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THE EFFECT OF XANTHINE/XANTHINE OXIDASE GENERATED REACTIVE OXYGEN SPECIES ON SYNAPTIC TRANSMISSION

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The effect of reactive oxygen species generated by the interaction of xanthine and xanthine oxidase on synaptic transmission was examined at the squid giant synapse and the lobster neuromuscular junction. Exposure of these synaptic regions to xanthine/xanthine oxidase produced a significant depression in evoked release, with no change in either resting membrane properties or in the action potential. Addition of catalase to the xanthine/xanthine oxidase-containing media partially blocked the synaptic depression, indicating that H_2O_2 contributes to the synaptic changes induced by exposure to xanthine/xanthine oxidase. H_2O_2 applied directly to the perfusing media also produced a decrease in synaptic efficacy. The results demonstrate that reactive oxygen species, in general, depress evoked synaptic transmission.

KEY WORDS: superoxide anion, hydrogen peroxide, synaptic transmission, glutamate.

INTRODUCTION

Reactive oxygen intermediates (such as superoxide anion, hydrogen peroxide and the hydroxyl free radical) have been implicated as causative factors in the tissue damage associated with various disease states.[1-4] In the central nervous system (CNS), as in virtually all other tissues studied, pathological changes are seen when the level of reactive oxygen species rises above normal values. For example, acute hypertension induced by intravenous norepinephrine injection in the cat is directly associated with abnormally high superoxide levels in brain parenchyma.[5,6] Using the Mongolian gerbil as a model system for stroke, Floyd[7,8] has shown that ischemia followed by reperfusion generates highly reactive free radicals. The beneficial action of antioxidants such as superoxide dismutase (SOD) or the 21 aminosteroids (lazarides) further re-inforces the idea that superoxide anion and other reactive oxygen species are involved in stroke-related CNS injury.[9-11] Reactive oxygen species have also been linked to other types of CNS disorders including autoimmune disease[12] and Downs syndrome.[13,14]

Although reactive oxygen species are implicated in CNS pathology, little is known about their direct action on neuronal function. Studies by Colton and Gilbert[15,16] and

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Pellmar[17,18] have elucidated the action of hydrogen peroxide on basic aspects of nerve function in both mammalian hippocampus and invertebrate synapses. Exposure to hydrogen peroxide at micromolar concentrations produced a depression of inhibitory and excitatory synaptic transmission in both systems. Axonal parameters such as the action potential amplitude were unaffected at these doses.[15,18] A similar phenomenon is seen in hippocampal slice when hydroxyl radicals are generated via irradiation.[19] However, recent biochemical studies suggest that reactive oxygen species generated by the interaction of xanthine with xanthine oxidase increase the release of L-glutamate, an excitatory neurotransmitter in the CNS.[20] Thus, different reactive oxygen species may produce diverse effects.

The primary reactive oxygen species produced by the interaction of xanthine with xanthine oxidase is superoxide anion, although H_2O_2 and hydroxyl radicals are secondarily formed as a result of dismutation and the metal-catalyzed Haber Weiss reaction.[21] Because xanthine/xanthine oxidase-generated oxyradicals may have unique actions and since no direct studies of the synaptic effect of xanthine/xanthine oxidase-generated oxyradicals have been done, we have examined the action of this system on glutamate-mediated synaptic transmission at the squid giant synapse and at the lobster neuromuscular junction.

MATERIALS AND METHODS

Preparation

Squid giant synapse. Experiments were carried out on the giant synapse of the squid stellate ganglion. Squid, *Loligo pealei*, were collected daily by the Marine Biological Laboratory, Woods Hole, MA and kept in cold, sea water tanks until used. The mantle from small squid (3 to 4 inches in length) was cut open and the stellate ganglion exposed. After careful isolation of the large pre-nerve, the aorta was cannulated and a perfused ganglion prepared as described by Stanley and Adelman.[22,23]

The ganglion was moved to a small, plexiglass chamber and stimulating electrodes placed on the presynaptic nerve trunk. Using a binocular microscope, the giant synapse was located and a KCl-filled, glass microelectrode for voltage recording was placed within the postsynaptic nerve adjacent to the synaptic region. A second glass microelectrode, for current passage, was placed within 100 microns of the first electrode. Ganglia were perfused with aerated, artificial sea water (ASW) containing 425 mM NaCl, 9 mM KCl, 10 mM $CaCl_2$, 24 mM $MgCl_2$, 23 mM $MgSO_4$ and 10 mM HEPES at pH 7.6 (18–20°C) and control measurements of resting membrane potential (V_m) and input resistance (R_0) of the postsynaptic axon were obtained. Postsynaptic action potentials and excitatory postsynaptic potentials (EPSP) were also recorded by stimulating the presynaptic axon at a frequency of 0.5 Hz. To measure EPSP amplitude, the ganglia were perfused with ASW containing only 2 mM Ca^{2+} instead of 10 mM Ca^{2+}. This low Ca^{2+} ASW was used to reduce the amount of transmitter released below that required to produce a postsynaptic action potential, allowing the observation of an EPSP.

Lobster nerve-muscle junction. In some experiments, loose patch clamp recordings of synaptic current were obtained from the L2 muscle of the deep abdominal extensor muscles of the lobster, *Homarus americanus*. The nerve innervating the L2 muscle was isolated and the muscle bundles were prepared as described by Grossman

and Kendig.[24] After equilibration for a minimum of 30 min in low calcium medium (450 mM NaCl, 9 mM $CaCl_2$, 10 mM KCl, 20 mM $MgCl_2$ and 5 mM HEPES at pH 7.4), a junction was found by extracellular probing of a muscle fiber surface with an ASW-filled patch electrode. Once located, a loose patch was formed at the junctional site and control synaptic currents measured using a Dagan patch clamp amplifier (Model 8800).

Data from both the squid and lobster preparations are presented as average values ($\pm$SEM) and normalized to percent change using each fiber as its own control. Statistical analysis was done using a paired students "*t*" test.

Xanthine and xanthine oxidase were obtained from Sigma Chemical Co. and catalase obtained from Calbiochem. Prior to use, xanthine oxidase was dialyzed for 6 h at 4°C in distilled water to remove any carrier. Xanthine (1 mM) and xanthine oxidase (0.3 U/ml or 0.6 U/ml) were mixed approximately 15 min prior to application to the cells. In some cases, the effect of hydrogen peroxide on synaptic parameters was examined. For these experiments, hydrogen peroxide was diluted into the ASW perfusing the preparation.

Measurements of Superoxide Anion

The amount of superoxide anion generated from the reaction of xanthine and xanthine oxidase in ASW at 22°C was measured prior to the synaptic experiments. A cytochrome C reduction assay was used to determine superoxide anion concentration.[25] These separate experiments showed that total superoxide anion concentration ranged from 5.8 to 8.5 μM over 50 min under our experimental conditions. It should be noted that the exact concentration of superoxide anion in the preparations is difficult to determine and the values presented probably represent the maximum level achievable.

RESULTS

Effect on the excitatory postsynaptic potential and synaptic current

A significant depression in the amplitude of excitatory postsynaptic potentials (EPSP) was seen when the squid giant synapse was perfused with xanthine/xanthine oxidase ASW. As shown in Figure 1, the EPSP amplitude obtained from a typical postsynaptic axon in 2 mM Ca^{2+} ASW decreased from 7 mV to 2 mV over 30 min of exposure to xanthine/xanthine oxidase, with an average decrease of 50 $\pm$ 8% (Table I). A similar decrease in synaptic current was seen at the lobster neuromuscular junction (NMJ). In this case, synaptic current was reduced to 50% ($\pm$4%, $n = 3$) of control values by 30 min of exposure to xanthine/xanthine oxidase. No recovery in the response was seen on washing either the giant synapse or the lobster neuromuscular junction with normal ASW.

In some experiments either xanthine oxidase or xanthine was omitted from the perfusion fluid to test for the possibility that xanthine or xanthine oxidase by itself caused the depression in EPSP amplitude or synaptic current. No significant changes were evident in resting membrane or synaptic properties.

Because the interaction of xanthine and xanthine oxidase can result in the production of hydrogen peroxide by dismutation of superoxide anion, we tested the effect of hydrogen peroxide on synaptic transmission. Exposure of the squid giant synapse to

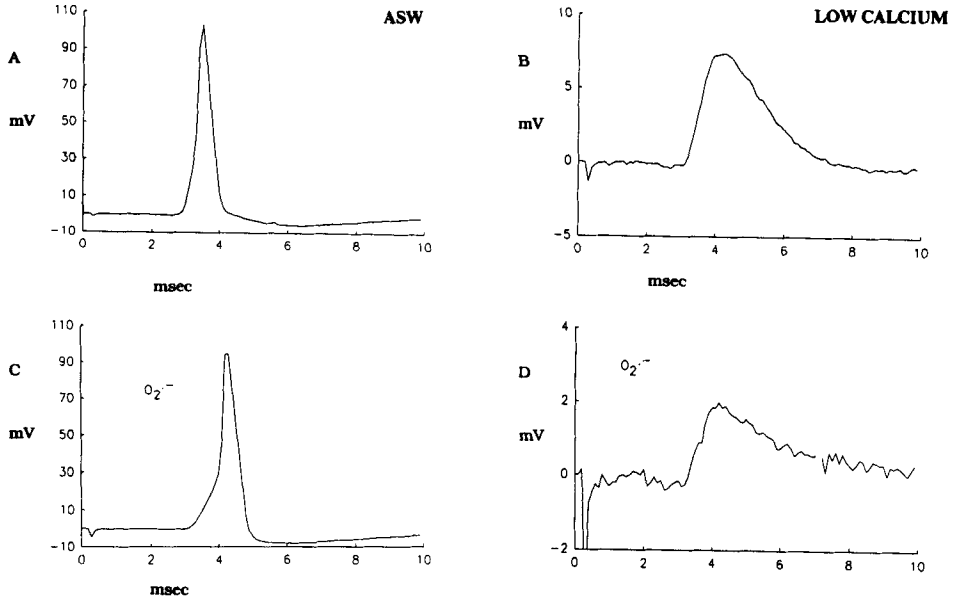

FIGURE 1 The effect of superoxide anion on synaptic transmission at the perfused squid giant synapse. Panel A – action potential recorded from a postsynaptic giant axon in ASW; Panel B – Excitatory postsynaptic potential (EPSP) in the same axon. External calcium concentration was lowered to 2 mM in order to decrease the amount of transmitter released below that needed for threshold of the postsynaptic action potential; Panel C – action potential recorded from the same axon after 30 min of exposure to 7 uM superoxide anion. Note action potential threshold is delayed due to the reduced EPSP; Panel D – EPSP in 2 mM calcium ASW from the same axon after 30 min of exposure to superoxide anion. Note higher amplification with increased baseline noise levels.

TABLE I
Effect of superoxide anion and H_2O_2 on EPSP amplitude

	(*n*)	EPSP amplitude (mV)	% control	*P*
Control	17	9.3 ± 1.1		
X/XO	12	4.8 ± 1.1	50 ± 8	< 0.001
Control	14	6.9 ± 0.5		
10^{-3} M H_2O_2	6	2.4 ± 0.6	35 ± 12	0.003
10^{-5} M H_2O_2	4	4.8 ± 1.3	76 ± 16	0.231

Data points are obtained from the pooled average values (±SEM) of EPSP amplitudes obtained in 2 mM Ca^{2+} ASW for a population of axons treated with either superoxide anion or H_2O_2. For each experimental group, each fiber in that group was taken as its own control and changes in EPSP amplitude after treatment compared to that before. Percent control was then taken as $EPSP_{exp}/EPSP_{control} \times 100$. For average values, controls from superoxide treated axons were analyzed separately from H_2O_2 treated axons. Significance was determined by the paired students' *t* test. (*n*) = number of individual postsynaptic axons.

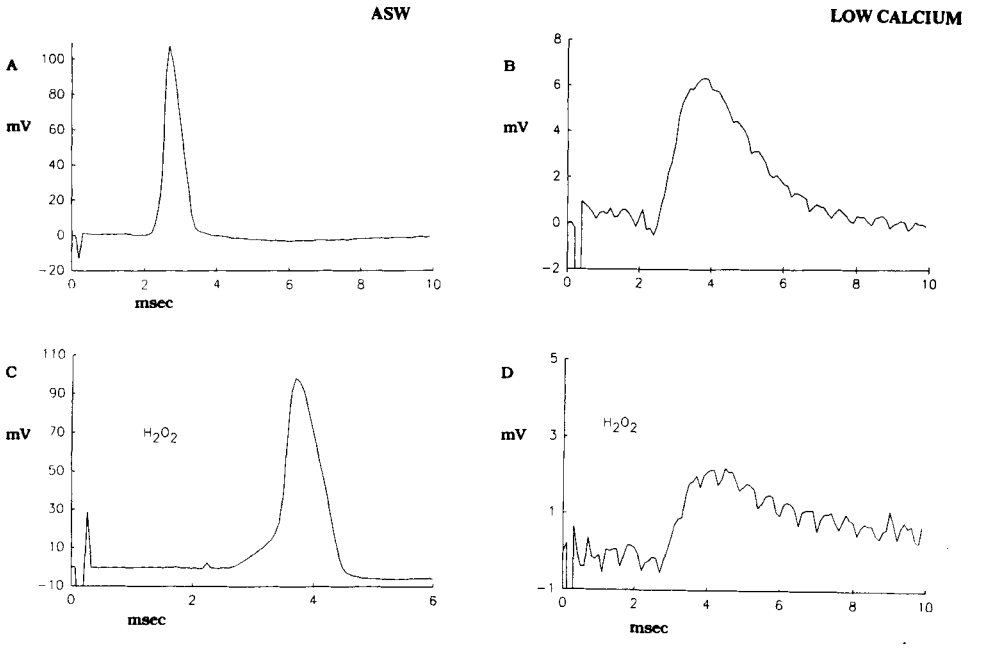

FIGURE 2 Effect of hydrogen peroxide on synaptic transmission of the giant synapse. Panel A – Normal action potential in ASW. Panel B – EPSP in 2 mM calcium ASW. Panel C – Action potential after 30 min in 10^{-3} M H_2O_2 ASW. Panel D – EPSP after 30 min in 10^{-3} M H_2O_2 in 2 mM calcium ASW. All recordings were taken from the same axon. Note change in scale in Panel C and D.

H_2O_2 also significantly depressed EPSP amplitude. As shown in Figure 2, EPSP amplitude from an individual axon declined from 6 mV to 4 mV after 30 min of treatment with 1×10^{-3} M H_2O_2. Average EPSP amplitude decreased to 35 $\pm$ 12% of control values and was significant at a P value of 0.003.

In some experiments, the contribution of H_2O_2 to the xanthine/xanthine oxidase induced changes was assessed using catalase, an enzyme which catalyzes the breakdown of H_2O_2. Preparations were pretreated with catalase at 10 U/ml (1 ug/ml) for 15 min prior to exposure to xanthine/xanthine oxidase. In addition, catalase was added to the xanthine/xanthine oxidase ASW. Synaptic current at 30 min in the xanthine/xanthine oxidase plus catalase solution was decreased by 33 $\pm$ 7% compared to a 50 $\pm$ 4% decrease in xanthine/xanthine oxidase alone.

Effect on resting membrane properties:

Exposure of the squid giant axon to xanthine/xanthine oxidase ASW produced no significant change in either membrane resting potential or resting input resistance. The same was true for 10^{-5} M H_2O_2. However, input resistance increased an average of 31 $\pm$ 10% from the control values with 1×10^{-3} M H_2O_2 ($P = 0.03$). Membrane potential at this concentration of H_2O_2 was unaffected.

No changes in either V_m or R_0 were produced by alteration of the calcium concentration in the ASW from 10 mM to 2 mM. Thus, decreases in EPSP amplitude reflected changes in presynaptic release parameters rather than a decrease due

to "short circuiting" of the response caused by a drop in R_0 or a depolarization of V_m.

Effect on the Action Potential

Xanthine/xanthine oxidase-generated reactive oxygen species did not appear to affect the postsynaptic action potential obtained from the squid synapse over the time period studied. As shown in Figures 1 and 2, nerve action potential conduction was not sensitive to reactive oxygen species at the concentrations used.

DISCUSSION

In both the squid giant synapse and the lobster neuromuscular junction, exposure to reactive oxygen species generated by the interaction of xanthine with xanthine oxidase produced an irreversible depression in glutamate-mediated synaptic transmission. This effect was not due to an independent action of xanthine or xanthine oxidase, since no change in synaptic parameters was seen with either component alone. Furthermore, because neither resting membrane properties nor the action potential were greatly changed, the action of these reactive oxygen species at the concentrations used is specific to synaptic transmission.

Which reactive oxygen species produces the depression is not entirely clear. Interaction of xanthine with xanthine oxidase has been shown to generate the superoxide anion.[2,21] In our system, independent measurements clearly demonstrated that superoxide anion was produced and thus, this anion may contribute to the observed decrease in synaptic efficacy. However, H_2O_2 and the hydroxyl radical are more likely candidates as the actual mediators of synaptic damage. H_2O_2 is readily produced by enzymatic and non-enzymatic dismutation of superoxide anion. Direct addition of H_2O_2, like exposure to xanthine/xanthine oxidase, decreased EPSP amplitude at the squid giant synapse. This, coupled with the fact that catalase reduced the xanthine/xanthine oxidase-induced depression in synaptic current, strongly suggests that H_2O_2 contributes to the functional changes. Part of the effect of xanthine/xanthine oxidase may also be attributed to the generation by hydroxyl radicals via the metal-catalyzed Haber Weiss reaction.

The depression of glutamate-mediated synaptic transmission at the invertebrate synapse by xanthine/xanthine oxidase generated oxyradicals is similar to changes seen in other synaptic preparations. For example, Pellmar[17,18] has shown in guinea pig hippocampal slice that H_2O_2 reduced EPSP amplitude by approximately 50% in the CA1 region. This effect was primarily on presynaptic i.e., nerve terminal, parameters because the post-synaptic response to iontophoretic application of glutamate was unchanged by H_2O_2. In some cases, however, post-synaptic function was altered. For example, N-methyl-D-aspartate (NMDA) receptor function in hippocampal brain slice was blocked by either H_2O_2[16] or xanthine/xanthine oxidase.[26] Depression of synaptic transmission was also seen when reactive oxygen species were generated by other means. Ionizing radiation produced a similar decline in EPSP amplitude in the CA1 region of the hippocampus.[19]

While it is clear that xanthine/xanthine oxidase generated reactive oxygen species decrease evoked (i.e., stimulated) glutamate release, biochemical studies in brain slice have shown an increase in calcium-independent release of glutamate and aspartate

during exposure to xanthine/xanthine oxidase.[20] Although apparently contradictory, these changes are also consistent with electrophysiological data from other synapses. Spontaneous release of transmitter, which occurs in the absence of extracellular calcium,[27,28] increased at the neuromuscular junction of the lobster walking leg preparation when treated with H_2O_2.[15] Exposure to high tensions of oxygen also produced an increase in spontaneous excitatory transmitter release in this preparation[29] as well as in the hippocampal slice.[30] Extracellular glutamate levels may also rise as a result of decreased glial metabolism of glutamate.[7,31] Reactive oxygen species have been shown to inhibit glutamate synthetase, a glial enzyme which catalyzes the degradation of glutamate.[31,32]

Reactive oxygen species, then, appear to behave in a similar manner on synaptic transmission regardless of how they are generated. Two general effects emerge; (1) a depression of evoked release and of postsynaptic transmitter action and (2) an increase in spontaneous release. Thus, although extracellcular glutamate levels may rise during exposure to reactive oxygen species, this potentially harmful effect is offset by a decrease in responsiveness of the NMDA receptor/ionophore, by the depression of evoked release and by desensitization of non-NMDA glutamate receptors. Zorumski *et al.*[33] have shown that non-NMDA glutamate receptors desensitize in the presence of maintained exposures to glutamate.

These data suggest that a significant portion of the neuronal damage seen during stoke and reperfusion injury may not be from oxyradical induced-glutamate release[34] and resultant glutamate-mediated neurotoxicity[35,36] but from the action of reactive oxygen species on non-synaptic sites. Reactive oxygen species are well known to affect a variety of membrane and intracellular proteins, including the Na^+/K^+ ATPase and the Ca^{2+} ATPase.[36–39] Inhibition of these processes could be a prime factor in the cellular changes seen with oxyradical associated pathology.

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