Mechanisms underlying $H_2O_2$-mediated inhibition of synaptic transmission in rat hippocampal slices

Marat V. Avshalumov, Billy T. Chen, Margaret E. Rice*

Departments of Physiology and Neuroscience and Neurosurgery, New York University School of Medicine, 550 First Avenue, New York, NY 10016, USA

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Abstract

Hydrogen peroxide ($H_2O_2$) inhibits the population spike (PS) evoked by Schaffer collateral stimulation in hippocampal slices. Proposed mechanisms underlying this effect include generation of hydroxyl radicals (·OH) and inhibition of presynaptic $Ca^{2+}$ entry. We have examined these possible mechanisms in rat hippocampal slices. Inhibition of the evoked PS by $H_2O_2$ was sharply concentration-dependent: 1.2 mM $H_2O_2$ had no effect, whereas 1.5 and 2.0 mM $H_2O_2$ reversibly depressed PS amplitude by roughly 80%. The iron chelator, deferoxamine (1 mM), and the endogenous ·OH scavenger, ascorbate (400 μM), prevented PS inhibition, confirming ·OH involvement. Isoascorbate (400 μM), which unlike ascorbate is not taken up by brain cells, also prevented PS inhibition, indicating an extracellular site of ·OH generation or action. We then investigated whether $H_2O_2$-induced PS depression could be overcome by prolonged stimulation, which enhances $Ca^{2+}$ entry. During 5-s, 10-Hz trains under control conditions, PS amplitude increased to over 200% during the first three–four pulses, then stabilized. In the presence of $H_2O_2$, PS amplitude was initially depressed, but began to recover after 2.5 s of stimulation, finally reaching 80% of the control maximum. In companion experiments, we assessed the effect of $H_2O_2$ on presynaptic $Ca^{2+}$ entry by monitoring extracellular $Ca^{2+}$ concentration ([Ca$^{2+}]_o$) during train stimulation in the presence of postsynaptic receptor blockers. Evoked [Ca$^{2+}]_o$ shifts were apparently unaltered by $H_2O_2$, suggesting a lack of effect on $Ca^{2+}$ entry. Taken together, these findings suggest new ways in which reactive oxygen species (ROS) might act as signaling agents, specifically as modulators of synaptic transmission. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Hydrogen peroxide; Oxidative stress; Transmitter release; Ion-selective microelectrode; $Ca^{2+}$; Ascorbate

1. Introduction

Reactive oxygen species (ROS) occur naturally during cell metabolism and are strongly regulated by the intracellular antioxidant network. Under pathological conditions, including ischemia, hyperoxia, and exposure to certain toxins, ROS can cause damage to the central nervous system [14,17]. Recent studies, however, have suggested that ROS, particularly hydrogen peroxide ($H_2O_2$), might also play a role in cellular signaling [12]. Among the most important signaling pathways modulated by $H_2O_2$ are those involving kinase and phosphatase enzymes, which are sensitive to this ROS [10,23,30,51].

It has also been shown that $H_2O_2$ can modulate neuronal activity. The effect of $H_2O_2$ on hippocampus slice physiology has been particularly well-studied [13,21,23,29,35–37,46]. Both the primary effect and the mechanism of action of $H_2O_2$ in hippocampal slices, however, remain uncertain. Several reports in the literature indicate that $H_2O_2$ causes a reversible depression of the population spike (PS) recorded in CA1 stratum pyramidale during stimulation of the Schaffer collaterals [13,35–37], although an augmentation in hippocampal PS amplitude has also been reported [21]. In addition, it is not yet certain whether PS depression is mediated directly by $H_2O_2$ or by hydroxyl radicals (·OH) that can be produced from the
interaction of $\text{H}_2\text{O}_2$ with tissue iron or copper through the Fenton reaction [7,16,17]:

$$\text{H}_2\text{O}_2 + \text{Me}^{n+} \rightarrow \text{Me}^{(n+1)+} + \cdot \text{OH} + \text{OH}^-, $$

where $\text{Me}^{n+}$ is the reduced form of the metal ion. Pellmar and colleagues concluded that PS depression was mediated by $\cdot \text{OH}$, because deferoxamine, an iron chelator, prevented $\text{H}_2\text{O}_2$-induced changes in PS amplitude [37]. In other experiments, however, although with lower concentrations of deferoxamine, Katsuki and colleagues [21] concluded that $\cdot \text{OH}$ was not involved in the effects of $\text{H}_2\text{O}_2$ on hippocampal slice physiology.

The site of action of $\text{H}_2\text{O}_2$ in mediating PS depression is also not yet clear, although there is strong evidence for a presynaptic location: $\text{H}_2\text{O}_2$ had no effect on antidromically evoked PS in CA1 stratum pyramidale [34]; nor did it alter EPSPs evoked by glutamate iontophoresis [35]. These data led Pellmar to propose that $\text{H}_2\text{O}_2$ might inhibit transmitter release by decreasing presynaptic Ca$^{2+}$ entry [35]. Recently, our laboratory showed that $\text{H}_2\text{O}_2$ can decrease stimulated dopamine release in striatal slices in a Ca$^{2+}$-dependent manner, consistent with a presynaptic site of action of $\text{H}_2\text{O}_2$ [6].

In the present studies, we tested the hypothesis that generation of $\cdot \text{OH}$ is required for the action of $\text{H}_2\text{O}_2$ and investigated whether decreased presynaptic Ca$^{2+}$ entry might be the mode of action. To ascertain whether PS depression is mediated by $\cdot \text{OH}$, the effect of $\text{H}_2\text{O}_2$ was assessed in the presence of deferoxamine [37], with concomitant determination of $\text{H}_2\text{O}_2$ concentration in the presence of this chelator. In addition, we tested whether the endogenous $\cdot \text{OH}$ scavenger, ascorbate [2,4,8,40], could modulate the effect of $\text{H}_2\text{O}_2$. To determine whether the action of ascorbate was intracellular or extracellular, $\text{H}_2\text{O}_2$ was applied in the presence of the stereoisomer of ascorbate, isoascorbate (D-ascorbate), which has similar electrochemical properties to ascorbate, but is not a substrate for the stereoselective ascorbate transporter [48]. Consequently, isoascorbate is not taken up by cells and remains in the extracellular compartment [4]. In companion experiments, we evaluated the effect of $\text{H}_2\text{O}_2$ on presynaptic Ca$^{2+}$ entry by monitoring extracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_o$) using ion-selective microelectrodes (ISMs) [31,42].

2. Materials and methods

2.1. Hippocampal slice preparation

Hippocampal slices (400-μm thickness) were prepared from young adult (50–60 days old), male Long–Evans rats. All animal experimentation was conducted following NIH guidelines and with approval by the NYU School of Medicine Institutional Animal Care and Use Committee. Rats were deeply anaesthetized with 50 mg kg$^{-1}$ pento-barbital sodium. After decapitation, the brain was rapidly removed and placed in ice-cold, oxygenated (95% $\text{O}_2$/5% $\text{CO}_2$) artificial cerebrospinal fluid (ACSF) for about 1 min, then bisected, blocked and the tissue mounted on the stage of a Vibratome (Ted Pella, St. Louis, MO, USA). Transverse hippocampal slices were cut in ice-cold ACSF that contained (in mM): 120 NaCl, 5 KCl, 35 NaHCO$_3$, 1.25 NaH$_2$PO$_4$, 1.5 CaCl$_2$, 1.3 MgCl$_2$, and 10 glucose. Slices were maintained in an incubation chamber at room temperature for at least 1 h before recording.

2.2. Extracellular recording

For the measurement of evoked potentials, an individual slice was transferred to a submersion-recording chamber (Warner Instrument, Hamden, CT, USA), where it was continuously superfused with ACSF at 1.5 ml min$^{-1}$ at 31°C. Extracellular PSs were elicited by stimulating the Schaffer collaterals and recorded in the stratum pyramidale of CA1 with conventional glass electrodes (2–7 MΩ tip resistance, backfilled with 1 M NaCl) connected to an Axoprobe 1A amplifier (Axon Instruments, Foster City, CA, USA). Pulse duration was 100 μs, with the stimulus intensity adjusted to the lowest level (0.2–1.3 V) required to evoke a PS of maximal amplitude. The stimulating electrode was a twisted bipolar electrode, made from Teflon-insulated platinum–iridium wire. In some experiments, 10-Hz pulse trains of 5-s duration were used, with the same pulse duration and amplitude as in single pulse experiments. In all slices, the evoked PS was monitored for 25–30 min in normal ACSF to ascertain that the response was stable; only slices with stable PS responses during this control period were tested further. For each experimental condition tested with single pulse stimulation, three evoked PS records were stored and averaged using locally written software. The amplitude (in mV) of these averaged PS records was measured from the mean of the positive peak preceding and the positive peak following the negative PS. Acquisition of pulse train experiments data was controlled by CLAMPED 7.0 software (Axon Instruments) which imported PS records in the computer via a DigiData 1200B D/A board (Axon Instruments).

2.3. Ca$^{2+}$ ion-selective microelectrode recording

Stimulated [Ca$^{2+}$]$_o$ shifts in hippocampal slices were monitored using Ca$^{2+}$-sensitive ion-selective microelectrodes (Ca$^{2+}$-ISMs). Ca$^{2+}$-ISMs were positioned in stratum radiatum of CA1; Ca$^{2+}$ entry was monitored during 10-Hz pulse trains of 5 s duration in the presence of postsynaptic receptor blockers: picrotoxin, 50 μM; AP5, 50 μM; and CNQX, 25 μM. Double-barreled Ca$^{2+}$-ISMs were prepared using theta glass (Warner Instrument) and calibrated as described previously [31,42]. The ion-sensing barrel contained Fluka 21048 (Fluka Chemical, Ronkon-
koma, NY, USA) as the ion exchanger and was backfilled with 100 mM CaCl₂. The reference barrel was backfilled with 150 mM NaCl. Extracellular potentials recorded with the reference barrel were subtracted from the ion signals using the AxoProbe 1A amplifier. Stimulated [Ca²⁺]ₐ shifts were recorded on a chart recorder and stored also on computer. The maximum amplitude of [Ca²⁺]ₐ shifts was measured from chart records and calculated with respect to the concentration of Ca²⁺ in ACSF using the Nernst equation [31].

2.4. Hydrogen peroxide and ascorbate analyses

Actual media concentrations of H₂O₂ were determined in these experiments using a commercially available kit (Oxis International, Portland, OR, USA). This quantitative assay is a spectrophotometric method based on the oxidation of ferrous ions (Fe²⁺) to ferric ions (Fe³⁺) by H₂O₂. The ferric ions bind to the indicator dye xylenol orange (3,3’-bis[N,N-di(carboxymethyl)-aminomethyl]-α-cresolsulfone-phthalain, sodium salt) to form a stable colored complex, which was monitored at 560 nm using a Turner 340 spectrophotometer (Barnstead/Thermolyne, Dubuque, IA, USA).

Ascorbate and isoascorbate concentrations in ACSF in the presence and absence of H₂O₂ were determined using a previously described HPLC method [4,41]. Media samples collected from the holding flask or from the recording chamber were immediately diluted 1:10 in ice-cold, deoxygenated eluent, then kept on ice until analysis. Under these conditions, ascorbate samples were stable for at least 1 h.

2.5. Chemicals

ACS-grade hydrogen peroxide solution, 8.8 M (30%), was obtained from Sigma (St. Louis, MO, USA). This stock solution was diluted in ACSF to the following experimental concentrations in ACSF immediately before slice application: 1.2 mM (0.004%); 1.5 mM (0.005%); 2.0 mM (0.007%). Deferoxamine mesylate, ascorbic acid and isoascorbic acid, glutathione (GSH), picrotoxin, AP5 (D,L-2-amino-5-phosphonopentanoic acid) were also purchased from Sigma; CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) was obtained from Tocris (Ballwin, MO, USA). Solutions containing these agents were also freshly prepared before application.

2.6. Experimental design and statistical analysis

In all experiments, the amplitude of the stabilized PS evoked by single pulse stimulation in ACSF alone was considered to be control (100%). Mean PS amplitude data are presented as percent of control±S.E.M.; concentrations of H₂O₂, ascorbate and isoascorbate, and [Ca²⁺]ₐ are presented as mean±S.E.M. For statistical analysis, either Students t-test or one-way ANOVA followed by Student–Newman–Keuls test was used as appropriate. The threshold of statistical significance was considered to be P < 0.05.

3. Results

3.1. Concentration dependence of H₂O₂-mediated PS depression

Previously it was shown that H₂O₂ (1.0–3.0 mM) can inhibit the Schaffer collateral-evoked PS in slices of guinea pig hippocampus [35–37]. We first determined whether H₂O₂ concentrations in this range were also effective in rat hippocampal slices. The effect of 15-min exposure to H₂O₂ on PS amplitude was sharply concentration dependent: 1.2 mM H₂O₂ had no significant effect (n=6), whereas 1.5 mM H₂O₂ caused a significant (P<0.001), reversible decrease in PS amplitude to 18±2% (n=19) of control (Fig. 1). Similarly, 2.0 mM H₂O₂ produced a reversible depression of the PS, but only to the same extent as 1.5 mM H₂O₂ (Fig. 1). In all subsequent experiments, therefore, 1.5 mM H₂O₂ was considered to be the minimum effective concentration.

3.2. Influence of iron chelation

It is known that deferoxamine, a metal ion chelator, prevents formation of ·OH from H₂O₂ [16]. To determine the involvement of ·OH in mediating the effect of H₂O₂ on PS amplitude in rat hippocampal slices, we investigated whether the effect persisted in the presence of this chelator. Deferoxamine (1 mM) alone did not alter PS amplitude (not illustrated); records obtained after 30 min of deferoxamine superfusion served as controls in these experiments. Application of nominally 1.5 mM H₂O₂ in the presence of 1 mM deferoxamine had no effect on PS amplitude (Fig. 2A). At face value, these data suggested that the PS decrease was mediated by ·OH.

An important control, however, which had not been reported in previous studies of the effect of H₂O₂ on hippocampal slice physiology, was to determine actual concentrations of H₂O₂ under varying conditions. To do so, we took samples of H₂O₂ solutions immediately after preparation and also from the recording chamber at the end of 15-min superfusion. Immediately after preparation, actual initial concentrations of H₂O₂ were the same as the nominal levels (Table 1). Concentrations of H₂O₂ in the recording chamber after 15-min superfusion were slightly lower than initial levels, with a decrease of 0.1 mM reaching significance (P<0.05; n=5) for 1.5 mM H₂O₂ in ACSF alone. For nominally 1.5 mM H₂O₂ + deferoxamine, initial H₂O₂ concentrations were not altered by this chelator (Table 1), however, in the presence of deferoxamine, H₂O₂ concentration in the recording chamber after
3.3. Effect of ·OH scavengers on the H$_2$O$_2$-induced depression of the evoked PS

Prevention of H$_2$O$_2$-induced PS suppression by deferoxamine confirmed the involvement of ·OH. We then assessed whether the endogenous ·OH scavenger, ascorbate, and its non-physiological stereoisomer, isoascorbate, could also afford protection from H$_2$O$_2$.

Initial studies with ascorbate and isoascorbate indicated that both caused a decrease in H$_2$O$_2$ concentration (P<0.05) (Table 1). To compensate for the decrease in H$_2$O$_2$ concentration and to be consistent with our deferoxamine studies, we used initially 2.0 mM H$_2$O$_2$ for further experiments with these antioxidants. Under these conditions, the concentration of H$_2$O$_2$ remained above 1.5 mM (Table 1). Somewhat surprisingly, ascorbate was relatively stable in the presence of H$_2$O$_2$. Ascorbate concentration in the chamber after 15-min superfusion with H$_2$O$_2$ was 360±22 μM (n=5), which was only 10% below the initial concentration of 400 μM. The stability of isoascorbate was similar, with 354±19 μM (n=5) after 15-min superfusion with H$_2$O$_2$.

Ascorbate markedly attenuated the effect of H$_2$O$_2$ (Fig.
Table 1

<table>
<thead>
<tr>
<th>Nominal H$_2$O$_2$ concentration (mM)</th>
<th>Initial [H$_2$O$_2$]</th>
<th>[H$_2$O$_2$] after 15-min superfusion</th>
</tr>
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<tbody>
<tr>
<td>1.5 H$_2$O$_2$</td>
<td>1.52±0.02</td>
<td>1.41±0.03$^*$</td>
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<tr>
<td>1.5 H$_2$O$_2$+Def$^b$</td>
<td>1.48±0.01</td>
<td>1.19±0.03$^{*e}$</td>
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<tr>
<td>1.5 H$_2$O$_2$+Asc$^b$</td>
<td>1.50±0.02</td>
<td>1.34±0.03$^c$</td>
</tr>
<tr>
<td>2 H$_2$O$_2$</td>
<td>2.02±0.01</td>
<td>1.95±0.01</td>
</tr>
<tr>
<td>2 H$_2$O$_2$+Def</td>
<td>1.94±0.06</td>
<td>1.62±0.04$^{*e}$</td>
</tr>
<tr>
<td>2 H$_2$O$_2$+Asc</td>
<td>2.02±0.01</td>
<td>1.83±0.01$^{*}$</td>
</tr>
<tr>
<td>2 H$_2$O$_2$+IsoAsc$^b$</td>
<td>2.02±0.02</td>
<td>1.82±0.01$^{*}$</td>
</tr>
</tbody>
</table>

$^a$Data are mean±S.E.M.; $n$=5. Samples were taken immediately after preparation of the H$_2$O$_2$ solution at room temperature and from the recording chamber at 31°C, after 15-min superfusion.

$^b$Def, deferoxamine; Asc, ascorbate; IsoAsc, isoascorbate.

$^cP<0.05$ with respect to the initial concentration.

$^dP<0.001$ with respect to the initial concentration.

$^eP<0.001$ compared with H$_2$O$_2$ concentration in the recording chamber in ACSF alone.

3A). Indeed, the average PS amplitude in the presence of ascorbate+H$_2$O$_2$ did not differ significantly from control (Fig. 3B). To determine whether the action of ascorbate was intracellular or extracellular, H$_2$O$_2$ was applied in presence of isoascorbate. Like ascorbate, isoascorbate also prevented PS suppression by H$_2$O$_2$, indicating an extracellular component to the effect.

Endogenous GSH, another biologically relevant scavenger of ·OH [8], has been shown previously to enhance recovery in this H$_2$O$_2$ model of oxidative stress [38]. When co-applied with H$_2$O$_2$, however, the concentration of GSH in the presence of H$_2$O$_2$ rapidly fell below detection limits (low micromolar levels). Unsurprisingly, therefore, GSH (initially 400 μM) did not protect against the effect of 2.0 H$_2$O$_2$ on the PS amplitude (not illustrated).

3.4. Pulse train stimulation and presynaptic Ca$^{2+}$ entry

Decreased presynaptic Ca$^{2+}$ entry has been proposed as a possible mechanism for the inhibitory effect of H$_2$O$_2$ on hippocampal PS amplitude [35]. To evaluate whether increased Ca$^{2+}$ influx during prolonged stimulation could overcome the H$_2$O$_2$-induced PS depression, we examined evoked PS during pulse train stimulation (5 s, 10 Hz) in the presence of 1.5 mM H$_2$O$_2$. We also assessed the effect of H$_2$O$_2$ on presynaptic Ca$^{2+}$ influx during train stimulation by monitoring [Ca$^{2+}$]$_o$ using Ca$^{2+}$-ISMs. Prior to the delivery of a pulse train, single pulses were used to elicit a PS with stable amplitude. The average amplitude of three single PSs served as the control against which PS amplitudes during pulse train stimulation were compared.

In ACSF alone, 10-Hz stimulation for 5 s caused a twofold increase in PS amplitude that was maximal after three to four pulses, then was constant until the end of the train (Fig. 4). The amplitude of the last pulse of the train was 213±14% of single pulse control ($n$=6; $P<0.001$). In the presence of H$_2$O$_2$, PS amplitude was depressed through much of the train (Fig. 4). The average PS amplitude evoked by the first pulse of the train in H$_2$O$_2$ was decreased to 18±2% ($n$=6; $P<0.001$) of the single pulse control in ACSF alone (Fig. 4). After 25 pulses (2.5

Fig. 3. Inhibition of H$_2$O$_2$-induced PS depression by ascorbate and isoascorbate. (A) Representative electrophysiological records of hippocampal PS in CA1 (each is the average of three records). The effect of H$_2$O$_2$ on PS amplitude in ACSF alone was prevented by co-application of ascorbate (Asc, 400 μM) or isoascorbate (IsoAsc, 400 μM) with nominally 2.0 mM H$_2$O$_2$. (B) Average changes in PS amplitude (as % control). The amplitude of the PS did not differ significantly from control when 2.0 mM H$_2$O$_2$ was applied together with ascorbate or isoascorbate ($n$=5), but did in ACSF alone ($n$=15; ***$P<0.001$). Data are mean±S.E.M.; dotted line indicates pre-H$_2$O$_2$ control amplitude (100%).
recovered to 150±13% (n=6; P<0.05) of single pulse control, although this still differed significantly from the amplitude of the last pulse of the train in ACSF alone (P<0.001) (Fig. 4).

To observe presynaptic Ca$^{2+}$ entry during 5-s, 10-Hz stimulation, Ca$^{2+}$-ISM measurements were made in the presence of a cocktail of postsynaptic receptor blockers (see Materials and methods). Under these conditions, the mean evoked decrease in [Ca$^{2+}$]$_i$ was 52±4 μM (n=6) (Fig. 5). These [Ca$^{2+}$]$_i$ shifts were unaffected by 1.5 mM H$_2$O$_2$, with an mean fall of 50±4 μM (n=6). The lack of difference between the size of the [Ca$^{2+}$]$_i$ decrease in the absence and presence of H$_2$O$_2$, as well as the apparently similar time course of the [Ca$^{2+}$]$_i$ shifts (Fig. 5) argued against an effect of H$_2$O$_2$ on presynaptic Ca$^{2+}$ entry.

4. Discussion

Previous studies of the effect of H$_2$O$_2$ on evoked PS amplitude in guinea pig hippocampal slices raised several unanswered questions. Here, we have addressed these questions in slices of rat hippocampus and confirm that H$_2$O$_2$-induced depression of the evoked PS is mediated by •OH, but that the mechanism does not appear to involve presynaptic Ca$^{2+}$ entry per se.

Consistent with previous reports [13,37], we found that H$_2$O$_2$ decreased the amplitude of the PS evoked by Schaffer collateral stimulation, with a minimum effective concentration of 1.5 mM H$_2$O$_2$ (Fig. 1). In contrast to these results, however, Katsuki and colleagues [21] reported that 0.3–3.0 mM H$_2$O$_2$ caused an augmentation of the PS amplitude in rat hippocampal slices. In the present studies, no enhancement was seen in any experiment (n=19). This difference in response to H$_2$O$_2$ might be explained by several differences in experimental conditions between the previous and present studies. The most important of these is likely to be that in the earlier study, slice incubation temperature during recovery after slicing
and in the incubation chamber was 37°C, whereas in our studies, recovery was at room temperature and recording was at 31°C. The lower temperatures of recovery and incubation presumably kept slices in more viable condition [11]. The fact that H$_2$O$_2$-induced PS depression was reversible upon washout of H$_2$O$_2$ is further consistent with maintained slice viability following H$_2$O$_2$ exposure.

4.1. The effect of H$_2$O$_2$ is mediated by ·OH

Many studies of the effects H$_2$O$_2$ on a variety of in vivo and in vitro systems have concluded that the effective agent is ·OH, because iron chelators, like deferoxamine, could prevent the H$_2$O$_2$-dependent effect [24,33,37,44,54]. In the present studies, H$_2$O$_2$ had no effect on PS amplitude in the presence of deferoxamine, confirming that ·OH, rather than H$_2$O$_2$ itself, was responsible for the observed PS depression. In these experiments, unlike previous studies, we monitored and maintained a normally effective concentration of H$_2$O$_2$ (Table 1). These findings again differed from those of Katsuki et al. [21], who found no effect of deferoxamine on the H$_2$O$_2$-mediated enhancement of the hippocampal PS. In that study, however, the concentration of deferoxamine tested was low (20 μM) and thus probably below an effective level.

Further evidence for ·OH involvement came from our finding that ascorbate also prevented H$_2$O$_2$-induced PS depression. This antioxidant is an effective scavenger of ·OH, as well as of peroxy radical and superoxide [2,8,40]. Importantly, ascorbate was effective within its normal range of extracellular concentration, which is 200–400 μM [28,40,45].

The site of ·OH generation in the present studies is not yet entirely clear, although data suggest an extracellular location. Because H$_2$O$_2$ is highly permeable through the membrane [25], an intra- or extracellular site would be possible. The concentration and duration of deferoxamine exposure in the present experiments, however, may not have been sufficient for this somewhat membrane-permeable iron chelator to enter cells [5,22,54], such that deferoxamine could have been acting in either compartment. An extracellular component of the H$_2$O$_2$ effect, however, was suggested by the results with isoascorbate. While ascorbate and isoascorbate have identical redox properties [19,32], only ascorbate is a substrate for the stereoselective ascorbate transporter [50] and can enter the intracellular compartment [4,40,48]; isoascorbate, therefore, remains extracellular. Because both stereoisomers prevented PS depression by H$_2$O$_2$, this suggests that either the generation of ·OH or its site of action is extracellular. Because ·OH is a neutral molecule, however, it would be expected to be membrane-permeable and thus not constrained to remain in the compartment where it was generated. In addition, it is relevant to consider that the synaptic cleft is part of the extracellular compartment.

4.2. Dissociation of the H$_2$O$_2$ effect on PS amplitude and Ca$^{2+}$ entry during pulse train stimulation

Presynaptic Ca$^{2+}$ influx through voltage-sensitive calcium channels is essential for the coupling of excitation and neurotransmitter release [15]. There is a strong, positive relationship between the extent of Ca$^{2+}$ entry and the amount of transmitter released, with enhancement of both during prolonged stimulation [20,39,47,53]. The present results are consistent with this basic relationship. Under control conditions during 10-Hz train stimulation, PS amplitude doubled within the first few pulses of the train, then remained constant for the rest of the stimulation (Fig. 4). With this same stimulus paradigm, [Ca$^{2+}$]$_o$ showed an initial rapid decrease, then continued to fall gradually through the stimulus train (Fig. 5).

One mechanism by which H$_2$O$_2$ might inhibit neurotransmission has been proposed to be decreased Ca$^{2+}$ entry at presynaptic terminals [35]. Consistent with this hypothesis, Ca$^{2+}$ influx evoked by K$^+$ depolarization was found to be depressed in retina cells under conditions of oxidative stress [1]. In the present studies, however, there was no obvious change in either the time-course or maximum amplitude of decreases in [Ca$^{2+}$]$_o$ during 10-Hz stimulation in the presence of H$_2$O$_2$ (Fig. 5), even though PS amplitude was depressed for over half of the stimulation period (Fig. 4). This suggests that H$_2$O$_2$ did not affect presynaptic Ca$^{2+}$-channels, but rather acted downstream from Ca$^{2+}$ entry. One possible mechanism might be oxidation of redox sensitive sites in vesicle fusion proteins, which could disrupt the interaction of components of Ca$^{2+}$-dependent vesicle fusion machinery [15,43]. The observation that PS depression begins to recover after a certain point during train stimulation might reflect conditions under which sufficient Ca$^{2+}$ entry has occurred to overcome such disruption, thus permitting transmitter release [43].

4.3. Physiological relevance of H$_2$O$_2$-dependent modulation of neurotransmission

The present results provide new information about the possible role of ROS in modulating synaptic transmission. Endogenously, H$_2$O$_2$ is produced during normal oxidative metabolism in the mitochondria, with levels that can exceed 2% of total O$_2$ consumption [3]. In presynaptic terminals, mitochondria are often found within 250 nm of the presynaptic membrane [9], where they provide a local supply of ATP needed for transmitter release, signal transduction, and regulation of [Ca$^{2+}$]$_o$ [18,26,49,52]. Consequently, the mitochondria are also well positioned to provide a source of endogenously produced H$_2$O$_2$ to modulate neurotransmission. Additionally, ·OH, which can be generated from H$_2$O$_2$, also has modulatory properties, including the ability to potentiate tyrosine kinase phos-
phorylation reactions [27], which might further alter synaptic processes.

In light of these recent findings, understanding processes that regulate both ROS generation and endogenous ROS-scavenging capacity takes on new importance. It has long been recognized that redox balance is critical for the prevention of oxidative damage. The present data show that regulation of ROS, for example by antioxidants like ascorbate, may also be critical for fine-tuning ROS-mediated modulation of normal physiological processes, including synaptic transmission.

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