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Research report

Thiol oxidation-mediated cell death in *Aplysia* cultured sensory neurons

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Abstract

Cellular thiol groups modulate various aspects of cellular function, including cell death. In this study, we found that a thiol oxidant, diamide, induced morphological changes such as cell swelling, membrane blebbing, and chromatin condensation in *Aplysia* cultured sensory neurons. Furthermore, diamide induced biphasic changes in the membrane potential, where hyperpolarization was followed by depolarization. Moreover, these diamide-induced cytotoxic effects were completely blocked by the equimolar addition of the disulfide reducing agent dithiothreitol (DTT). We also found that during H_2O_2-induced cell death, DTT attenuated cell swelling and membrane blebbing, but not DNA breakage, whereas the vitamin E analogue trolox attenuated DNA breakage, but not cell swelling and membrane blebbing. These results demonstrate that during H_2O_2-induced cell death, apoptotic features such as DNA breakage are mediated in part by free radical generation, whereas necrotic features such as cell swelling and membrane blebbing are primarily mediated by the oxidation of cellular thiol groups.

Theme: Development and regeneration
Topic: Neuronal death

Keywords: Aplysia; Diamide; Hydrogen peroxide; Thiol oxidation; Cell death

1. Introduction

Cellular redox status plays an important role in the regulation of various cellular functions. Cellular redox imbalance induced by the oxidation of cellular antioxidants such as glutathione, as a result of oxidative stress, causes free radical reactions to become uncontrollable and leads to cell death. Moreover, growing evidence indicates that the oxidation of cellular thiol groups induces cell death unrelated to free radicals [11,16,19]. For example, a specific thiol oxidant diamide, which directly oxidizes cellular thiol groups (e.g., glutathione and thioredoxin), can induce cell death through mitochondrial signaling and the activation of caspase-3 [17]. Cell death occurs via necrosis or apoptosis. Necrosis typically arises as a result of acute pathological stimuli. In contrast, apoptosis is typically associated with mild toxic stimulation over extended periods. Moreover, necrotic cells show many cytoplasmic changes such as cellular swelling, surface blebbing and membrane leakage. On the other hand, apoptotic cells show morphological changes such as cytoplasmic shrinkage, the formation of apoptotic bodies, nuclear shrinkage, chromatin condensation, and DNA fragmentation.

Highly reactive oxygen species (ROS) such as H_2O_2 and O_2^- may cause cell death in various cell types including neurons [2,3,15]. For example, ROS generation is associated with the neurodegenerative diseases, Parkinson's and Alzheimer's disease. Cell death induced by H_2O_2 depends on free radicals such as the hydroxyl radical (OH) [12,13]. H_2O_2 is converted in vivo to hydroxyl radicals (OH), via the catalytic action of reduced iron and copper within neurons. The hydroxyl radicals so evoked, attack membranes, proteins, DNA, and lipid, causing lipid peroxidation

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and DNA damage, and eventual cell death [21]. Furthermore, H_2O_2 can also induce cellular redox imbalance, by inducing the oxidation of cellular thiol groups such as glutathione and thioredoxin, and this too leads to cell death [17]. Previously we showed that H_2O_2 can induce cell death in cultured sensory neurons, and that it has both apoptotic and necrotic effects [9]. Treatment with H_2O_2 caused not only nuclear DNA breakage, but also neurite fragmentation, cell body disintegration, and a change in the resting membrane potential. However, it is unclear how these apoptotic and necrotic events are produced by H_2O_2, because it can presumably act by producing hydroxyl radicals and/or by causing a cellular redox imbalance, which raises the question as to which of these possible alternatives is actually responsible for H_2O_2-induced cell death.

To address this question, the thiol oxidant diamide was used to oxidize cellular thiol groups. Treatment with diamide was found to induce both necrotic and apoptotic cell death in *Aplysia* sensory neurons. Moreover, the necrotic features in H_2O_2-induced cell death were found to be mediated predominantly by the oxidation of thiol groups. In contrast, apoptotic features in H_2O_2-induced cell death were found to be mediated primarily as a result of the deleterious actions of free radicals.

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) 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

Resting membrane potentials were recorded as described previously [4]. Briefly, the voltage recordings and current injections were carried out in conventional single electrode current clamp mode using an Axoclamp 2B (Axon Instruments, CA). Data were stored on a PC running pCLAMP software (Axon Instruments). The culture medium was replaced with 50% isotonic L15/50% ASW (isotonic 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 $NaHCO_3$ A; ASW: 450 mM NaCl, 10 mM KCl, 11 mM $CaCl_2$, 29 mM $MgCl_2$, 10 mM HEPES at pH 7.6). 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. The input resistance was determined by injecting a hyperpolarizing current (−0.4 nA) into the sensory neurons for 500 ms, and the electrode was then removed. Neurons were treated with diamide or vehicle for 3 or 6 h, and the resting membrane potentials and the input resistances were re-recorded in the same cells. Hydrogen peroxide (H_2O_2) (Merck), DL-dithiothreitol (DTT) (Sigma), and diamide (Sigma) solutions were freshly made-up in L15/ASW. Trolox (1 M) (Sigma) dissolved in dimethyl sulfoxide (DMSO) was added to L15/ASW solution to a final concentration of 1 mM.

2.3. Measurement of cell morphological changes

To detect changes in the cell shape, each sensory neuron in the culture dish was photographed with a digital camera (Olympus, C-3030) connected to an inverted microscope (Nikon, Diaphot) before and at various time points after treatment with 1 mM hydrogen peroxide (H_2O_2). DTT or trolox was pretreated for 5 min before the addition of diamide or H_2O_2.

2.4. Propidium iodide staining

Fixed sensory neurons in dissociated cultures were propidium iodide (P.I., 20 μg/ml in PBS, Sigma) stained for 5 min at room temperature in the dark. After staining solution had been removed by washing, the nuclear morphologies were observed under a confocal microscope (Radiance2000, Bio-Rad).

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

TUNEL staining was performed as previously described [9]. 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. TUNEL reaction mixture (Roche Molecular Biochemicals, Mannheim) was then added, and the cells were incubated in a humidified chamber for 1 h at 37 °C. The cells were then thoroughly rinsed with PBS, treated with anti-fluorescein antibodies conjugated with horseradish peroxidase for 30 min at 37 °C, and 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. Diamide-induced cell death in Aplysia sensory neurons

To examine effects of the oxidation of cellular thiol groups on cell death, we treated cultured *Aplysia* sensory neurons with a thiol-oxidizing diamide. As shown in Fig. 1, treatment with 50 μM or higher concentrations of diamide for 9 h induced necrotic morphological changes, i.e., cell swelling and membrane blebbing (Fig. 1). To determine whether diamide could produce apoptotic cell death, we used propidium iodide (PI), which detects nuclear condensation. PI staining showed that nuclear condensation was induced by diamide ($\geq$50 μM) (Fig. 1). Diamide also induced DNA breakage, another apoptotic nuclear event, as determined by TUNEL staining (data not shown). Taken together, these findings show that diamide ($\geq$50 μM) induces both necrotic and apoptotic events. However, lower concentrations (i.e., $<$50 μM) of diamide were not effective at inducing necrotic changes in *Aplysia* cultured sensory neurons (data not shown).

3.2. Diamide-induced changes in the membrane potential and input resistance of Aplysia sensory neurons

We also examined whether diamide affects the membrane potential and the input resistance of *Aplysia* sensory neurons. Diamide ($\geq$50 μM) treatment produced both an early hyperpolarization and a late depolarization (Fig. 2A), and treatment with higher concentrations of diamide de-

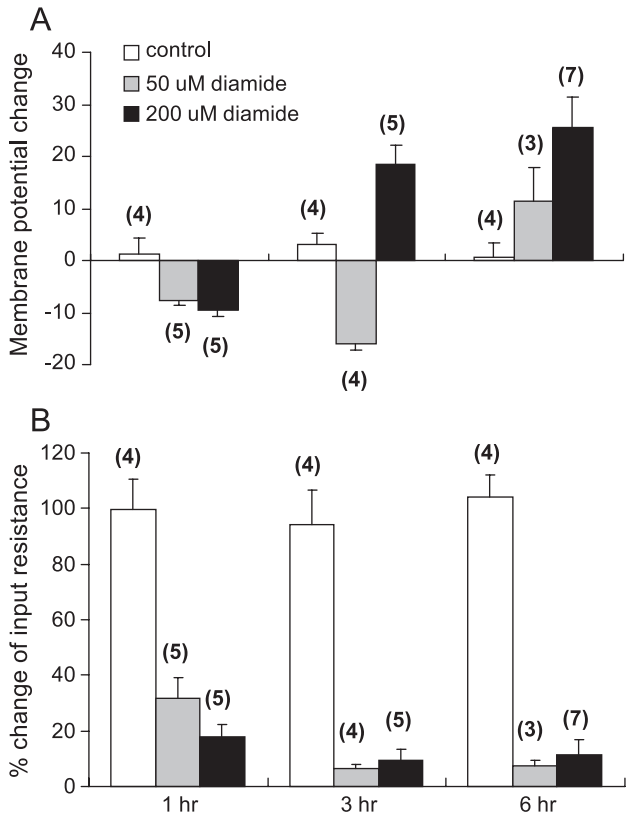

Fig. 2. Effects of diamide on the membrane potential (A) and percentage changes in input resistance (B) after 1, 3 and 6 h of treatment in *Aplysia* cultured sensory neurons. The data presented are means ± S.E.M., and the values in parentheses are the numbers of cells examined.

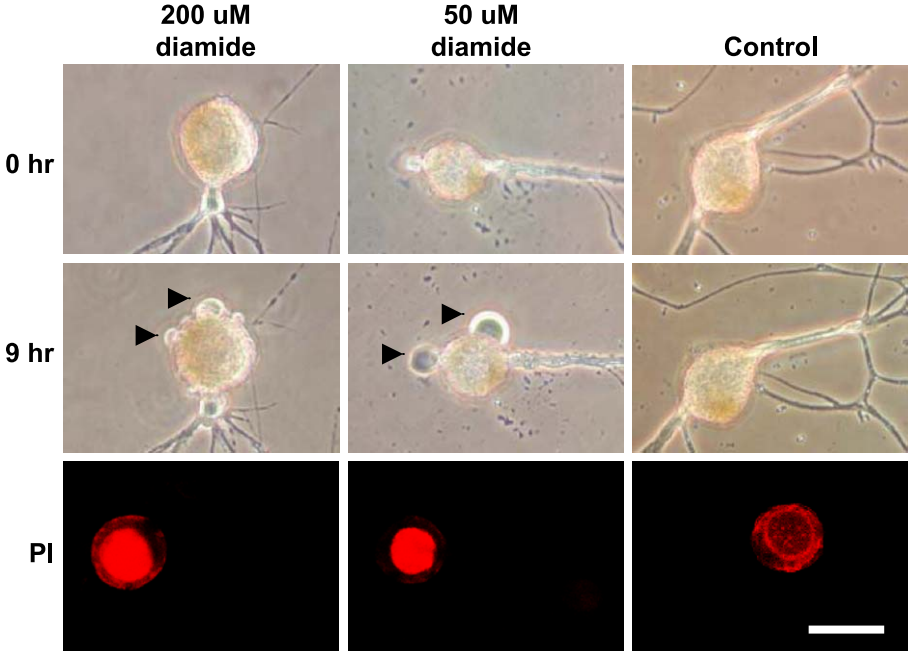

Fig. 1. Diamide-induced morphological changes in cultured sensory neurons. (Upper panel) Phase contrast micrographs were obtained for 0 and 9 h after diamide treatment (at 200, 50 and 0 μM, respectively). Arrowheads indicate membrane blebs. (Lower panel) Representative examples of PI staining after exposing cells to various concentrations of diamide for 12 h. PI, propidium iodide. Scale bar: 50 μm.

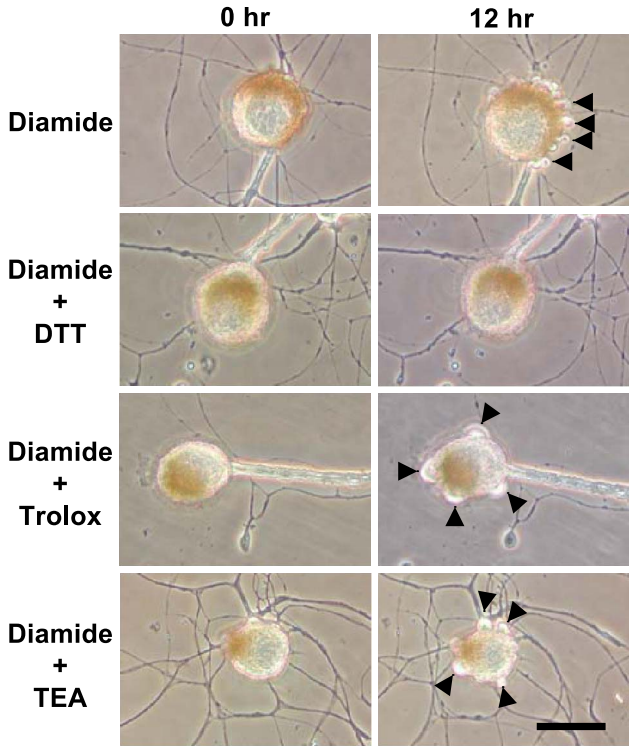

Fig. 3. DTT completely blocked the cell swelling and membrane bubbling induced by diamide in sensory neurons. Phase contrast photography showing morphological changes in sensory neurons 12 h after treatment with 200 μM diamide, 200 μM diamide plus 200 μM DTT, 200 μM diamide plus 500 μM of trolox, and 200 μM diamide plus 50 mM TEA, respectively. DTT or trolox was pretreated for 5 min before the addition of diamide. Arrowheads indicate membrane blebs. Scale bar: 50 μm.

creased the time required for these biphasic changes (Fig. 2A). We previously reported upon similar changes in membrane potential induced by H_2O_2 treatment [4,9]. Unlike biphasic changes of membrane potential, the input resistance gradually decreased (Fig. 2B). As shown in Fig. 2, the relative input resistance 6 h after diamide treatment was less than 10% of the initial level, indicating the membrane leakage. In a control experiment, membrane potential and input resistance were unchanged in mock treated cells (Fig. 2). These results show that diamide induces biphasic membrane potential changes and reduces the input resistance, eventually leading to membrane leakage and necrotic cell death.

3.3. DTT blocks diamide-induced cell death, whereas trolox and TEA do not

To examine whether the disulfide reducing agent DTT inhibits diamide-induced cell death, we added DTT at the same concentration as diamide. As shown in Fig. 3, DTT completely blocked diamide-induced cell swelling and membrane blebbing. In addition, it also blocked the membrane potential changes. The changes were not statistically significant from -44.0 ± 0.6 mV ($n=3$, 0 h) to -45.0 ± 0.6 mV ($n=3$, 9 h). However, the vitamin E analog trolox did not block the diamide-induced morphological changes (Fig. 3). These results demonstrate that sulfhydryl modifications of cellular thiol groups or proteins, but not free radicals play an important role in diamide-induced morphological changes.

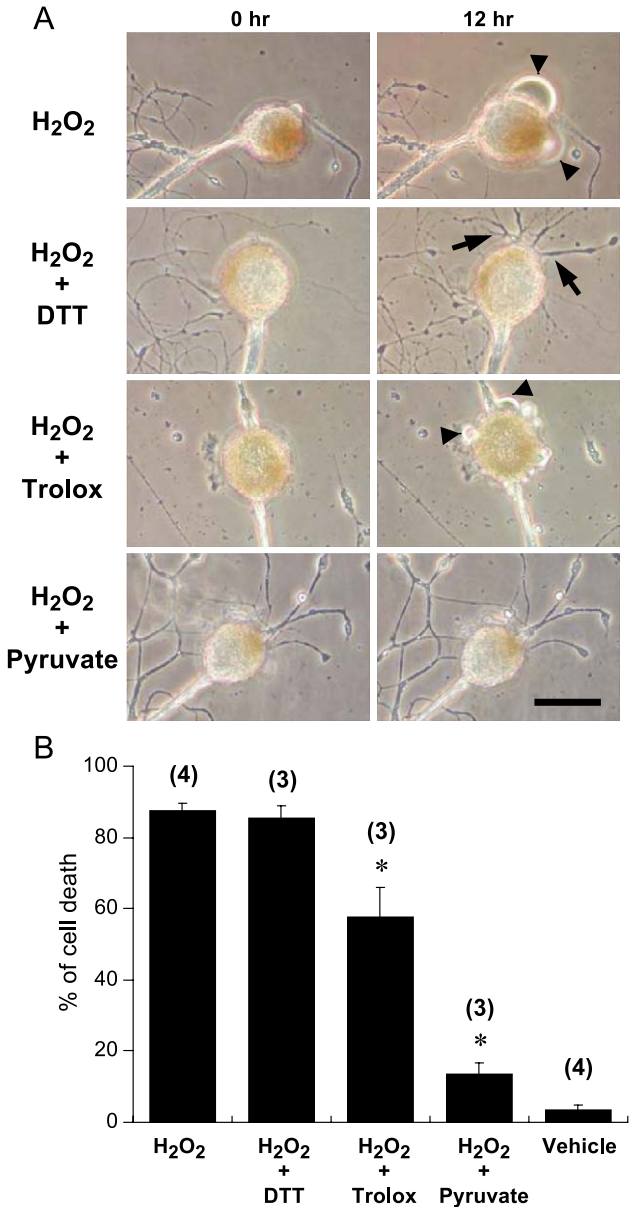

Fig. 4. (A) DTT, but not trolox, blocked H_2O_2-induced cell swelling. Phase contrast photomicrograph showing morphological changes in sensory neurons 12 h after treatment with 1 mM H_2O_2, 1 mM H_2O_2 plus 1 mM DTT, 1 mM H_2O_2 plus 1 mM trolox, and 1 mM H_2O_2 plus 3 mM pyruvate. Arrowheads indicate membrane blebs. Arrows indicate neurite processes newly generated from the cell body. (B) Trolox, but not DTT, attenuated H_2O_2-induced DNA fragmentation. Data showing the percentage changes of TUNEL-positive cells after treatment with 1 mM H_2O_2, 1 mM H_2O_2 plus 1 mM DTT, 1 mM H_2O_2 plus 1 mM trolox, and 1 mM H_2O_2 plus 3 mM pyruvate. *$P<0.01$ (unpaired t-test) compared to 1 mM H_2O_2. The data presented are means ± S.E.M. Scale bar: 50 μm.

Previously, it was reported that K^+ efflux plays an important role in cell death [23]. In addition, we demonstrated that the hyperpolarization induced by the oxidation of cellular thiol groups is mediated by the activation of K^+ channels [4]. To determine whether the blocking of diamide-induced hyperpolarization has any effect on diamide-induced cell death, we treated cultured neurons with a non-specific K^+ channel blocker TEA (50 mM) before diamide treatment. TEA did not attenuate the diamide-induced cell swelling or membrane blebbing (Fig. 3). In a control experiment, TEA alone was found not to affect the morphology of sensory neurons (data not shown). These results show that K^+ efflux is not a direct cause of diamide-induced morphological changes in *Aplysia* sensory neurons, though K^+ efflux per se is produced by diamide.

3.4. DTT attenuates necrotic features but not apoptotic features in H_2O_2-induced cell death

Consistent with previous reports [9], prolonged treatment of *Aplysia* sensory neurons with 1 mM H_2O_2 for 12 h produced cytological changes, such as cell swelling, membrane blebbing (Fig. 4A), and endonucleolysis (Fig. 4B). To examine whether cellular redox imbalance underlies the necrotic morphological changes induced by 1 mM H_2O_2, *Aplysia* sensory neurons was pretreated for 5 min with 1 mM DTT. It was found that the DTT treatment attenuated the cell swelling and membrane blebbing induced by treatment with H_2O_2 for 12 h (Fig. 4A), but did not affect H_2O_2-induced endonucleolysis after 3 h of H_2O_2 treatment (Fig. 4B). Unexpectedly, in many of the cells (12 of 20 cells), lamellipodia or neurite processes were observed to protrude from the cell body after 12 h of H_2O_2 treatment in DTT pretreated cells (Fig. 4A, arrows). DTT alone did not induce any morphological changes (data not shown).

It is well known that free radicals also play key roles in H_2O_2-induced cell death. To examine whether free radicals are involved in H_2O_2-induced cell death, *Aplysia* sensory neurons were pretreated for 5 min with the free radical scavenger trolox. Under these conditions, 1 mM trolox did not block H_2O_2-induced necrotic morphological changes. However, unlike DTT, trolox attenuated H_2O_2-induced DNA breakage (Fig. 4B). In a control experiment, the H_2O_2 scavenger pyruvate blocked H_2O_2-induced morphological changes and DNA breakage. Taken together, these results suggest that 1 mM of H_2O_2 induces apoptotic cell death events via free radical generation and necrotic events via sulfhydryl modification (Fig. 5).

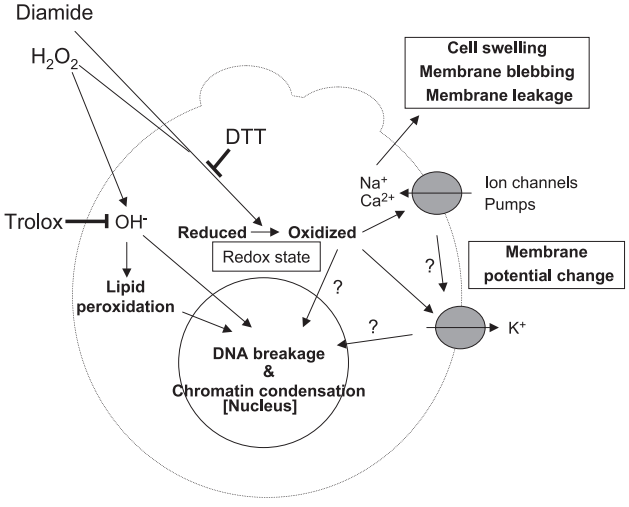

Fig. 5. Schematic diagram of the cell death pathway induced by H_2O_2 or diamide in *Aplysia* sensory neurons. Exogenous H_2O_2 or diamide penetrates cell membranes and changes cellular redox states, eventually leading to biphasic membrane potential changes, chromatin condensation and necrotic morphological changes such as cell swelling, membrane blebbing, and membrane leakage. H_2O_2 is converted to the hydroxyl radical ($OH^{\cdot}$) by reduced iron and copper within neurons. $OH^{\cdot}$ can cause lipid peroxidation within membranes and DNA damage. DTT, dithiothreitol.

4. Discussion

This study shows that the oxidation of cellular thiol groups by diamide induces both necrotic and apoptotic cell death in *Aplysia* sensory neurons, which is blocked by antioxidant DTT. Moreover, H_2O_2-induced necrotic changes such as cell swelling and membrane blebbing are mainly mediated by the oxidation of cellular thiol groups in *Aplysia* sensory neurons, whereas H_2O_2-induced apoptotic changes such as DNA breakage are mediated in part by free radical generation (Fig. 5).

The necrotic morphological and electrical changes were found to be produced by a higher concentration (≥ 50 μM) of diamide in a dose-dependent manner. This is consistent with other studies, which showed that necrosis arises from acute pathological stimuli, whereas apoptosis is related to relatively mild toxic stimuli [10,19]. These necrotic changes, induced by diamide, appear to be mediated by sulfhydryl modification of cellular thiol groups, because they are completely blocked by DTT. Moreover, it is well known that diamide directly oxidizes glutathione (GSH) to glutathione disulfide (GSSG) and induces a GSH-to-GSSG imbalance within cells, and leads uncontrolled endogenous free radical generation and cell death. However, our experiments suggest that diamide-induced cell death may be unrelated to free radical generation, because trolox did not block diamide-induced cell death. In aggrement with our results, a cellular redox imbalance induced by diamide in PC-12 cells was found to initiate cell death through mitochondrial signaling and the activation of caspase-3 independently of ROS production [17]. It has also been reported that monocyte necrosis is mediated by the activation of caspase-like proteases [22]. Therefore, a cellular redox imbalance in *Aplysia* sensory neurons may induce necrosis through the activation of specific proteases unrelated to free radical generation.

Alternatively, ion channels or pumps may be involved in this diamide-induced necrotic process, by causing the accumulation of ions within the cells and thus changing the osmotic pressures. A number of reports have suggested that cations are accumulated within cells by the actions of cation channels or Na^+ pumps that are activated by free radicals [1,5,6,18,20]. However, our results suggest that membrane potential changes induced by H_2O_2 or diamide appear to be mediated by sulfhydryl modification—not by free radicals. *Aplysia* sensory neurons may provide a good model for the study of how the sulfhydryl modification of certain channels or pumps can produce ion accumulations that lead to necrotic events.

Although the activation of K^+ efflux has been suggested to be actively or passively implicated in necrosis [1,22], the activation of K^+ channels during the early phase in diamide-induced or H_2O_2-induced cell death in the present study, did not appear to be necessary for cell death, because a TEA blockade of this hyperpolarization did not protect against diamide-induced or H_2O_2-induced cell death. Thus, membrane hyperpolarization could be a protective mechanism against membrane depolarization underlying oxidant-induced cell death.

In addition to necrotic events, diamide also induced apoptotic changes in the nucleus, i.e., chromatin condensation and DNA breakage. It is possible that membrane hyperpolarization may play a key role in the induction of such apoptotic-like features by diamide in *Aplysia* sensory neurons, because a depletion in cellular K^+ can trigger apoptotic cell death, as has been shown in various cell types [23]. However, more study is required if we are to understand how apoptotic nuclear events like chromatin condensation and DNA breakage are produced by the diamide-induced sulfhydryl modification of cellular thiol groups.

H_2O_2, like diamide, can perturb the cellular redox imbalance caused by glutathione (GSH)–glutathione disulfide (GSSG) redistribution, and there are common aspects of diamide-induced cell death and H_2O_2-induced cell death in *Aplysia* cultured sensory neurons. First, they are associated with similar morphological changes, i.e., cell swelling, membrane blebbing, and membrane leakage. Second, they cause similar biphasic membrane potential changes, i.e., a hyperpolarization followed by a depolarization. Third, these morphological and membrane potential changes are inhibited by DTT. Moreover, trolox was found not to attenuate the diamide-induced or the H_2O_2-induced morphological changes. Thus, it is likely that, in *Aplysia* cultured sensory neurons, acute cellular redox imbalance induced by H_2O_2, but not lipid peroxidation by hydroxyl radical might be responsible for the observed H_2O_2-induced necrotic changes. In addition, we observed that the lamellipodia or neurite processes extended out from cell body in the presence of DTT in the case of H_2O_2-induced cell death but not in diamide-induced cell death. H_2O_2 can activate various signal pathways and is useful if intracellular concentrations are maintained within physiological levels [7]. Therefore, it is possible that the reduction in the cytotoxic effect of H_2O_2 by DTT promotes cell survival.

In the case of H_2O_2-induced cell death, free radical generation plays a key role in DNA breakage. DTT was found to block changes in the membrane potential [4], but not to inhibit H_2O_2-induced DNA breakage in *Aplysia* sensory neurons. H_2O_2 reacting via the Fenton reaction with reduced iron or copper produces hydroxyl radicals, and leads to DNA breakage. Moreover, in the present study trolox was found to attenuate H_2O_2-induced DNA breakage in *Aplysia* cultured sensory neurons. Furthermore, we detected DNA breakage soon after H_2O_2 treatment. It has been shown that 84% of the sensory cells treated with 1 mM H_2O_2 for 3 h were TUNEL-positive, but that membrane hyperpolarization mediated by the activation of K^+ channels was produced some 3–6 h after H_2O_2 treatment [9]. This differential indicates that H_2O_2 activates two separate pathways that lead to cell death. One is a nuclear apoptotic pathway through free radical generation, which produces chromosomal DNA breakage, whereas the other is a cytosolic necrotic pathway that occurs via redox state dysregulation, which produces membrane blebbing and cell swelling (Fig. 5). However, we cannot exclude the possibility that the cellular redox imbalance induced by H_2O_2 promotes DNA breakage and chromatin condensation, because trolox treatment did not completely block the nuclear apoptotic pathway. It is well known that poly (ADP-ribose) polymerase (PARP) activation via DNA breakage is involved in H_2O_2-induced cell death in many cell lines [8,14]. Therefore, it remains to be determined whether the activation PARP is involved in diamide or H_2O_2-induced cell death in *Aplysia* sensory neurons.

Acknowledgements

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