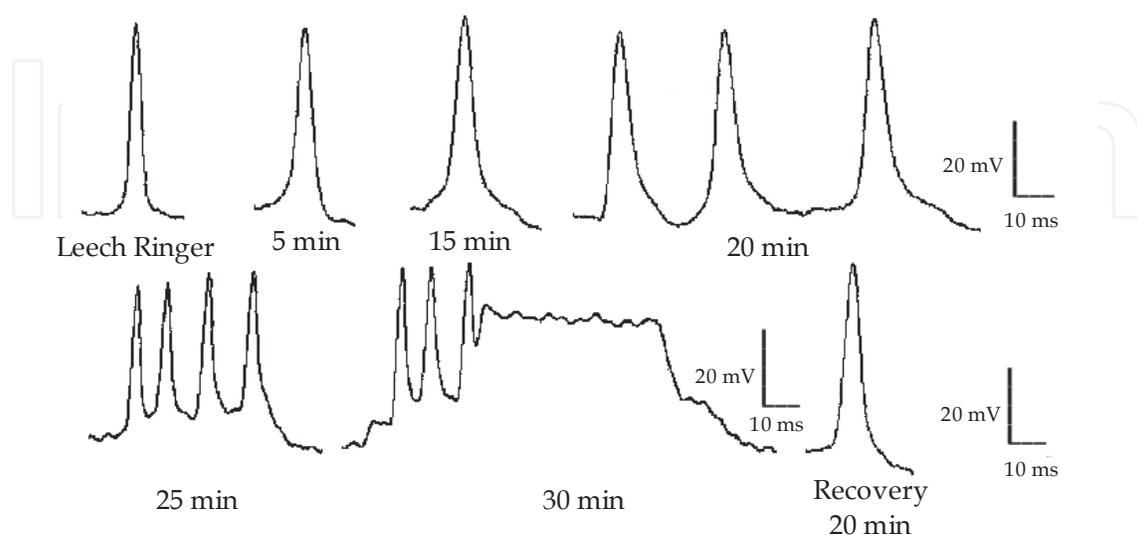


Fig. 1. Early after depolarization and repetitive firing recorded in LRNC after exposure of isolated ganglion to 1 mM CHP

Table 1 summarizes the values of the APD in a leech Ringer and after adding CHP (0.25, 1 and 1.5 mM). Table 1 shows that CHP caused a concentration-dependent increase in APD.

	Leech Ringer	5 min	15 min	20 min	30 min	Recovery 20 min
0.25 mM CHP n= 6	10.45±0.98	12.33±1.74	13.50±2.13	13.50±1.39	14.4±0.80 P>0.05	15.66±1.05
1 mM CHP n= 6	9.66±0.52	13.17±4.02	25.17±2.91	31.05±5.96	35.33±13.60 P≤0.01	26.50±8.38
1.5 mM CHP n= 11	9.66±2.18	16.09±3.15	41.64±8.27*	68.72±13.4*	127.80±15.95* P≤0.01	23.43±4.61

Table 1. Values of the APD (in ms) of LRNC before, 5, 15, 20 and 30 minutes after the adding of CHP (0.25, 1 and 1.5 mM) and during the recovery. Data are expressed as mean ± SEM; n=number of cells. * *repetitive firing*

3.2 Effects of hydrogen peroxide on the spontaneous spike potential of leech Retzius nerve cells

Previous investigations have shown that H₂O₂ is involved in cascades of pathological events affecting neural cells. The background of this study were the findings that 1 mM CHP treatment caused an extreme change in the duration of the action potential and suppression of Ca²⁺ activated outward K⁺ currents of LRNC. The aim of the present experiments was to examine the effect of the higher concentrations of H₂O₂ on LRNC. H₂O₂ in concentrations up to 10 mM in the reaction mixture had no effect on spontaneous spike potential. Extracellular application of H₂O₂ (1, 5 and 10 mM) did not significantly change (P > 0.05) the duration of the action potential of the LRNC. H₂O₂ is ineffective in generating either cardiac-like action potential or early after depolarization in LRNC.

3.3 Effects of glutathione on the cumene hydroperoxide-induced change of the spontaneous spike potential of leech Retzius nerve cells

The background of this study were the findings that the hydroxyl radical scavenger, mannitol (5 mM) significantly reduced neurotoxic effect of CHP on the spontaneous spike electrogenesis of LRNC (Jovanović, 2010).

Taking in to consideration that it has been proved that CHP affects the lasting action potentials of Retzius nerve cells, the possibility of recovering the changes by the effect of antioxidant, GSH was also examined. Firstly, Retzius cells were exposed to the effect of CHP (1 mM) then GSH was added in a concentration of 0.2 mM to the Leech Ringer solution. The application of GSH, a free radical scavenger, to a bathing solution reverses the CHP effects. The GSH, largely inhibited the effects of CHP on the APD. In the presence of the GSH the APD has been extended by 9.22±1.14 ms (in controlled conditions, before the application of

CHP and GSH) 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 2). In the presence of GSH repetitive firings has not been registered in any examined cells.

	Leech Ringer	5 min	15 min	20 min	30 min	Recovery 20 min
CHP+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

Table 2. Effects of GSH on CHP-induced prolongation of the APD in LRNC.

The APD of LRNC before and after the adding of CHP (1 mM) and GSH (0.2 mM). Data are expressed as mean \pm SEM; n=number of cells

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. These observations point out the significance of GSH in the protection of SH groups of membrane proteins as well as lipids in oxidative stress caused by CHP.

3.4 Modulation of Ca^{2+} activated K^+ current in leech Retzius nerve cells 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 **leech** Retzius nerve cells elongated after the exposure to 1mM 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. Several studies have reported the possibility that ROS alter ionic channel function. The K^+ channels, key regulators of neuronal excitability, are targets of ROS. It is well known that outward K^+ current are essential for maintaining normal APD. The K^+ currents contributing to the resting membrane potential and repolarization of the action potential were studied in voltage-clamped Retzius neurones. Modifications of voltage-sensitive K^+ channel activity by ROS would lead to changes in APD and the electrical excitability of neuronal cells. To test this hypothesis, the effect of CHP on Ca^{2+} activated K^+ current was studied.

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 the sodium free leech Ringer in order to induce fast and slow K^+ outward currents. Figure 2 illustrates the typical outward currents elicited in a CHP responsive neuron, depolarized in steps from a holding potential of -56 mV to $+4$ mV. Both components of the delayed outward K^+ current, I_{kr} and I_{ks} were inhibited by external CHP.

Application of CHP (in a concentration of 1 mM) caused suppression of fast and slow Ca^{2+} activated outward K^+ currents. The fast and slow steady part of the K^+ outward current was reduced by 40% and 31%, respectively. Figure 2 shows K^+ current amplitudes measured before and during exposure to CHP.

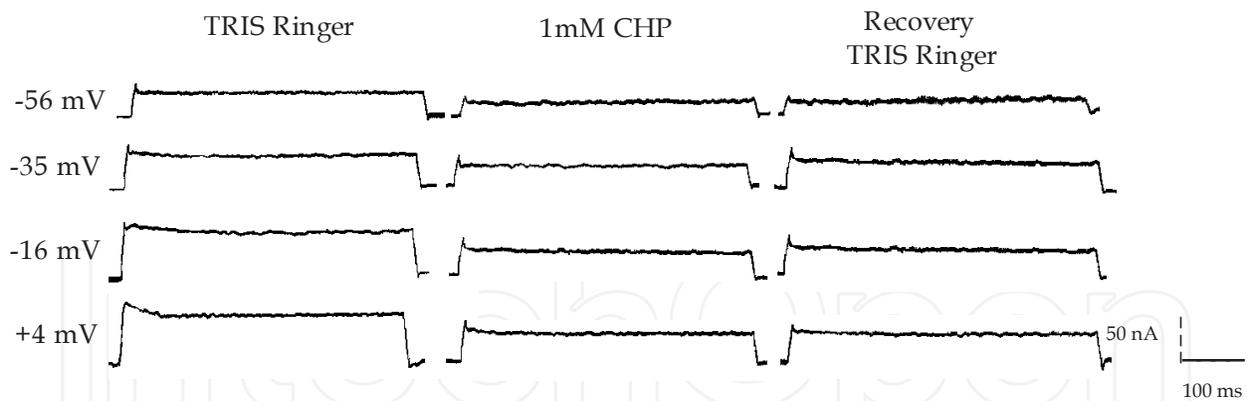
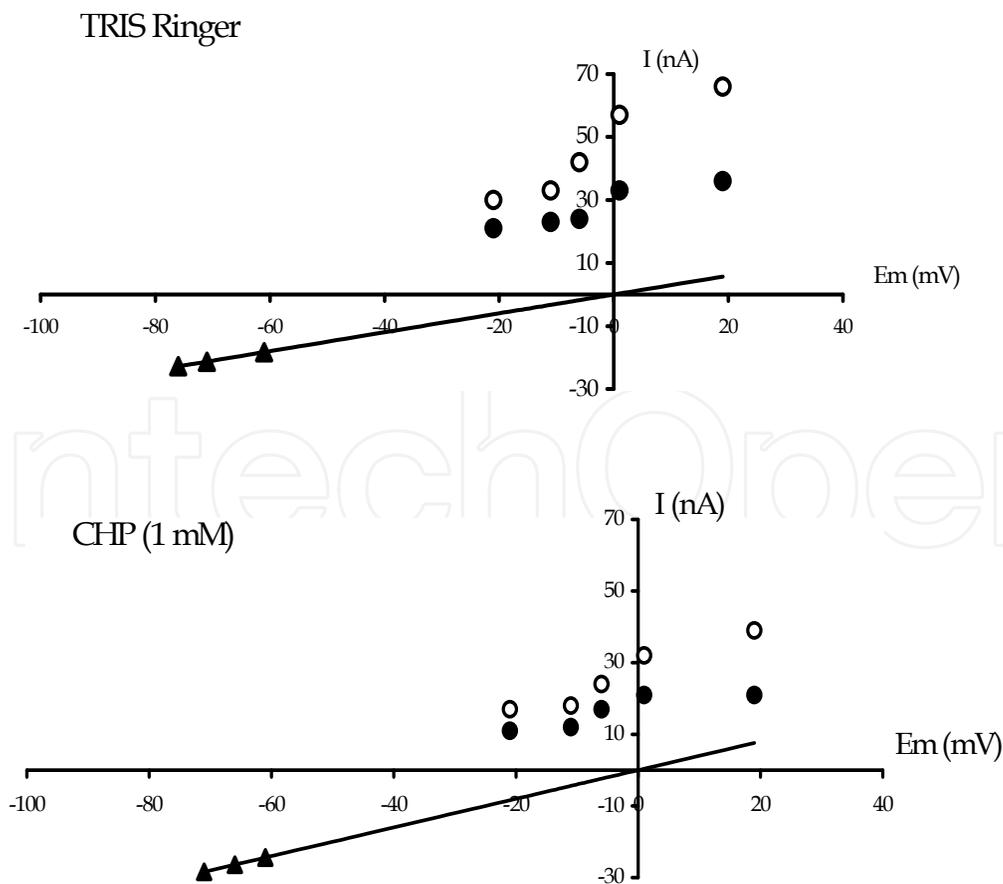


Fig. 2. Patterns of voltage clamp current record obtained from Retzius nerve cell in Tris-Ringer, after adding 1 mM CHP and again in an Na-free fluid (recovery) during displacement of holding potential from -56 mV to +4 mV.

Figure 3 shows the current-voltage relationship, separately, for the peak and established a steady level of depolarizing K^+ outward current. At the test potential of +4 mV the fast and late steady part of the K^+ outward current dropped from 60 to 36 nA (40%) and from 33 to 23 nA (31%). These results demonstrate the marked electrophysiological effects of CHP in leech Retzius nerve cells. Upon washout of the CHP, the fast and slow steady part of the K^+ outward current recovered by approximately 30% and 40% within 15 min.



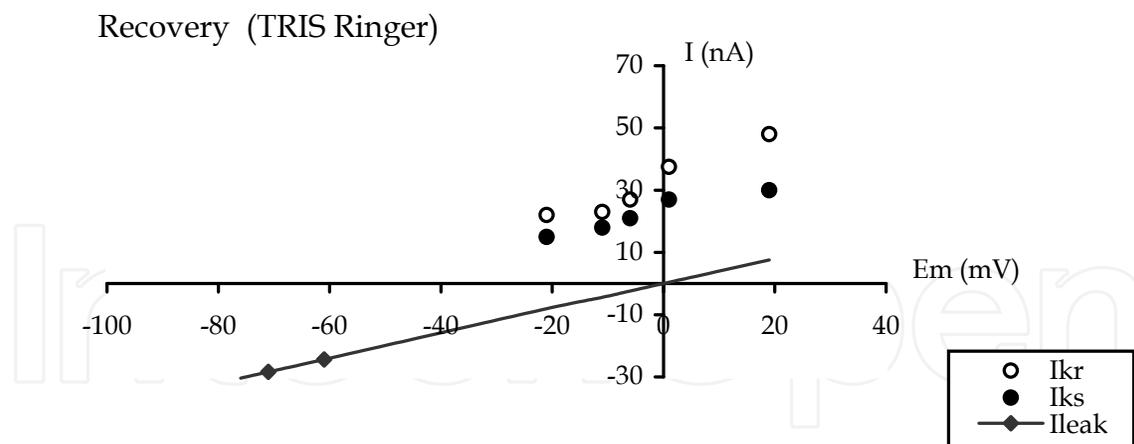
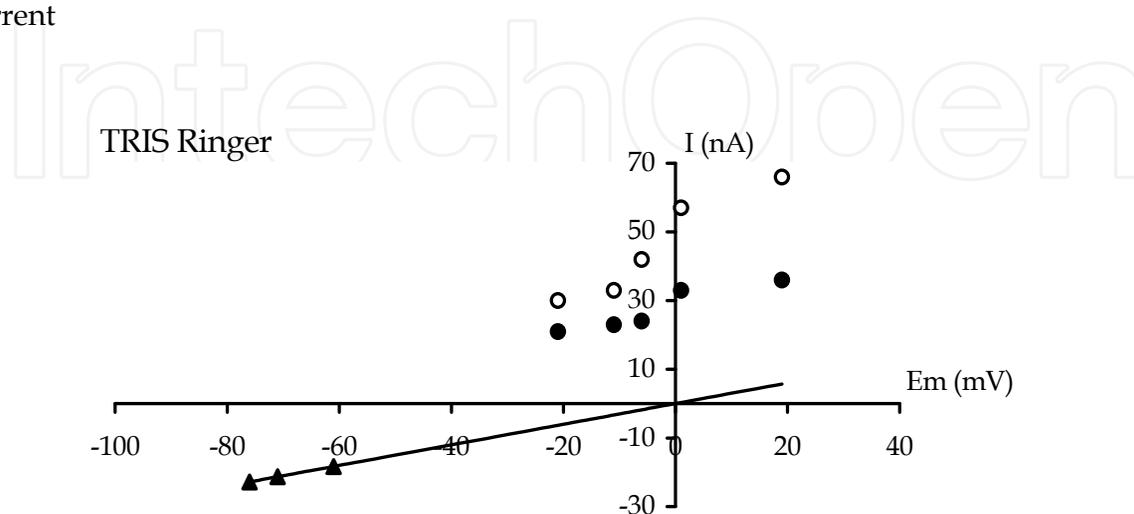


Fig. 3. The current-voltage relationship for the same cell, measured at the peak of the K^+ outward current (open circles) and at the end of stimulation (solid circles) in the Tris-Ringer, 25 min after adding 1 mM CHP and during the recovery.

3.5 Effects of hydrogen peroxide on the Ca^{2+} activated K^+ current of leech Retzius nerve cells

Modification of on membrane potassium currents by H_2O_2 , a membrane-permeable form of ROS, in LRNC was examined using the voltage clamp technique. Using a two-electrode voltage clamp, we examined the H_2O_2 effect on the K^+ outward current. In contrast to the effect of CHP, application of the H_2O_2 failed to inhibit fast and slow outward K^+ currents in leech Ringer. At the test potential of -14 mV from holding potential of -77 mV, the fast and late steady part of K^+ outward current dropped by 7.48% and 6.07%, respectively. 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 H_2O_2 . Voltage clamp experiments using double microelectrode methods revealed that H_2O_2 reduced a fast and slow K^+ outward current by 7.48% and 6.07% respectively at the test potential of -14 mV, after 25 min.

Ikr- rapid Ca^{2+} activated K^+ current; Iks- slow Ca^{2+} activated K^+ current; Ileak - passive leak current



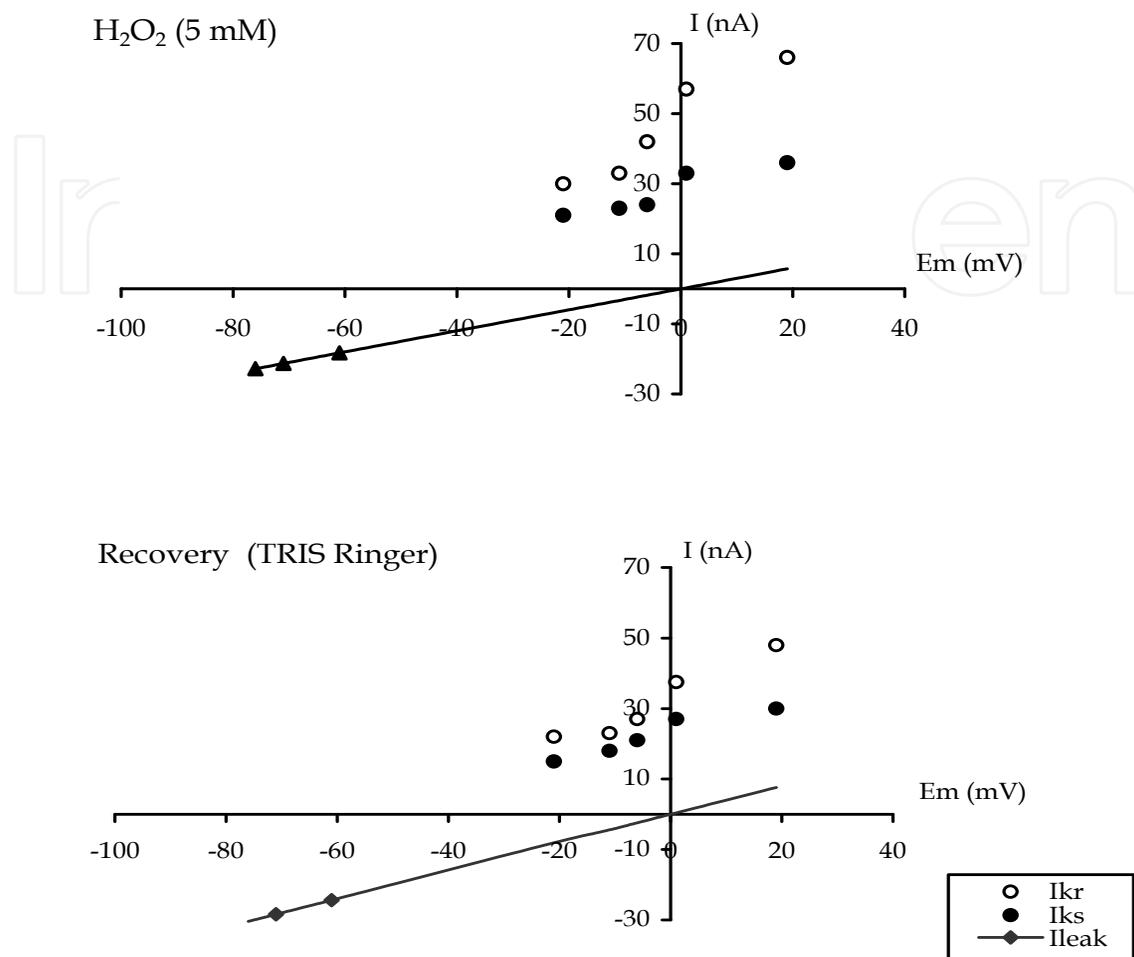


Fig. 4. Current-voltage relationship for the same cell measured at the peak of the K⁺ outward current (open circles) and at the end of stimulation (solid circles) in Tris-Ringer, 25 min after adding 5 mM H₂O₂ and during the recovery.

I_{kr} - rapid Ca²⁺ activated K⁺ current; I_{ks} - slow Ca²⁺ activated K⁺ current; I_{leak} - passive leak current

3.6 Effects of glutathione on cumene hydroperoxide-induced suppression of the Ca²⁺ activated K⁺ current of leech Retzius nerve cells

Reduced glutathione applied in a concentration of 0.2 mM partially blocked the effect of CHP on Ca²⁺ activated outward K⁺ currents. Figure 5 illustrates the effect of GSH on Ca²⁺ activated K⁺ currents. The application of the GSH reduced fast and slow K⁺ outward currents in the leech Ringer. At the test potential of -17 mV from the holding potential of -57 mV, the fast and late steady part of the K⁺ outward current dropped by 21% and 12%, respectively.

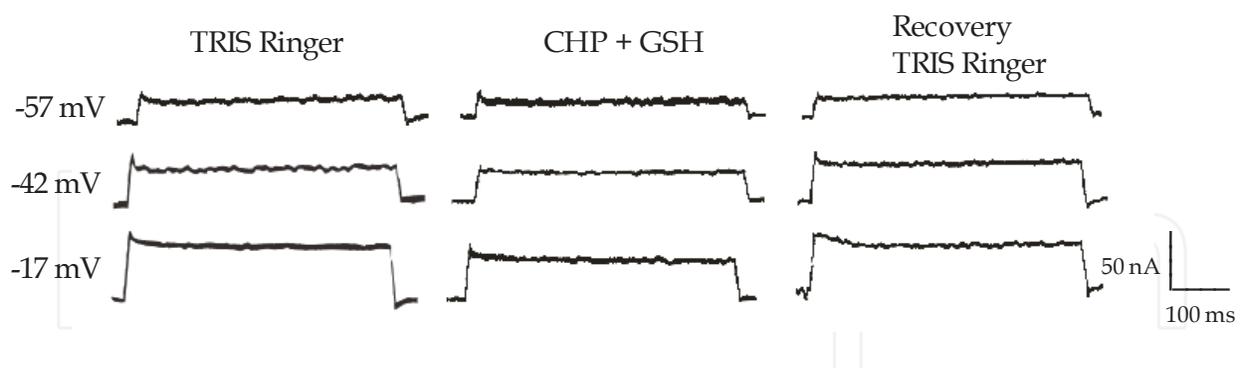
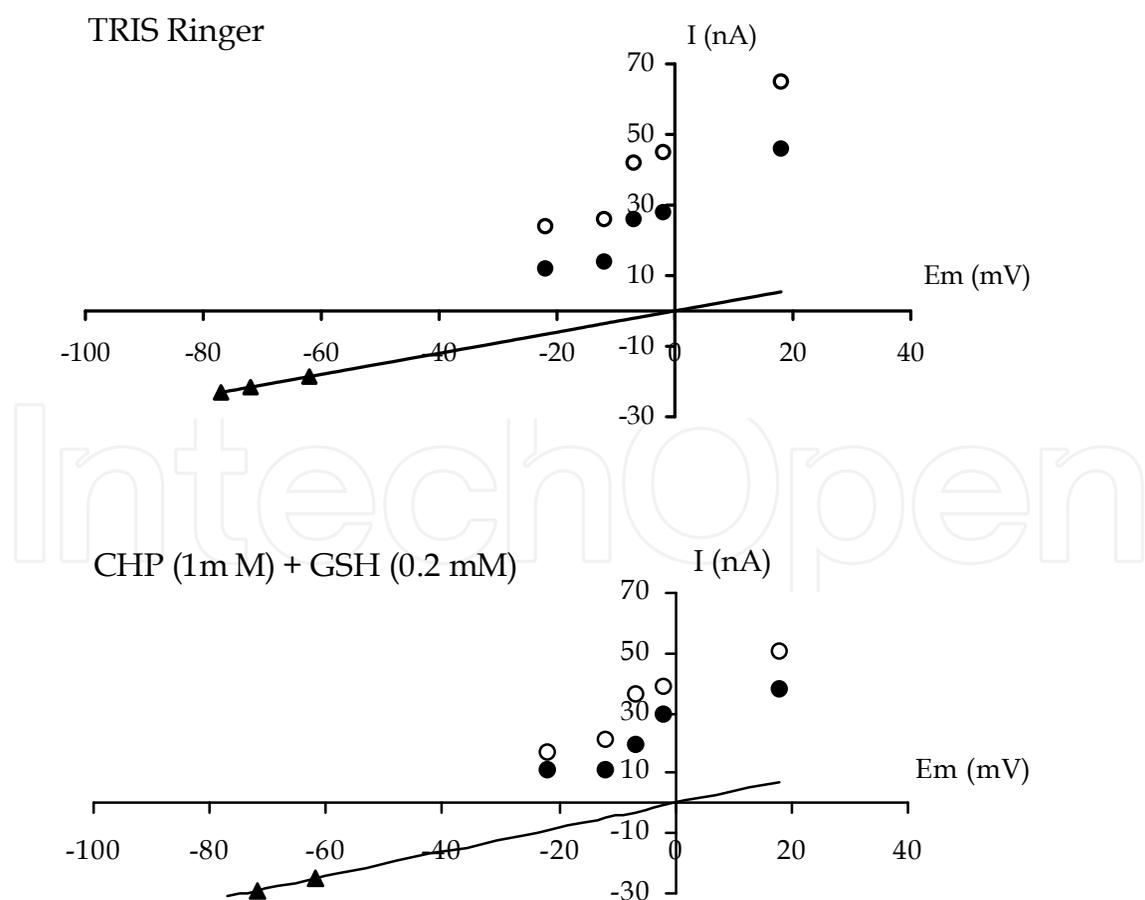


Fig. 5. Patterns of the voltage clamp current record obtained from Retzius nerve cell in the Tris-Ringer, after adding 1 mM CHP and 0.2 mM GSH, and in Na-free fluid (recovery) during displacement of the holding potential from -57 mV to -17 mV.

In the corresponding current-voltage relationship (Fig. 6) there were no significant changes on the early or late part of the K^+ outward current in the presence of 1 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 51 nA (21%) and from 46 to 38 nA (12%).



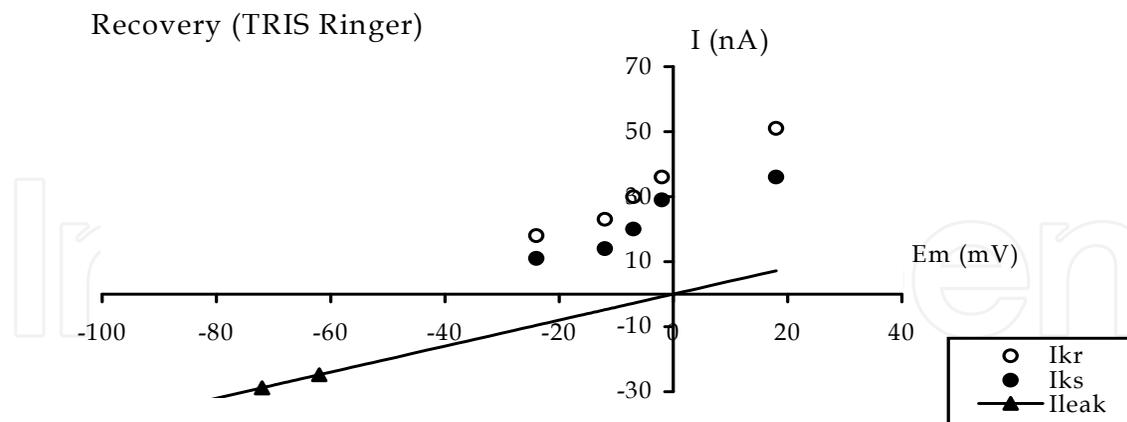


Fig. 6. Current-voltage relationship for the same cell measured at the peak of the potassium outward current (open circles) and at the end of stimulation (solid circles) in the Tris-Ringer, 25 min after adding 1 mM CHP and 0.2 mM GSH, and during the recovery.

Ikr- rapid Ca^{2+} activated K^+ current; Iks- slow Ca^{2+} activated K^+ current; Ileak - passive leak current

4. Discussion

The interest in H_2O_2 as a biologically active oxygen-derived intermediate is evident, because it is associated to a series of alterations and effects in different types of cells. The present data show that H_2O_2 did not significantly change, within 30 min, the shape of the amplitude of spontaneous spike potentials of LRNC. In the voltage clamp experiments, H_2O_2 was ineffective in the suppression of fast and slow Ca^{2+} activated K^+ currents. The background of this study were the findings that a 1 mM H_2O_2 treatment with or without FeCl_2 did not significantly change the resting membrane potential of LRNC (Jovanović & Beleslin, 1996; Jovanović & Beleslin, 1997; Jovanović & Beleslin, 2004).

The present results suggest that leech ganglia have an efficient system against oxidative stress. There are several explanations why leech Retzius nerve cells should be resistant to H_2O_2 . The simplest could be that leech Retzius nerve cells have a low concentration of polyunsaturated fatty acids, which are very sensitive to radical injury. This possibility is unlikely since neuronal membrane are rich in lipids (Whittemore et al., 1995; Wilson, 1997). Another explanation could be that they have an efficient scavenging enzyme system which reacts rapidly with H_2O_2 . Peroxidation of lipids that inactivates membrane-associated enzymatic protein, increases membrane permeability. However, since we have insignificant changes in action potential with H_2O_2 , it is reasonable to suppose that lipid membrane peroxidation did not occur. On the other hand, since changes in the action potential were not significant, it could further be expected that H_2O_2 was decomposed by a number of enzymatic and nonenzymatic antioxidant systems. A possible explanation for weak responses of LRNC to H_2O_2 is that the extracellular H_2O_2 application, results in an intracellular concentration some 7–10-fold below that of extracellular (Stone, 2004). It is well known that concentration as well as time of exposure plays an important role in the

response generated by ROS. The range of $[H_2O_2]$ used by different authors varies from 0.1 to 50 mM (Kourie, 1998).

When cells are exposed to external H_2O_2 , the intracellular consumption of H_2O_2 catalyzed by anti-oxidants and enzymes is able to generate a gradient of H_2O_2 across membranes, which makes the intracellular H_2O_2 concentration lower than the extracellular one. Previous studies have reported that H_2O_2 did not affect channel activity when added to the extracellular side (Soto, 2002). In particular, oxidation of free SH groups of cysteines, present in a greater proportion at the intracellular side, could explain the observed difference. These results provide evidence for an intracellular site(s) of H_2O_2 action. It has been recently demonstrated that H_2O_2 activates TRPC6 channels via modification of thiol groups of intracellular proteins (Graham et al., 2010). H_2O_2 is a weaker oxidizing agent than other ROS. H_2O_2 is not by itself reactive enough to oxidize organic molecules in an aqueous environment. Nevertheless, the biological importance of H_2O_2 stems from its participation in the production of more reactive chemical species such as HO^\bullet and its role as a source of free radicals has been emphasized rather than its chemical reactivity. However, because of its extremely short half-life, HO^\bullet is effective only near the locus of its production.

The results reported in this paper show that an alkyl-hydroperoxide, CHP is a more efficient oxidant than H_2O_2 . In contrast to H_2O_2 , CHP, induced dose and time dependent membrane depolarization with a marked prolongation of spontaneous repetitive activity. These actions appear to underlie the prolongation of the action potential, and contribute to repetitive firing. Several mechanisms have been proposed for the plateau of the prolonged action potential, such as sustained inward Na^+ current, block of Ca^{2+} activated K^+ channels, modification of Ca^{2+} channels or its transformation in Na^+ channels. Our findings support the second proposal. A possible explanation is that CHP form free radicals that are more stable than the HO^\bullet . H_2O_2 and organic hydroperoxides, generate distinct ROS. HO^\bullet generated by one-electron reduction of the H_2O_2 , damages adjacent molecules at diffusion controlled rates. By contrast, the organic hydroperoxide triggers the generation of the free radical intermediates peroxy and alkoxy radicals, which can cross cellular membranes and evoke the production of the HO^\bullet (Hwang et al., 2009). It was well known that HO^\bullet generated from H_2O_2 could cause peroxidation of lipids that inactivates membrane-associated protein, increasing membrane permeability. This metabolic and physico-chemical alteration of a cell membrane would produce intracellular Ca^{2+} overload. CHP is more hydrophobic than H_2O_2 . 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. The present results support the view that CHP stimulates lipid peroxidation, as the mechanism of ROS-induced cell membrane injury.

Although several previous investigations have described electrophysiological effects of H_2O_2 and CHP, the literature describing these effects is sometimes contradictory. For example, Cerbai et al. (1991) and Ward and Giles (1997) did not observe any effect, in contrast to Tarr and Valzeno (1989) who obtained a decrease in the rectifying current. Vega-Saenz de Miera and Rudy (1992) reported that H_2O_2 inhibited three cloned voltage-gated K^+ channels expressed in *Xenopus* oocytes. 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 (Dyavanapalli et

al., 2010; Whyte et al., 2009). Nakaya et al. (1992) examined the mechanism of membrane depolarization induced by CHP in guinea-pig papillary muscles, using ion-selective microelectrode and patch clamp techniques. They demonstrated that the depolarization of the resting membrane is, at least in part, due to the inhibition of inward rectifier K^+ channel activity, and may play an important role in the genesis of reperfusion-induced arrhythmias.

There are conflicting descriptions of the current changes induced by ROS, and an incomplete understanding of which is responsible for the observed changes in action potential duration. For example, the inward rectifying K^+ current has been reported to be either unaffected (Cerbai et al., 1991) or decreased (Tarr & Valenzeno, 1991). The electrophysiological effects of ROS generally consist of a reduction in action potential amplitude and an increase in action potential duration followed by a reduction (Tarr and Valenzeno, 1989; Barrington, 1994; Satoh and Matsui, 1997), although either only a reduction (Goldhaber et al., 1989; Hayashi et al., 1989; Coetzee et al., 1990) or only an increase in action potential duration (Barrington, 1994) have also been reported. Nakaya et al. (1991) reported that ROS-induced shortening of the action potential duration of guinea-pig isolated ventricular myocytes. The underlying mechanisms of the action potential shortening are undoubtedly complex, and changes in membrane currents other than the outward current through the ATP-sensitive K^+ channels may also contribute to the action potential shortening. Matsuura and Shattock (1991) demonstrated that oxidant stress induces a decrease in the resting potassium conductance and an increase in Ca^{2+} activated membrane conductance. Both factors may underlie the depolarization of resting membrane potential, prolongation of the action potential and automaticity. Tokube et al. (1996) reported biphasic changes in the action potential duration, with initial lengthening of the action potential and subsequent shortening. In voltage-clamp experiments, ROS suppressed the L-type calcium current, the delayed rectifier K^+ current and the inward rectifier K^+ current. A recent paper reported that relatively low concentrations of CHP (100 μ M) led to a significant decrease in the cellular content of ATP and reduced glutathione (Vimard et al., 2010).

K^+ channels are a family of ion channels that govern the intrinsic electrical properties of neurons in the brain (Lujan, 2010). K^+ currents control action potential duration, Ca^{2+} dependent synaptic plasticity, the release of neurotransmitters and epileptiform burst activity. Enhanced excitability of K^+ channels (via downmodulation, or changes in biophysical properties such as inactivation kinetics and voltage dependence), could all result in enhanced Ca^{2+} responses to firing activity (Pongs, 1999). Ca^{2+} activated K^+ channels are a large family of K^+ channels that are found throughout the central nervous system and in many other cell types, but its *in vivo* physiological functions have not been fully defined. Ca^{2+} dependent K^+ channels are activated by both depolarization and increases in intracellular free $[Ca^{2+}]$. Ca^{2+} dependent K^+ currents contribute to the repolarization of neurons to resting membrane potential (Hille, 2001). Thus, Ca^{2+} dependent K^+ channels determine the shape of the action potential and help in regulating cell excitability (Goodman, 2008). Outward currents play a principal but not a sole role in repolarization in many types of excitable cells. The excitability of neurons is governed by the input they receive from their neighbours and the intrinsic excitability of the neuron. Electrophysiological studies have revealed that the voltage gated ion channels are important in determining the intrinsic excitability of neurons in the CNS. The voltage gated ion channels are critical in producing hyperexcitability such as that associated with seizure discharges (Errington, 2005) and causing epilepsy in humans (Du et al., 2005; Ez-Sampedro

et al., 2006; Jorge et al., 2011). Ca^{2+} activated K^+ channels are essential for the production of bursting activity in mammalian cortical neurons (Jin et al., 2000) and they can also influence rhythmic firing patterns and bursting output (Gu et al., 2007). Voltage gated K^+ currents play crucial roles in modifying neuronal cellular and network excitability and activity (Muller & Connor, 1991).

Results of our study demonstrate that SH reducing agent, GSH, incompletely inhibited the effect of CHP on calcium-activated potassium channels in LRNC. The SH groups are known to be important for the function of many membrane transport systems. These include also various potassium channels (Lee et al., 1994; Han et al., 1996). Redox modification of cysteine SH groups may also be an important mechanism of controlling ion channel function. There are several explanations for the incomplete recovery of calcium-activated potassium channels. The simplest could be that CHP treatment must be modifying other amino acid residues, e.g., as methionine or tryptophan, besides cysteine. Cysteine and methionine residues are particularly sensitive to oxidation by almost all forms of ROS. In addition, the localization of the critical SH groups (responsible for the inhibitory action of the oxidative agents), could explain the observed partly protective effects of glutathione against cumene hydroperoxide-induced toxicity. The part of changes in channel properties depend on cysteine residues located on the cytoplasmic domains of the calcium-activated potassium channels in LRNC. The activity of potassium channels is dependent on the redox status of one or more SH groups on the channel protein, or an associated regulatory protein. Of course, it is possible that the oxidant agent affects other components associated to the membrane or to the channel (the target of the oxidizing agent could be a β -subunit or some membrane-bound enzyme able to promote channel phosphorylation).

5. Conclusions and implications

The present data show that the CHP is a more efficient neurotoxin and oxidant than H_2O_2 , as well as that the suppression of Ca^{2+} activated outward K^+ currents is responsible for the prolongation of spike potential in leech Retzius nerve cells. Here we discuss the implications that free radicals can have a significant role in the appearance of spontaneous repetitive activity in Retzius nerve cells by interrupting the process of repolarization.

What is the pathophysiological relevance of a block of voltage-gated K^+ channels? In the past few years it has become more appreciated that a block of voltage-gated K^+ channels by ROS contributes to increased neuronal excitability and repetitive firing. These data indicate that a block of voltage gated K^+ channels contributes to an increase in neuronal excitability such as that associated with seizure discharges and causing epilepsy in humans. The interaction of ROS with K^+ channels may cause modifications of membrane currents and potentials thereby leading to neuronal dysfunction. The suppression of Ca^{2+} dependent K^+ currents proposed in this paper might have a broader significance, pertaining not only to leeches, but mammalian neurons as well. Leech ganglia are good models for studying the cellular basis for epileptiform activity. The largest neurons in the leech nervous system are Retzius cells which exhibit stable resting membrane potential and which are non-bursting neurons with a low spontaneous firing rate. An understanding of ion mechanisms epilepsy will provide insight into the molecular events of epileptogenesis, improve molecular diagnostic utility, and identify novel therapeutic targets for improved treatment of human epilepsy.

Electrophysiological analyses showed that oxidative modification of K⁺ channels might represent a fundamental pathogenic mechanism in the mammalian brain during normal aging, as well as in neurodegenerative diseases such as Alzheimer's and Parkinson's. Therefore, it is probable that the action of ROS on K⁺ channels might play a role in changes in electrical identity of neurons produced by ischemia and of course in neuronal death.

Considering that K⁺ channels and ROS are universal players in the biological game, we put forward the hypothesis that the oxidation of voltage-gated K⁺ channels may represent a general pathogenic mechanism in biological organisms. Obviously, more work is needed to establish the possible involvement of ion channels and of their modulation by ROS as important mechanisms in several pathological conditions in the brain. In addition, such knowledge may help to explain pathophysiological alterations in neurological diseases and to develop new strategies for therapeutic intervention that aim at preventing oxidative stress in the brain.

6. References

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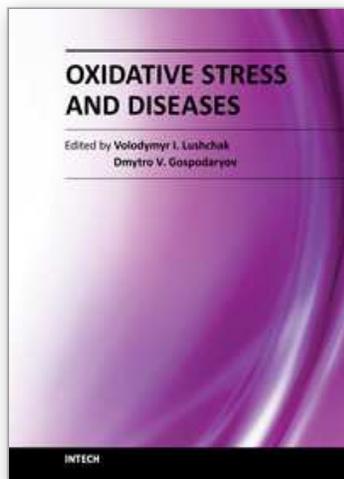
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Oxidative Stress and Diseases

Edited by Dr. Volodymyr Lushchak

ISBN 978-953-51-0552-7

Hard cover, 610 pages

Publisher InTech

Published online 25, April, 2012

Published in print edition April, 2012

The development of hypothesis of oxidative stress in the 1980s stimulated the interest of biological and biomedical sciences that extends to this day. The contributions in this book provide the reader with the knowledge accumulated to date on the involvement of reactive oxygen species in different pathologies in humans and animals. The chapters are organized into sections based on specific groups of pathologies such as cardiovascular diseases, diabetes, cancer, neuronal, hormonal, and systemic ones. A special section highlights potential of antioxidants to protect organisms against deleterious effects of reactive species. This book should appeal to many researchers, who should find its information useful for advancing their fields.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Zorica Jovanović (2012). Effects of Oxidative Stress on the Electrophysiological Function of Neuronal Membranes, *Oxidative Stress and Diseases*, Dr. Volodymyr Lushchak (Ed.), ISBN: 978-953-51-0552-7, InTech, Available from: <http://www.intechopen.com/books/oxidative-stress-and-diseases/effects-of-oxidative-stress-on-the-electrophysiological-function-of-neuronal-membranes>

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