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Brain Research 970 (2003) 159–168
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Research report

Hydrogen peroxide modulates K^+ ion currents in cultured *Aplysia* sensory neurons

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Accepted 8 January 2003

Abstract

Hydrogen peroxide (H_2O_2) causes oxidative stress and is considered a mediator of cell death in various organisms. Our previous studies showed that prolonged (>6 h) treatment of *Aplysia* sensory neurons with 1 mM H_2O_2 produced hyperpolarization of the resting membrane potential, followed by apoptotic morphological changes. In this study, we examined the effect of H_2O_2 on the membrane conductance of *Aplysia* sensory neurons. Hyperpolarization was induced by 10 mM H_2O_2 within 1 h, and this was attributed to increased membrane conductance. In addition, treatment with 10 mM H_2O_2 for 3 min produced immediate depolarization, which was due to decreased membrane conductance. The H_2O_2-induced hyperpolarization and depolarization were completely blocked by dithiothreitol, a disulfide-reducing agent. The later increase of membrane conductance induced by H_2O_2 was completely blocked by 100 mM TEA, a K^+ channel blocker, suggesting that H_2O_2-induced hyperpolarization is due to the activation of K^+ conductance. However, the inhibition of K^+ efflux by TEA did not protect against H_2O_2-induced cell death in cultured *Aplysia* sensory neurons, which indicates that the signal pathway leading to H_2O_2-induced cell death is more complicated than expected.

Theme: Development and regeneration

Topic: Neuronal death

Keywords: Aplysia; K^+ channel; Hydrogen peroxide; Tetraethylammonium; Membrane potential; Cell death; Sulfhydryl modification

1. Introduction

Hydrogen peroxide (H_2O_2) is ubiquitously generated during normal cellular metabolism, but, when present in excess, H_2O_2 may induce the destruction of many cell types through necrotic or apoptotic processes [6,23]. We previously showed that H_2O_2 can induce cell death in *Aplysia* sensory neurons, which has both apoptotic (neurite fragmentation, cell body disintegration, apoptotic nuclear shrinkage, and chromatin condensation) and necrotic (swelling of organelles) characteristics [22].

H_2O_2 causes a change in the resting membrane potential (by depolarization or hyperpolarization) of various cells in many organisms [12,13,15,18,20]. In many cases, H_2O_2 modulates the resting membrane potential by activating K^+ channels. For example, in LLC-PK1 cells, H_2O_2-induced hyperpolarization is due to the activation of an ATP-sensitive, Ca^{2+}-independent K^+ channel, which is mediated by ATP depletion [12]. In human endothelial cells, 1 mM H_2O_2 elicited hyperpolarization by activating a Ca^{2+}-activated K^+ channel mediated by an increased intracellular Ca^{2+} concentration, whereas low concentrations of H_2O_2 induced membrane depolarization, mediated by the inhibition of inward-rectifying K^+ channels via intracellular messengers [4]. Moreover, H_2O_2 also directly modulates K^+ channels by oxidizing the sulfhydryl groups of channel proteins [1,25,31]. These channels include voltage-dependent K^+ channels [25,31], inward-rectifier K^+ channels [1], ATP-regulated K^+ channels [16,33], and Ca^{2+}-activated K^+ channels [32]. Vega-Sbenz and Rudy [31] showed that the inactivation of cloned voltage-activated K^+ channels (KV1.4, KV3.3 and KV3.4) in *Xenopus*

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0006-8993/03/$ – see front matter
doi:10.1016/S0006-8993(03)02316-3

oocytes is inhibited by the application of H_2O_2. Furthermore, diamide, a sulfhydryl oxidizing agent, inhibits, whereas the sulfhydryl reducing agent dithiothreitol (DTT) augments, the channel activity of Ca^{2+}-activated K^+ channels in a dose-dependent manner [32]. Several studies have also shown that K^+ efflux leading to a decrease in $[K^+]_i$ is implicated in cell death; conversely, cell death was found to be inhibited when K^+ efflux was inhibited [3,24,34,35].

In a previous study, we demonstrated that *Aplysia* sensory neurons showed hyperpolarization of the membrane potential after prolonged treatment (>6 h) with 1 mM H_2O_2. However, it was not clear how prolonged treatment with H_2O_2 could hyperpolarize the membrane potential in *Aplysia* sensory neurons [22]. In this study, we investigated the effects of H_2O_2 on ion currents and the effects of the inhibition of K^+ efflux on cell death in *Aplysia* sensory neurons. As a result, we found that the application of 10 mM H_2O_2 produced biphasic responses of the membrane potential, immediate depolarization and later hyperpolarization, both of which were primarily mediated by K^+ currents and by sulfhydryl modification in cultured sensory neurons. However, the blocking of K^+ efflux by treatment with 100 mM TEA, did not reduce H_2O_2-induced cell death.

2. Materials and methods

2.1. Dissociated culture of Aplysia sensory neurons

Mechanosensory neurons of *Aplysia* were dissociated from the pleural ganglia of adult *Aplysia kurodai* (60–180 g), and plated on poly-L-lysine (Sigma, St. Louis, MO, USA) coated culture dishes (P50G-0-14-F) containing L15 media and filtered hemolymph (1:1). Cultures were maintained at 18 °C and the culture media were replaced every 2 days. After 3–5 days, when the neurites had grown substantially, they were used for the experiments described below.

2.2. Electrophysiological recordings

The resting membrane potential was recorded as described previously [7]. Briefly, the voltage recordings and current injections were carried out in a conventional single electrode current clamp mode using an Axoclamp 2B (Axon Instruments, CA, USA). The culture medium was replaced with 50% isotonic L15/50% ASW (L15: Leibowitz L15 (Sigma) with 400 mM NaCl, 27 mM $MgSO_4$, 27 mM $MgCl_2$, 11 mM $CaCl_2$, 10 mM KCl, and 2 mM $NaHCO_3$; ASW: 450 mM NaCl, 10 mM KCl, 11 mM $CaCl_2$, 29 mM $MgCl_2$, 10 mM HEPES at pH 7.6) [21]. Cultured *Aplysia* sensory neurons (3–5 days old) were impaled with a microelectrode (8–13 MΩ) filled with 0.5 M KCl, 2 M K-acetate, and 10 mM K-HEPES (pH 7.4). The resting potential was measured 5–10 min after impalement. Only those cells with a resting potential more negative than −40 mV were used. Hydrogen peroxide (Merck), 5-hydroxytryptamine (5-HT) (Sigma), DL-dithiothreitol (DTT) (Sigma), and diamide (Sigma) solutions were freshly made by dissolving in L15/ASW. Ouabain (1 M; Sigma) dissolved in dimethyl sulfoxide (DMSO) was diluted in L15/ASW solution to a final concentration of 0.1 mM. Membrane excitability was measured as the number of action potentials elicited during 1 s by a depolarizing current pulse (0.05–0.3 nA), which produced one to three spikes before drug application. Sensory neurons were voltage-clamped at a holding potential of −60 mV in L15/ASW solutions and then clamped at potentials between −90 and −30 mV for 400 ms in 10 mV steps with two electrodes (5–15 MΩ) filled with 0.5 M KCl, 2 M K-acetate, and 10 mM K-HEPES (pH 7.4). In some cases, shown in Figs. 2 and 5, the membrane potential and the conductance were alternatively measured in the same neuron by using a two-electrode voltage clamp or current clamp.

2.3. TdT-mediated dUTP nick end labeling (TUNEL) staining

TUNEL staining was performed as previously described [22]. Briefly, 120–250 sensory neurons were plated and cultured for 4–5 days. After being treated with H_2O_2 and TEA for 30 min at 18 °C, cultured *Aplysia* sensory neurons were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 30 min at room temperature. After rinsing with PBS, endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol, and the samples were again rinsed, three times with PBS. The TUNEL reaction mixture (Roche Molecular Biochemicals, Mannheim, Germany) was then added, and the cells were incubated in a humidified chamber for 1 h at 37 °C. After being thoroughly rinsed with PBS, the cells were treated with anti-fluorescein antibodies conjugated with horseradish peroxidase for 30 min at 37 °C, and then washed three times with PBS. Diaminobenzidine substrate solution (Roche Molecular Biochemicals) was then added and incubation was continued for 15 min at room temperature. The cells were then washed out with PBS, and examined under a light microscope.

3. Results

3.1. Effects of H_2O_2 on the membrane potential of cultured Aplysia sensory neurons

We have previously shown that prolonged (6–18 h) treatment with 1 mM H_2O_2 of *Aplysia* sensory neurons produces a hyperpolarization of the membrane potential.

We also found that the application of a higher concentration of H_2O_2 (10 mM) could produce a hyperpolarization within 1 h, which allowed us to perform continuous intracellular recordings for an hour or so. The application of 10 mM H_2O_2 to cultured sensory neurons also led to a transient depolarization of the resting potential from -44.0 ± 0.7 to -39.6 ± 0.8 mV (Fig. 1A and B). However, in the continuous presence of 10 mM H_2O_2, hyperpolarization followed within 1 h, as demonstrated by a resting potential shift from -44.0 ± 0.7 to -63.3 ± 1.8 mV (Fig. 1A and B). This hyperpolarization reached a maximum approximately 1 h after the addition of H_2O_2, and was eventually replaced by a depolarization, indicative of cell deterioration [22] (Fig. 1A). In untreated cells, the resting membrane potential was not significantly changed within 1 h, from -44.0 ± 1.6 to -43.5 ± 4.4 mV (Fig. 1B). Together these results show that 10 mM H_2O_2, over the space of 1 h, produced two types of resting membrane potential responses, i.e. an immediate depolarization and a later hyperpolarization in cultured sensory neurons.

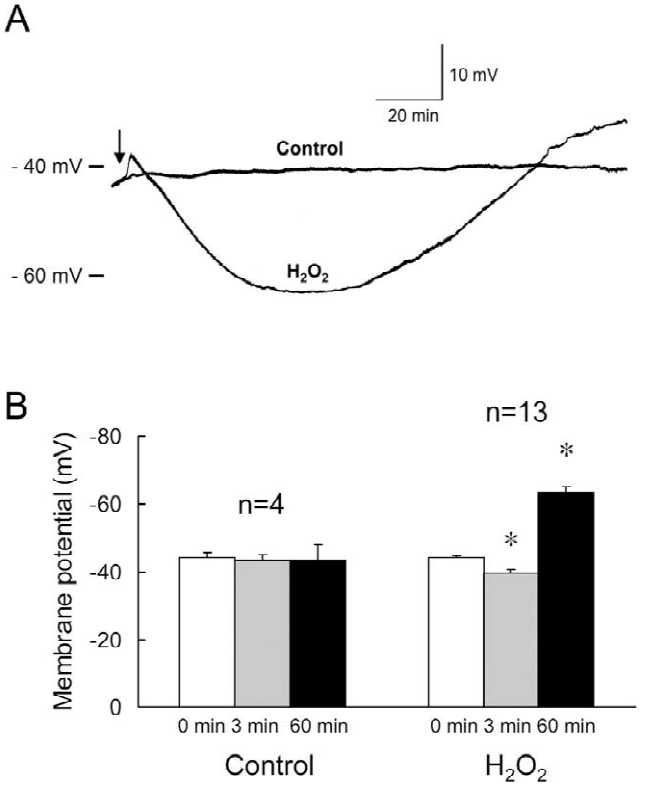

Fig. 1. Effects of H_2O_2 on the membrane potential of *Aplysia* cultured sensory neurons. (A) Representative traces of the membrane potential of sensory neurons exposed either to 10 mM H_2O_2 or mock treated (control). The two traces of the membrane potential are superimposed. The arrow indicates H_2O_2 or mock application. (B) Summary bar graph displaying the mean±S.E.M. of the membrane potential at set times, i.e. before (0 min) and 3 and 60 min after H_2O_2 treatment. *, $P<0.001$ compared with cells before H_2O_2 treatment (0 min), two-tailed paired *t*-test. *n*, number of cells treated.

3.2. Effects of H_2O_2 on the membrane conductance of Aplysia sensory neurons

To investigate the ionic basis of the H_2O_2-induced depolarization and hyperpolarization in cultured sensory neurons, we conducted conventional two-electrode voltage clamping in cultured sensory cells. As shown by the *I–V* curves (Fig. 2B), 10 mM H_2O_2 decreased the slope conductance within 3 min from 37.9 ± 5.3 to 31.4 ± 5.0 nS ($P<0.05$, two-tailed paired *t*-test, $n=7$), and this was followed by an increase in the slope conductance ca. 1 h after treatment from 37.9 ± 5.3 to 102.7 ± 10.7 nS ($P<0.001$, two-tailed paired *t*-test, $n=7$) (Fig. 2). Thus, H_2O_2-induced depolarization correlates with a decrease in membrane conductance, and H_2O_2-induced hyperpolarization is mediated by an increased membrane conductance in cultured sensory neurons.

To examine whether the holding currents (I_h) required to maintain the normal resting membrane potential are modified by H_2O_2 treatment, we measured the steady state currents at -50 mV in the same cells before and 3 and 60 min after H_2O_2 treatment (Fig. 2C). A paired analysis showed that I_h was transiently decreased 3 min after H_2O_2 treatment, but increased 1 h after H_2O_2 treatment. Considering the fact that the equilibrium potentials of Na^+, K^+ and Cl^- are close to $+55$, -80 and -55 mV, respectively, these results suggest that these two membrane potential responses induced by treatment with 10 mM H_2O_2 are probably mediated by the closing and opening of K^+ or Cl^- channels.

3.3. Effects of sulfhydryl modification on H_2O_2-induced depolarization and hyperpolarization

Several studies have shown that H_2O_2 can modulate channel activity by sulfhydryl modification [1,25,31]. To examine whether H_2O_2 affects the membrane potential in this manner, we treated cultured *Aplysia* sensory cells with DTT, a disulfide-reducing agent. Pretreatment with 30 mM DTT for 30 min slightly hyperpolarized the resting membrane potential (1–2 mV) of *Aplysia* sensory neurons ($n=2$). In this condition, the application of 10 mM H_2O_2 did not produce a change in the resting membrane potential (-45.8 ± 1.9 mV (0 min) vs. -44.8 ± 2.9 mV (3 min) vs. -46.3 ± 1.9 mV (60 min), $n=4$) (Fig. 3A), suggesting that H_2O_2-induced changes in the membrane potential are completely blocked by DTT.

To examine whether the thiol-oxidizing agent, diamide, can mimic the effect of H_2O_2 on the membrane potential, we treated cultured *Aplysia* sensory neurons with diamide. The application of diamide at 0.1–10 mM induced membrane hyperpolarization in a dose-dependent manner (Fig. 3B). Diamide at 1 mM produced a hyperpolarization similar to that produced by 10 mM H_2O_2, and induced hyperpolarization within 1 h from -44.0 ± 1.2 to

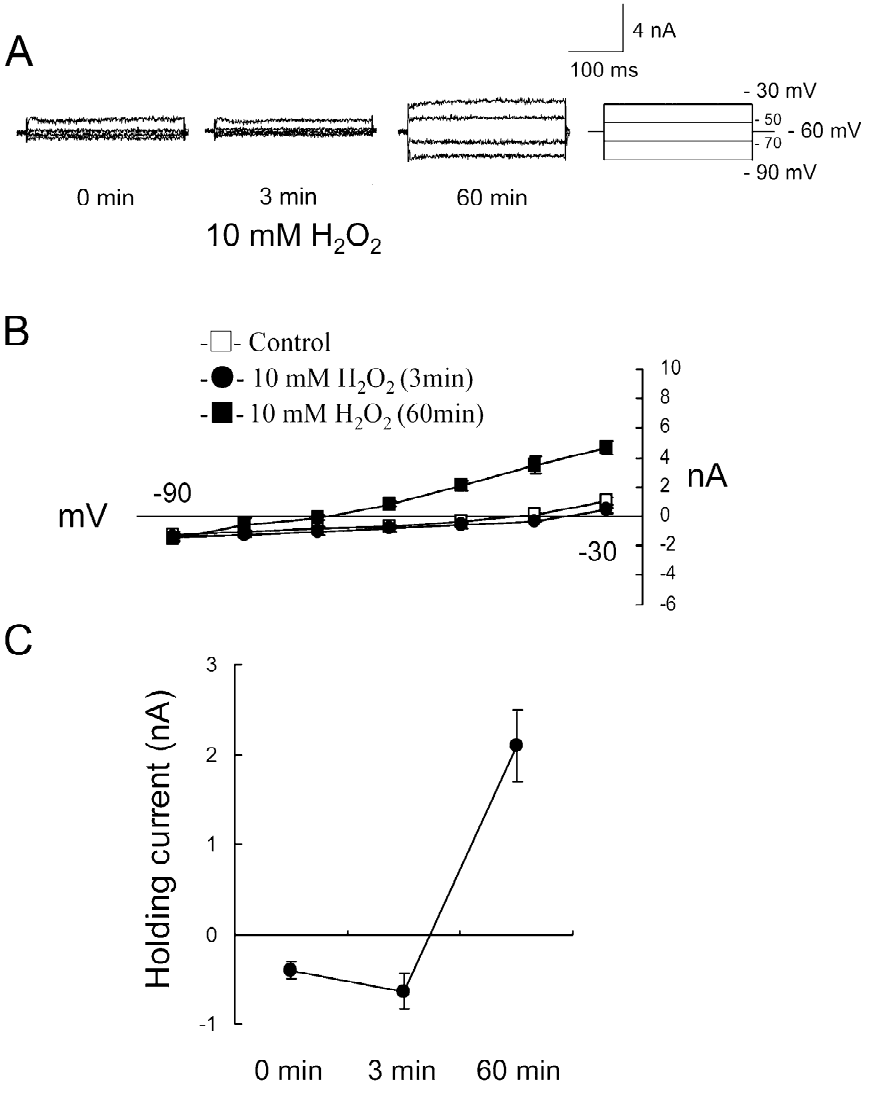

Fig. 2. Effects of H_2O_2 on membrane conductance in *Aplysia* cultured sensory neurons. (A) Representative traces of membrane currents in an untreated cell (control) and in a cell exposed to 10 mM H_2O_2. (B) Current–voltage (*I–V*) curves before (control), and 3 and 60 min after the application of 10 mM H_2O_2. (C) Steady-state holding currents during voltage-clamping at −50 mV. Bars represent mean±S.E.M.

−74.0±2.4 mV, but did not induce immediate depolarization (−44.0±1.2 vs. −44.0±0.9 mV, $n=4$) (Fig. 3C). However, this diamide-induced hyperpolarization was blocked by 10 mM DTT, a disulfide-reducing agent (−41.3±0.9 vs. −43.3±1.8 mV, $n=3$) (Fig. 3C). These data suggest that sulfhydryl oxidation can hyperpolarize *Aplysia* sensory neurons.

3.4. TEA blocked H_2O_2-induced hyperpolarization but not H_2O_2-induced depolarization

TEA, a non-selective K^+ channel blocker, was used to characterize currents activated during H_2O_2-induced depolarization and hyperpolarization. To determine whether TEA inhibits H_2O_2-induced depolarization, we added 100 mM TEA to sensory neurons 5 min before the H_2O_2 treatment. On applying 100 mM TEA the membrane potential was slightly depolarized from −43.6±1.3 to −39.3±1.7 mV ($n=7$) (Fig. 4). In this condition, treatment with 10 mM H_2O_2 produced a further depolarization of the membrane potential from −39.3±1.7 to −34.6±1.5 mV (Fig. 4). These results show that H_2O_2-induced depolarization is not blocked by a high concentration of TEA (100 mM).

Next, we examined the effect of TEA on H_2O_2-induced hyperpolarization (Fig. 5A). Treatment with 10 mM H_2O_2 of sensory neurons for 1 h induced a hyperpolarization from −42.4±0.6 to −73.2±2.7 mV ($n=5$) (Fig. 5A), and increased membrane conductance from 24.6±5.0 to 111.8±14.7 nS ($n=5$) (Fig. 5B and C). A low concentration of TEA (10 mM) applied to the hyperpolarized membrane was found to partially block the H_2O_2-induced hyperpolarization (−73.2±3.0 to −61.0±2.3 mV, $n=5$) by reducing the membrane conductance (111.8±14.7 to

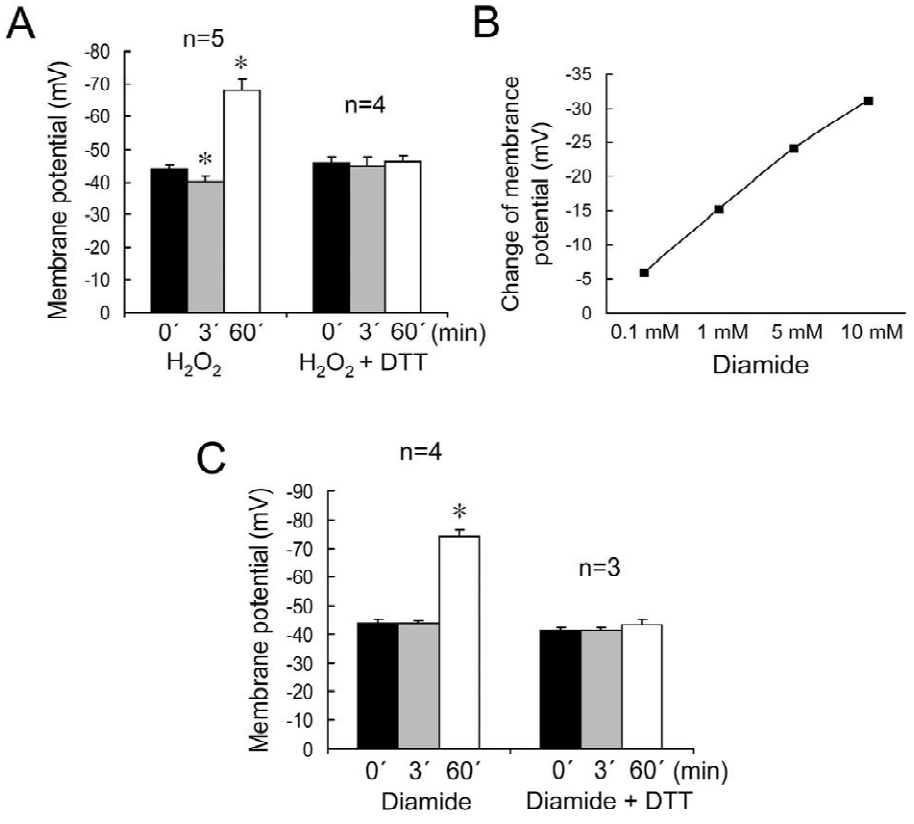

Fig. 3. (A) Effects of DTT on H_2O_2-induced depolarization and hyperpolarization. H_2O_2-induced depolarization and hyperpolarization were completely blocked by 30 mM DTT. Bars represent mean±S.E.M. of the membrane potential. (B, C) Effects of diamide on the membrane potential of *Aplysia* sensory neurons. (B) A summary bar displays the mean membrane potential changes induced by diamide treatment (0.1–10 mM) for 20 min ($n=2$). (C) Effects of diamide (1 mM) on the membrane potential of *Aplysia* sensory neurons. Diamide-induced hyperpolarization was completely blocked by 10 mM DTT. Bars represent mean±S.E.M. of the membrane potential. *, $P<0.001$, two-tailed paired *t*-test. *n*, number of cells treated.

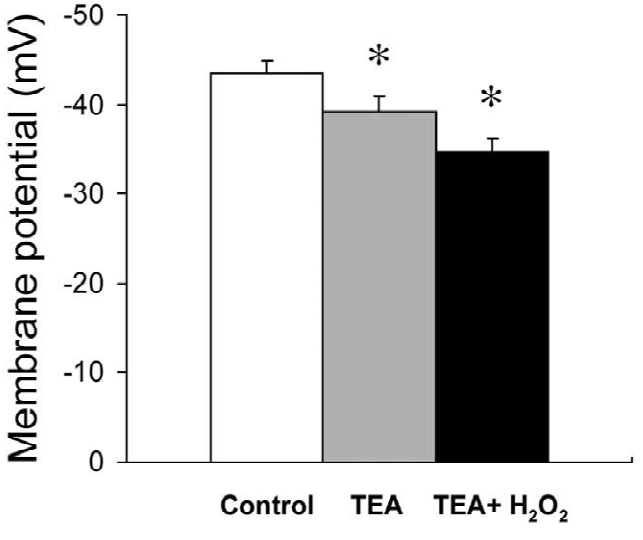

Fig. 4. Effects of TEA on H_2O_2-induced depolarization. 100 mM TEA failed to block H_2O_2-induced depolarization. Data are mean±S.E.M. ($n=7$). *, $P<0.001$, two-tailed paired *t*-test. *n*, number of cells treated.

49.3±9.7 nS, $n=5$), which was completely blocked by 100 mM TEA (membrane potential: −61.0±2.3 to −41.6±2.7 mV; membrane conductance: 49.3±9.7 to 22.9±7.6 nS, $n=5$). These results show that TEA blocked the H_2O_2-induced hyperpolarization in a dose-dependent manner and that the change in membrane potential due to TEA is proportional to the membrane conductance. Thus, we suggest that the H_2O_2-induced increase in membrane conductance is primarily mediated by increased K^+ conductance, considering that this conductance increase is mainly blocked by TEA and that the resting membrane potential induced by 10 mM H_2O_2 is very close to E_K (−80 mV).

The H_2O_2-induced hyperpolarization was unaffected by 0.1 nM apamin, a Ca^{2+}-dependent K^+ channel blocker ($I_{K(Ca)}$), and 100 μM glibenclamide, an ATP-dependent K^+ channel ($I_{K(ATP)}$) blocker (data not shown). These results indicate that different types of K^+ channels, other than apamin-sensitive Ca^{2+}-dependent K^+ channels and glibenclamide-sensitive ATP-dependent K^+ channels, may play a role in the H_2O_2-induced hyperpolarization of cultured *Aplysia* sensory neurons.

To examine whether external Ca^{2+} is important for this

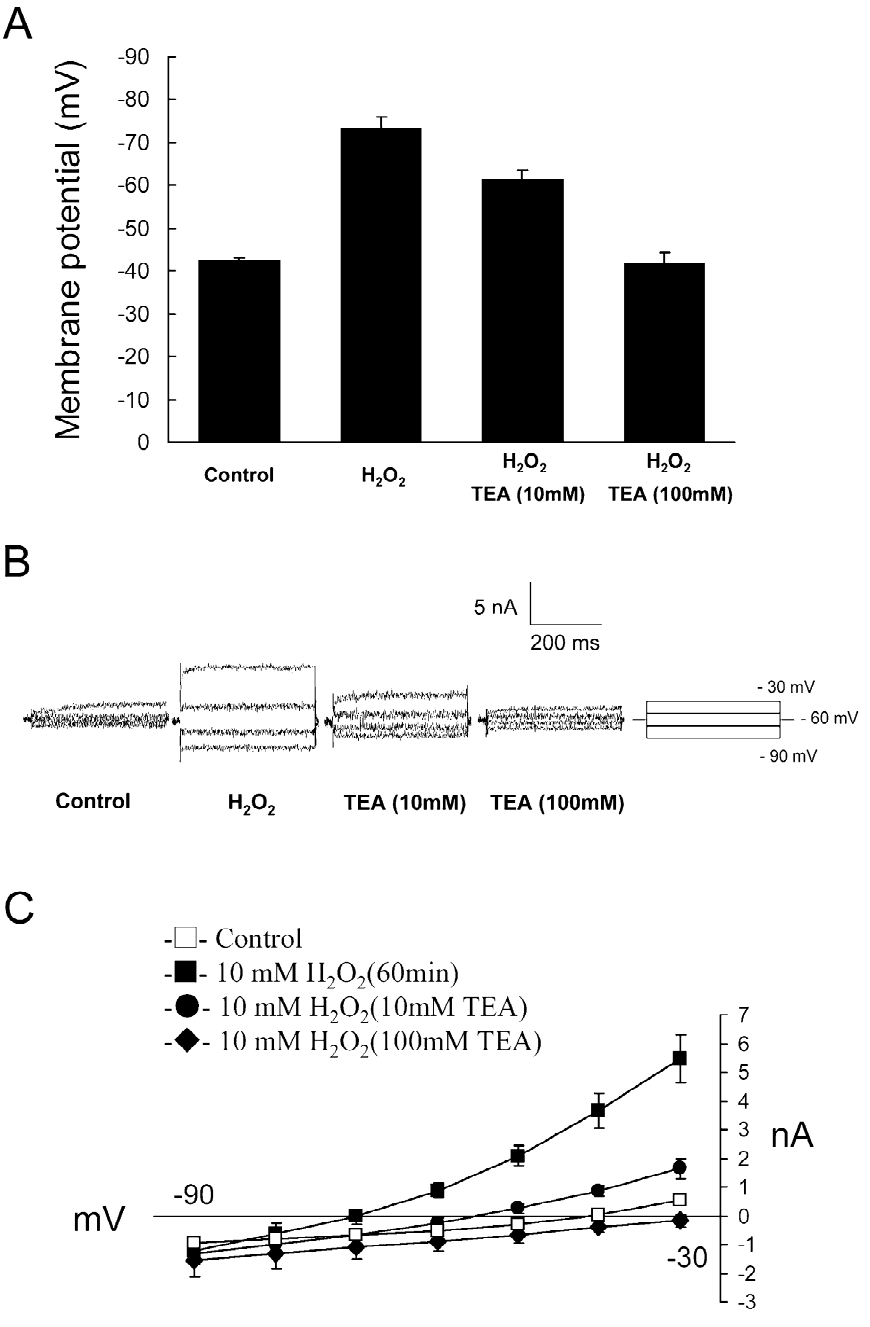

Fig. 5. Effects of K^+ channel blockers on H_2O_2-induced hyperpolarization. (A) Bar graph summarizing the effects of K^+ channel blockers on the membrane potential. Bars represent mean±S.E.M. of the membrane potential ($n=4$). (B) Representative traces of membrane currents in sensory cells exposed to 10 mM H_2O_2 for 60 min. (C) Effects of TEA on current–voltage relations in the absence (control) and presence of 10 mM H_2O_2. Bars represent mean±S.E.M. of the percentage change in membrane conductance. *n*, number of cells treated ($n=5$).

H_2O_2-induced hyperpolarization, we used Ca^{2+}-free L15/ASW instead of L15/ASW as a bath solution. In this condition, H_2O_2-induced hyperpolarization was normally observed from -42.5 ± 1.5 to -62.0 ± 2.4 mV ($n=4$), thus indicating that H_2O_2-induced hyperpolarization is not dependent on external Ca^{2+}.

The activation of electrogenic Na^+–K^+-ATPase may induce a hyperpolarization of the membrane potential [18]. We examined this possibility by applying 0.1 mM ouabain, a selective blocker of Na^+–K^+-ATPase, to sensory neurons 5 min before treating with H_2O_2. Under these conditions, treatment with 10 mM H_2O_2 produced an immediate depolarization of the membrane potential from -44.0 ± 2.1 to -40.7 ± 2.4 mV ($P<0.003$, two-tailed paired *t*-test, $n=4$), and a prolonged hyperpolarization from -44.0 ± 2.1 to -66.0 ± 1.8 mV ($P<0.001$, two-tailed

paired *t*-test, $n=4$). This result indicates that H_2O_2-induced depolarization and hyperpolarization do not involve the activation of ouabain-sensitive Na^+–K^+-ATPase.

3.5. Effects of H_2O_2 on the membrane excitability of *Aplysia* sensory neurons

To examine whether immediate depolarization, induced by 10 mM H_2O_2, is correlated with increased membrane excitability, we applied a depolarizing current pulse step (0.05–0.3 nA) to produce one to three spikes during a 1 s depolarization. Treatment of cultured sensory neurons with 10 mM H_2O_2 for 3 min produced a significant change in the number of spikes from 2.1±0.3 to 6.4±1.0 ($n=7$) ($P<0.005$, two-tailed paired *t*-test) (Fig. 6). However, this increase was lower than that induced by 10 μM 5-HT treatment ($P<0.001$, two-tailed unpaired *t*-test) (Fig. 6). In mock-treated control cells, the number of spikes did not change significantly (i.e. 2.3±0.3 to 2.5±0.3, $P>0.3$, two-tailed paired *t*-test, $n=4$). These results imply that treatment with 10 mM H_2O_2 produces increased membrane excitability in cultured sensory neurons, although this is less than the increase in membrane excitability induced by 5-HT treatment.

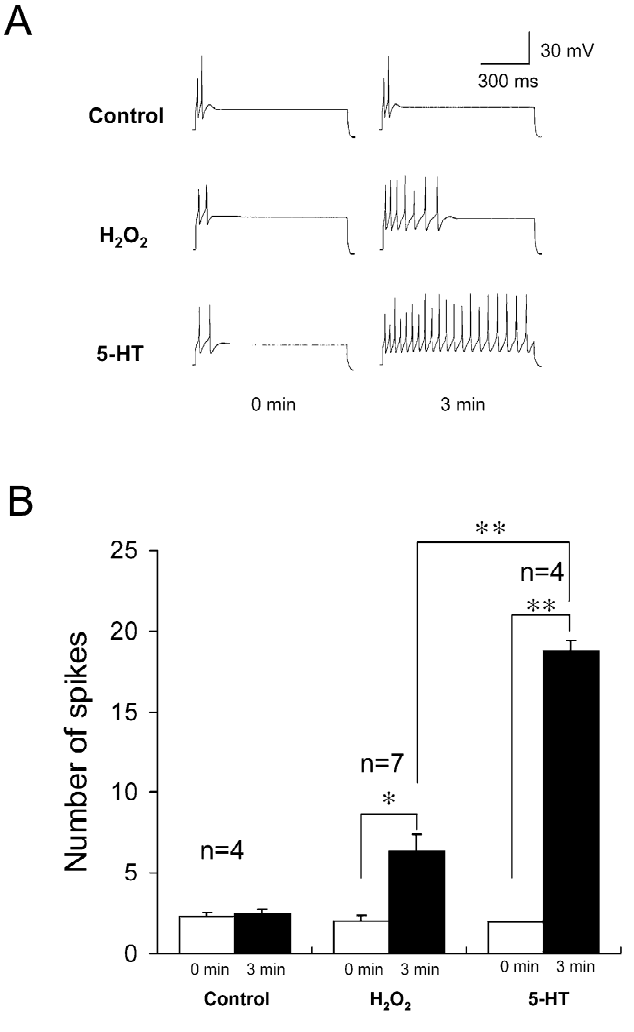

Fig. 6. Effects of 10 mM H_2O_2 on membrane excitability in *Aplysia* sensory neurons. (A) Membrane excitability was measured before (0 min) and 3 min after treatment. (B) Group data showing that treatment with 10 mM H_2O_2 caused an increase in membrane excitability in cultured sensory cells. Membrane excitability is described as numbers of spikes (action potentials) produced by a fixed step command over a period of 1 s. Data are presented as mean±S.E.M. *, $P<0.005$; **, $P<0.001$. *n*, number of cells treated.

3.6. TEA did not block H_2O_2-induced cell death in cultured sensory neurons

The inhibition of K^+ efflux is known to partially prevent cell death after hypoxia [24,34]. We determined whether the inhibition of K^+ efflux by TEA could prevent H_2O_2-induced cell death in cultured sensory neurons. We induced sensory neuron cell death by treating them with 10 mM H_2O_2 for 30 min. Cell death was analyzed by TUNEL assay as previously described [22]. In this condition, the membrane potential was hyperpolarized from −44.8±0.9 to −59.1±3.1 mV ($P<0.01$, two-tailed paired *t*-test, $n=5$) and approximately 80% of cells turned out to be TUNEL-positive (Fig. 7). Treatment with 10 mM H_2O_2 plus 100 mM TEA for 30 min did not induce hyperpolarization (−37.8±1.7 to −36.5±2.1 mV; $P>0.5$, two-tailed paired *t*-test, $n=4$). However, we did not observe a blocking effect of TEA (at 10 or 100 mM) on H_2O_2-induced cell death (Fig. 7). These results indicate that the inhibition of K^+ efflux did not exert a protective effect on H_2O_2-induced cell death in cultured sensory neurons.

4. Discussion

The present study showed that oxidative stress caused by H_2O_2 can influence the membrane potential and the membrane conductance of cultured *Aplysia* sensory neurons in a relatively short period of time (~1 h).

It is known that the redox modulation of sulfhydryl groups regulates the functions of various ion channels, such as K^+ channels, Ca^{2+} channels, and nonselective cation channels [10,17,25]. This study shows that H_2O_2-induced hyperpolarization is completely blocked by DTT. In addition, we also found that the oxidizing agent diamide induces membrane hyperpolarization. It is possible that H_2O_2-induced hyperpolarization is due to the oxidation of the thiol status of membrane proteins, primarily K^+ channels. However, immediate membrane depolarization is not induced by diamide up to 10 mM, indicating that the regulatory mechanisms underlying H_2O_2-induced depolarization and hyperpolarization differ. Consistent with our findings, it has been reported that, in rat mesangial cells, diamide did not completely mimic the biphasic membrane voltage response induced by H_2O_2 [13]. It is well known

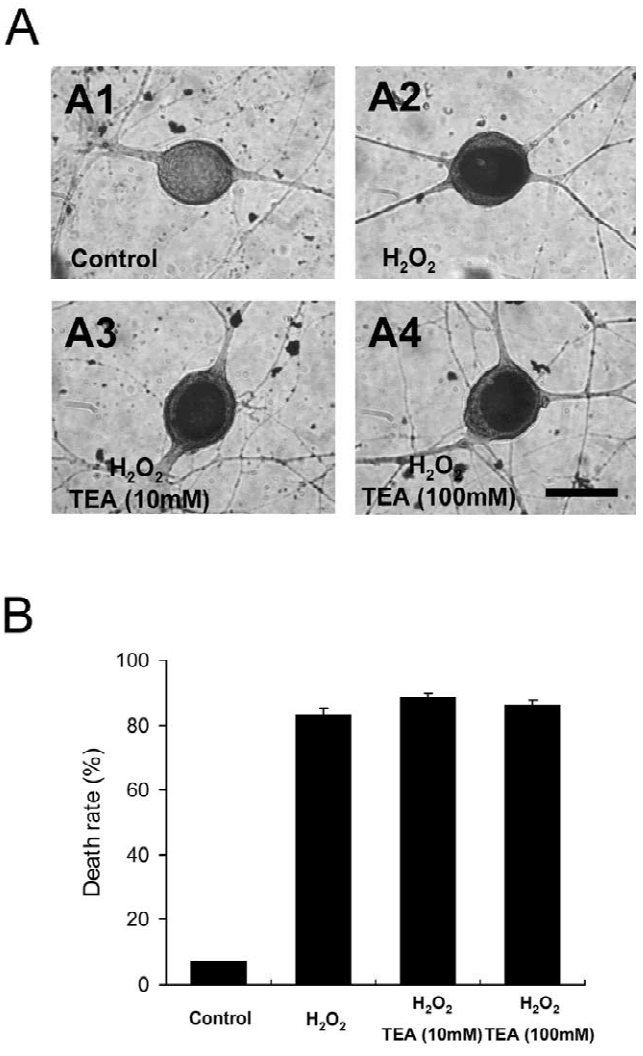

Fig. 7. Effects of TEA on H_2O_2-induced apoptosis. (A) Examples of TUNEL staining of *Aplysia* sensory neurons. The nuclei were stained by TdT-mediated dUTP nick end labeling. (A1) Normal *Aplysia* sensory neuron without H_2O_2 treatment (negative control). Cells were treated with 10 mM H_2O_2 for 30 min in the absence of TEA (A2) or in the presence of 10 mM TEA (A3) and 100 mM TEA (A4). Scale bar, 50 μm. (B) The bar graph shows that neither 10 nor 100 mM TEA inhibited H_2O_2-induced cell death, as assayed by TUNEL staining. Data are presented as mean±S.E.M. ($n=3$ independent determinations).

that both diamide and H_2O_2 produce oxidative stress, but their intracellular effects differ. Whereas diamide depletes glutathione pools and oxidizing thiol groups [19], H_2O_2 induces several different cytotoxic effects such as lipid peroxidation, protein oxidation, and DNA damage [6]. Therefore, it is possible that H_2O_2-induced depolarization is mediated by complex pathways. In many cases, H_2O_2 can regulate the activities of K^+ channels, which results in a changed membrane excitability. In our experiments, prolonged treatment with H_2O_2 activated K^+ channels, and this resulted in a decreased membrane excitability (data not shown). However, acute treatment with H_2O_2 increased membrane excitability, resulting from a decreased membrane conductance. These findings are reasonable if it is accepted that closure of K^+ currents, which are normally active at the resting membrane potential and which are relatively insensitive to TEA, may be involved in these H_2O_2-induced changes in membrane excitability. In *Aplysia* sensory neurons, it is well known that S-type K^+ currents ($I_{K,S}$) are relatively active at the resting potential, moderately voltage dependent, and insensitive to various K^+ channel blockers, including TEA (K_d, 90 mM), 4-aminopyridine (4-AP) and external Ba^{2+} [2,14,27,28]. Therefore, although we have no direct evidence, it is possible that S-type K^+ currents may be involved in the membrane depolarization and the increased membrane excitability induced by H_2O_2.

H_2O_2 can activate multiple signal pathways, such as PKA, PKC and MAPK [6]. In *Aplysia* sensory neurons, it is well known that the membrane depolarization and the increased membrane excitability induced by the application of 5-HT are primarily mediated by cAMP-dependent protein kinase (PKA) [2,5,14]. It has also been shown that the protein kinase C (PKC) activators, phorbol esters, can also induce increased membrane excitability, possibly by activating adenylyl cyclase in *Aplysia* sensory neurons [29]. Zabouri and Sossin [36] reported that, in *Aplysia* homogenate, autonomous PKC, which is active in the absence of activators, is formed due to the oxidation of PKC mediated by xanthine/xanthine oxidase, which produce both hydrogen peroxide and superoxide. Therefore, there is a possibility that either PKA or PKC may be involved in the H_2O_2-induced increases in membrane excitability.

On more prolonged application of 10 mM H_2O_2 for 1 h, the membrane potential underwent a transition from a depolarized to a hyperpolarized state. This change in the membrane potential was paralleled by an increase in the membrane conductance. In our experiments, H_2O_2-induced hyperpolarization has been reported in other cases and is known to be mediated by the activation of various K^+ channels, such as the ATP-sensitive K^+ channel, which is mediated by intracellular ATP depletion [12,20], or the Ca^{2+}-activated K^+ channel, mediated by a rise of intracellular Ca^{2+} [13,18]. Our data also show that hyperpolarization is likely to be mainly due to the activation of TEA-sensitive K^+ channels, which were blocked by ca. 71.7% by 10 mM TEA; moreover, this TEA sensitivity was independent of voltage. However, $I_{K(Ca)}$ and $I_{K(ATP)}$ are unlikely to be involved in the H_2O_2-induced hyperpolarization of *Aplysia* sensory neurons, because apamin, reduced external Ca^{2+}, a low concentration (10 mM) of TEA [11], Ba^{2+} and glibenclamide all failed to block the hyperpolarization. Therefore, the ion currents responsible for H_2O_2-induced hyperpolarization seem to be K^+ currents other than $I_{K(Ca)}$ and $I_{K(ATP)}$. It would be interesting to characterize the K^+ channels, $I_{K(H_2O_2)}$, which are relatively TEA-sensitive and which are activated by prolonged application of H_2O_2. However, we could not

exclude the possibility that S-type K^+ channels are to some extent responsible for H_2O_2-induced hyperpolarization.

Indeed, the S-type K^+ channel is weakly dependent on voltage, independent of internal Ca^{2+}, and relatively insensitive to external TEA (K_d, 90 mM) and external Ba^{2+} (K_d, >10 mM) [2,14,27,28]. S-type K^+ currents are classified into two types: a steady-state and a slowly activating current [14,26]. Steady-state S-type K^+ currents are voltage-independent and are primarily involved in 5-HT-induced increases in membrane excitability. On the other hand, slowly activating S-type K^+ currents, which are active at hyperpolarized potentials (less than or equal to −70 mV), are relatively insensitive to cAMP and are moderately voltage-dependent [14]. Therefore, it is possible that steady-state S-type K^+ currents are involved in H_2O_2-induced depolarization, and slowly activating S-type K^+ currents are involved in H_2O_2-induced hyperpolarization.

Although a high concentration of TEA (100 mM) completely blocks both H_2O_2-induced hyperpolarization and increased membrane conductance, it is possible that Cl^- channels and ionic pumps, other than K^+ channels, may be involved in H_2O_2-induced hyperpolarization, after considering the possible non-specific effects of high concentrations of TEA (100 mM). In *Aplysia* neurons, Chesnoy-Marchais [8,9] first described voltage-dependent, hyperpolarization-activated Cl^- currents, which were slowly activated by hyperpolarization (greater than −50 mV) and by increased intracellular Cl^- concentration. In fact, this current is not detected in *Aplysia* sensory neurons, except when the intracellular Cl^- concentration is elevated [30]. Therefore, it is unlikely that hyperpolarization-activated Cl^- currents are involved in H_2O_2-induced hyperpolarization. Similarly, ouabain-sensitive ion pumps do not seem to contribute to the membrane potential changes induced by H_2O_2.

It has been postulated that H_2O_2 disrupts cell membrane integrity in a nonspecific manner through lipid peroxidation. However, this was not the case in our study because H_2O_2-induced hyperpolarization was selectively mediated by a changed K^+ conductance.

It was previously reported that the activation of K^+ channels is an early response in cell death, and, therefore, reducing K^+ efflux attenuates cell death [24,34,35]. In mouse neocortical neurons, the attenuation of K^+ efflux by either TEA or by elevated extracellular K^+ levels reduced the apoptosis induced by serum deprivation or by staurosporine [34]. In proximal tubules, the inhibition of K^+ efflux, with either glibenclamide or TEA, was found to markedly reduce the extent of DNA damage and partially prevent cell death [24]. However, in our experiments, TEA inhibited H_2O_2-induced hyperpolarization, but did not block H_2O_2-induced cell death, indicating that the inhibition of K^+ efflux by TEA is insufficient to protect against H_2O_2-induced cell death in cultured *Aplysia* sensory neurons. In addition, these findings indicate that the signal pathway leading to H_2O_2-induced cell death in *Aplysia* sensory neurons is more complicated than previously believed.

Acknowledgements

This work was supported by grant R01-1999-000-00147-0 from the Basic Research Program of KOSEF and NRL M1-0104-00-0140 and M1-0108-00-0075 from MOST. D.-J.C. and S.-H.L were supported by a BK21 Fellowship from the Ministry of Education and Human Resource Development.

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