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Modulation of Presynaptic GABA Release by Oxidative Stress in Mechanically-isolated Rat Cerebral Cortical Neurons

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Reactive oxygen species (ROS), which include hydrogen peroxide (H_2O_2), the superoxide anion ($O_2^{-}\cdot$), and the hydroxyl radical (OH·), are generated as by-products of oxidative metabolism in cells. The cerebral cortex has been found to be particularly vulnerable to production of ROS associated with conditions such as ischemia-reperfusion, Parkinson's disease, and aging. To investigate the effect of ROS on inhibitory GABAergic synaptic transmission, we examined the electrophysiological mechanisms of the modulatory effect of H_2O_2 on GABAergic miniature inhibitory postsynaptic current (mIPSCs) in mechanically isolated rat cerebral cortical neurons retaining intact synaptic boutons. The membrane potential was voltage-clamped at -60 mV and mIPSCs were recorded and analyzed. Superfusion of 1-mM H_2O_2 gradually potentiated mIPSCs. This potentiating effect of H_2O_2 was blocked by the pretreatment with either 10,000-unit/mL catalase or 300-μM N-acetyl-cysteine. The potentiating effect of H_2O_2 was occluded by an adenylate cyclase activator, forskolin, and was blocked by a protein kinase A inhibitor, *N*-(2-[p-bromocinnamylamino] ethyl)-5-isoquinolinesulfonamide hydrochloride. This study indicates that oxidative stress may potentiate presynaptic GABA release through the mechanism of cAMP-dependent protein kinase A (PKA)-dependent pathways, which may result in the inhibition of the cerebral cortex neuronal activity.

Key Words: Gamma-aminobutyric acid, Hydrogen peroxide, Oxidative stress, Inhibitory postsynaptic potentials

INTRODUCTION

Reactive oxygen species (ROS), which include hydrogen peroxide (H_2O_2), the superoxide anion ($O_2^{-}\cdot$), and the hydroxyl radical (OH·), are generated as by-products of oxidative metabolism in cells. Increased intracellular production of ROS results in oxidative stress and damage to the central nervous system [1,2]. ROS-induced oxidative stress to the brain has been implicated in normal aging and in various neurodegenerative disorders, including Parkinson's disease, amyotrophic lateral sclerosis, Alzheimer's disease, and ischemia-reperfusion injury [3-8]. The cerebral cortex is the one of the brain areas most susceptible to ROS and is particularly vulnerable to generation of ROS associated with ischemia-reperfusion.

Numerous neurotransmitter systems are sensitive to ROS, including adrenergic [9], dopaminergic [10], serotonergic [11] and GABAergic [12,13] systems. The effects of ROS on neurotransmission can occur via various mechanisms [14]. For example, ROS may interact with neurotransmitter receptors and ion transport proteins such as channels, pumps, and transporters, leading to changes in receptor activity and ionic homeostasis. In addition, ROS can alter ligand-receptor interactions or ion transport indirectly via actions within the lipid environment of cell membranes. ROS, particularly H_2O_2, might also play a role in cellular signaling [15]. Among the most important signaling pathways modulated by H_2O_2 are those involving kinase and phosphatase enzymes, which are sensitive to ROS [16-19]. For GABAergic neurotransmission, ROS have been shown to increase release and decrease uptake of GABA in a variety of neuronal preparations [12,20,21]. A recent study indicated that H_2O_2 increased GABAergic mIPSCs through presynaptic inositol-1,4,5-trisphosphate receptor (IP3R)- sensitive Ca^{2+} release mechanism [22]. However, it is not clear whether cAMP-dependent protein kinase A is involved in the effect of ROS on GABAergic mIPSCs in central neurons. Thus, we examined the role of PKA in the modulatory effect of H_2O_2 on GABAergic miniature inhibitory postsynaptic currents (mIPSCs) in mechanically-isolated cerebral cortical neurons of the rat. As a result, we have found that H_2O_2 potentiates the presynaptic GABA release through the mechanism of cAMP-dependent protein kinase A (PKA)-dependent pathways, which may result in the inhibition of the cerebral cortical neuronal activity.

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ABBREVIATIONS: ROS, reactive oxygen species; NAC, N-acetylcystein; H-89, N-(2-[p-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide hydrochloride.

METHODS

Isolation of single cerebral cortical neurons with intact synaptic boutons

All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Kyung Hee University and all efforts were made to minimize the number of animals utilized, as well as animal suffering.

Single cerebral cortical neurons with presynaptic boutons were isolated using techniques described previously [23,24]. In brief, 10- to 15-day-old Sprague-Dawley rats of both sexes were decapitated under Zoletil 50® anesthesia (50 mg kg^{-1}, i.p.). Brains were quickly removed and transversely sliced at a thickness of 400 μm using a vibratome (Series 1500, Vibratome, St. Louis, MO, USA). Before mechanical dissection, slices were preincubated for at least 1 h at room temperature (22~25°C) in a solution well saturated with 95% O_2 and 5% CO_2. For mechanical dissociation, slices were transferred to a 35-mm plastic culture dish (Falcon Primaria 3801, Becton Dickinson, Rutherford, NJ, USA), and the cerebral cortex was identified under a binocular microscope (SZ-ST, Olympus, Tokyo, Japan). Mechanical dissociation was accomplished using a custom-built vibration device and a fire-polished glass pipette oscillating at approximately 50~60 Hz. The tip of the pipette was lightly placed on the surface of the cerebral cortex and vibrated horizontally (0.1~0.2 mm displacement) for approximately 2 min. Slices were removed, and the mechanically dissociated neurons were allowed to settle and adhere to the bottom of the dish for at least 15 min before commencement of recording. The neurons undergoing dissociation retained short portions of their proximal dendrites.

Electrical measurements

Electrophysiological recordings were performed in the conventional whole-cell patch-clamp recording mode [25] under voltage-clamp conditions. Patch pipettes were prepared from glass capillaries (1B150F-4, World Precision Instruments Inc., Sarasota, FL, USA) on a vertical pipette puller (PP-830, Narishige, Tokyo, Japan) in 2 stages. The patch pipette was positioned on the neuron using a water-driven micromanipulator (WR-60, Narishige, Tokyo, Japan). The resistance between the recording electrode filled with the internal pipette solution and the reference electrode was 5~7 MΩ. The neurons were visualized with phase-contrast equipment on an inverted microscope (IX-70, Olympus, Tokyo, Japan). Electrical stimulation, current recordings, and current filtration (at 1 kHz) were obtained with an EPC-10 patch-clamp amplifier (HEKA Electronik, Lambrecht, Germany) linked to an IBM-compatible PC controlled by HEKA software. Data were digitized with a LIH 1600 board (HEKA Electronik, Lambrecht, Germany) and stored on a hard disk. The currents were monitored on a thermal linearcorder (WR3320-3CL, Graphtec Co., Yokohama, Japan). All experiments were performed at room temperature (22~25°C).

Solutions

The ionic composition of the incubation solution was (in mM): NaCl 124, KCl 5, KH_2PO_4 1.2, $MgSO_4$ 1.3, $CaCl_2$ 2.4, glucose 10, and $NaHCO_3$ 24. The pH was adjusted to 7.4 by continuous bubbling with 95% O_2 and 5% CO_2. The standard external solution was (in mM): NaCl 150, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, glucose 10, and *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid (HEPES) 10. The pH was adjusted to 7.4 with tris-hydroxymethyl aminomethane (Tris-base). The composition of the internal pipette solution for mIPSC recording was (in mM): CsCl 110, TEA-Cl 30, EGTA 5, adenosine 5'-triphosphate magnesium salt (Mg-ATP) 5, guanosine 5'-triphosphate trisodium salt (Na3-GTP) 0.4, and HEPES 10. The pH was adjusted to 7.2 with Tris base. For mIPSC recording, external solutions contained 300 nM TTX, 3 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and 10 μM DL-2-amino-5-phosphonovaleric acid (AP5) to block voltage-dependent Na^+ channels and glutamatergic excitatory synaptic currents.

Data analysis

Spontaneous miniature inhibitory postsynaptic currents (mIPSCs) were counted and analyzed using the Mini-Analysis program (Synaptosoft Inc., Leonia, NJ, USA). Kaleida Graph software (Synergy Software, Reading, PA, USA) was used for curve fitting. To set the adequate threshold for mIPSCs, we analyzed mIPSCs at the threshold of 1 pA, and then manually accepted or rejected each event according to the characteristics of rise time and decay time kinetics. Then, we plotted the histogram of amplitude distribution of mIPSCs. Almost all events of mIPSCs were larger than 10~20 pA. Thus, we set the threshold for event detection at 10~20 pA for mIPSC. Spontaneous events were initially detected automatically using an amplitude threshold of 10~20 pA for the recording of mIPSCs at the holding voltage (V_H) of −60 mV. After automatic detection, all events were manually accepted or rejected on the basis of the rise and decay times. Events with brief rise times (0.5~1.5 ms) that were well-fitted by a single-exponential function were selected for analysis. The amplitude and frequency of large numbers of mIPSCs obtained from a single neuron were examined by constructing all-point cumulative probability distributions and compared using the Kolmogorov-Smirnov (K-S) test with Stat View software (SAS Institute Inc., Cary, NC, USA). Mean amplitudes and frequencies of mIPSCs were normalized to the control conditions and were reported as means±SEM. Differences in mean amplitude and frequency of mIPSCs were tested with Student's paired two-tailed *t*-test using absolute rather than normalized values. Values of $p < 0.05$ were considered significant.

Drugs

Zoletil 50® (tiletamine HCl 125 mg/5 mL + zolazepam HCl 125 mg/5 mL) was purchased from Virbac laboratory (06516 Carros, France). Tetrodotoxin, (-)-bicuculline methochloride, CNQX, AP5, ethylene glycol-bis (β-aminoethyl ether)-*N,N,N'N*-tetraacetic acid (EGTA), Mg-ATP, Na_3GTP, methanesulfonic acid, HEPES, dimethyl sulfoxide, *N*-methyl-D-glucamine, $BaCl_2$, CsCl, $CdCl_2$, cesium methanesulfonate, 4-aminopyridine (4-AP), forskolin, *N*-(2-[p-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide hydrochloride (H-89), tetraethyl ammonium chloride (TEA-Cl), $BaCl_2$, catalase, N-acetyl-L-cysteine, and clotrimazole were purchased from Sigma (St. Louis, Missouri). CNQX, bicuculline, and forskolin were dissolved in dimethyl sulfoxide at 10 mM as a stock solution. Drugs were added to external solutions at the final concentrations shown in the text and the vehicle concentrations never exceeded 0.01%. Drugs were applied

using a rapid application system termed the "Y-tube method" as described elsewhere [26].

RESULTS

GABAergic mIPSCs in isolated cerebral cortical neurons with presynaptic boutons

Mechanically isolated cerebral cortical neurons retained short portions of their proximal dendrites. When the neurons were voltage-clamped at V_H -60 mV in the presence of 300 nM TTX, 3 μM CNQX, and 10 μM AP5, spontaneous mIPSCs were observed. After rupture of the patch membrane, it took 10~20 min for synaptic currents to stabilize. Upon stabilization, recording of mIPSCs were started and the amplitudes of almost all mIPSC in the present study were larger than 10~20 pA. In most neurons, mIPSCs were stable for approximately 60 min. These results indicate that presynaptic nerve terminals attached to the dissociated neurons were functional and their spontaneous activity stable within 60 min. Superfusion of 50 μM bicuculline completely blocked spontaneous mIPSCs frequency (8.9±4.3% of the control, $p<0.01$, n=3, Fig. 1). After washing out bicuculline, the frequency of mIPSC was restored to the control level (103.1±5.7% of the control, p=0.644). These results indicate that the spontaneous mIPSCs are mediated by $GABA_A$ receptors.

Effect of H_2O_2 on GABAergic mIPSCs in isolated cerebral cortical neurons

Superfusion of 1 mM H_2O_2 potentiated the frequency and amplitude of GABAergic synaptic events in all cerebral cortical neurons (Fig. 2). H_2O_2 increased the mean mIPSC frequency to 267.3±37.8% of the control ($p<0.01$, n=9) and the mean amplitude to 131.0±8.9% of the control ($p<0.01$, n=9). Washing out H_2O_2 for at least 10 min could not completely restore the frequency and amplitude of mIPSC to the control level (182.8±52.2% of the control, p=0.151 for the frequency; 117.2±11.4% of the control, p=0.169 for the amplitude). Superfusion of H_2O_2 did not significantly change the rise time and the decay time of the mIPSC (96.9±3.2% of the control, p=0.565 for the rise time; 115.5±6.2% of the control, p=0.302 for the decay time). The results indicate that superfusion of H_2O_2 presynaptically potentiates the release probability of GABA at the presynaptic terminals and postsynaptically potentiates the GABA-activated Cl^- currents without any alteration of kinetics. These results suggest that inhibitory GABAergic synaptic transmission in the cerebral cortical neurons is increased by an increase in presynaptic ROS concentration.

Effect of N-acetylcystein and catalase on the potentiating effect of H_2O_2 on mIPSCs

To elucidate whether the effect of H_2O_2 on the presynaptic GABA release is due to the oxidative stress induced by increased intracellular ROS, we examined the blocking effects of N-acetylcystein (NAC) and catalase on the H_2O_2 potentiating of mIPSCs. Changes in the frequency and amplitude of mIPSCs were recorded before and after the superfusion of 1 mM H_2O_2 with the pretreatment of 300 μM NAC (Fig. 3) or 1,000-unit/ml catalase (Fig. 4). NAC is well known as a ROS scavenger, whereas catalase activates the decomposition of H_2O_2 into water and oxygen. Superfusion of NAC for 10 min did not alter the mIPSCs frequency (108.7±30.1% of the control, n=5, p=0.787) and the mIPSC amplitude (113.3±6.8% of the control, n=5, p=0.106). However, pretreatment with NAC suppressed the potentiating effect of 1 mM H_2O_2 on the mIPSC frequency (125.9±23.5% of the control, n=5, p=0.666) and the mIPSC amplitude (128.2±11.3% of the control, n=5, p=0.291). In addition, pretreatment with catalase, an enzyme catalyzing H_2O_2 to H_2O and O_2, for 10 min did not alter the mIPSCs frequency (87.1±8.3% of the control, n=11, p=0.152) and the mIPSC amplitude (106.1±3.8% of the control, n=11, p=0.141). However, pretreatment with catalase significantly blocked the potentiating effect of 1 mM H_2O_2 on the mIPSC frequency (96.1±14.1% of the control, n=11, p=0.592) and the mIPSC amplitude (105.8±5.1% of the control, n=11, p=0.973). The result indicates that the potentiating effect of H_2O_2 on the mIPSCs frequency and amplitude is blocked by NAC and catalase.

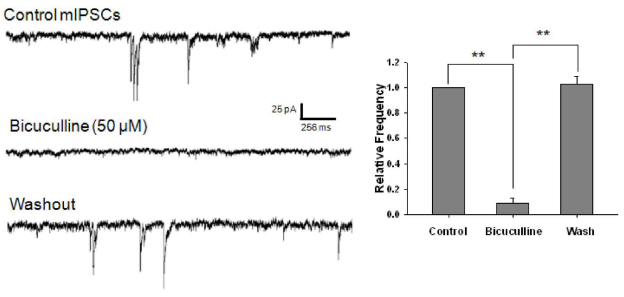

Fig. 1. GABAergic mIPSCs recorded from mechanically dissociated cerebral cortical neurons. In the presence of 300 nM TTX, 3 μM CNQX, and 10 μM AP5, superfusion of 50 μM bicuculline completely and reversibly blocked mIPSCs in isolated cerebral cortical neurons. Holding voltage was -60 mV. Intracellular and extracellular Cl^- concentrations were 140 mM and 161 mM, respectively. Asterisks represent a statistically significant difference (**$p<0.01$).

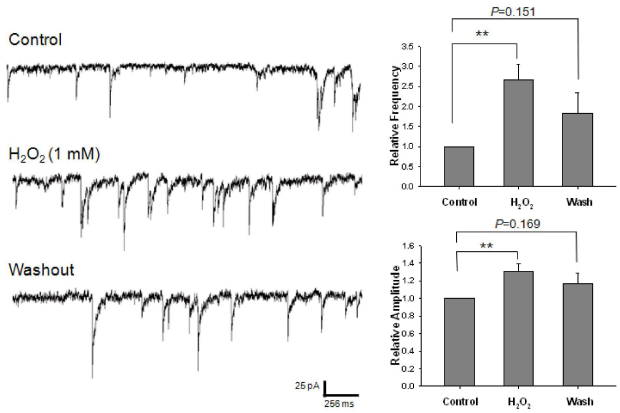

Fig. 2. Effect of H_2O_2 on GABAergic mIPSCs. Each recording trace is the representative current trace of GABAergic mIPSCs recorded before (control), during and after the superfusion of 1 mM H_2O_2. The bar histograms show the mean±S.E.M. of the relative frequency and amplitude of mIPSCs. All frequencies and amplitudes are normalized to those of control mIPSCs. **$p<0.01$.

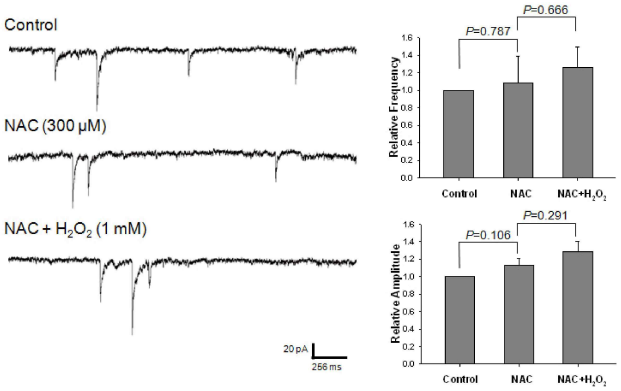

Fig. 3. Effect of NAC on the potentiating of H_2O_2 on GABAergic mIPSCs. Each recording trace shows the control current trace, the current trace recorded during the pretreatment with 300 μM NAC, and the current trace recorded during the superfusion of 1 mM H_2O_2 in the presence of NAC. Bar histograms show the mean± S.E.M. of the relative frequency and amplitude of mIPSCs recorded in the above conditions. The pretreatment with NAC suppressed the potentiating effect of H_2O_2 on the frequency and amplitude of mIPSCs.

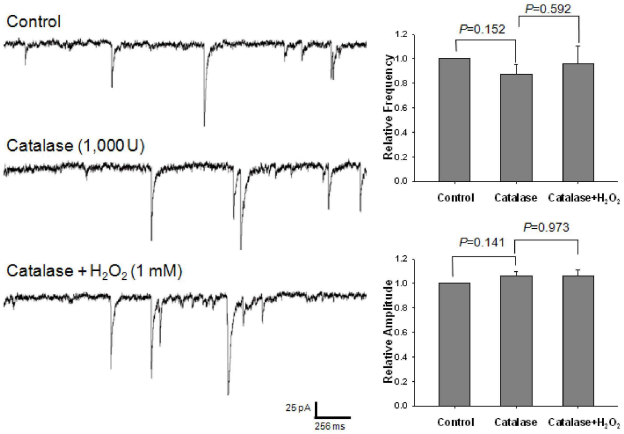

Fig. 4. Effect of catalase on the potentiating of H_2O_2 on GABAergic mIPSCs. Each recording trace shows the control current trace, the current trace recorded during the pretreatment with 1,000-unit catalase, and the current trace recorded during the superfusion of 1 mM H_2O_2 in the presence of catalase. Bar histograms show the mean±S.E.M. of the relative frequency and amplitude of mIPSCs recorded in the above conditions. The pretreatment with catalase suppressed the potentiating effect of H_2O_2 on the frequency and amplitude of mIPSCs.

Effect of adenylate cyclase activation on the potentiating effect of H_2O_2 on mIPSCs

Activation of adenylate cyclase can increase cAMP formation and then potentiates presynaptic neurotransmitter release. Thus, we examined the effect of forskolin, an adenylyl cyclase activator, on the potentiating effect of H_2O_2 on GABAergic mIPSCs. Forskolin (10 μM) significantly increased the mIPSC frequency to 147.8±15.6% of the control ($p<0.05$, n=6), without affecting the mIPSC amplitude

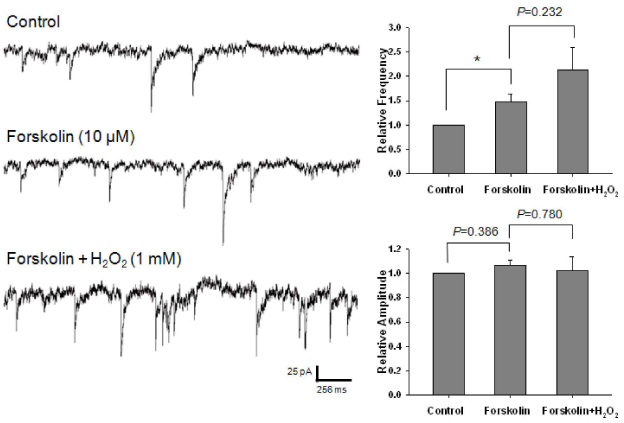

Fig. 5. Effect of forskolin on the potentiating of H_2O_2 on GABAergic mIPSCs. Each recording trace shows the control current trace, the current trace recorded during the superfusion of 10 μM forskolin, and the current trace recorded during the superfusion of 1 mM H_2O_2 in the presence of forskolin. Bar histograms show the mean± S.E.M. of the relative frequency and amplitude of mIPSCs recorded in the above conditions. Forskolin significantly potentiated the frequency of mIPSCs without any alteration of amplitude. In the presence of forskolin, H_2O_2 did not significantly alter the frequency and amplitude of mIPSCs. *$p<0.05$.

(106.0±6.3% of the control, p=0.386, n=6, Fig. 5). In the presence of forskolin, superfusion of H_2O_2 slightly increased the mIPSC frequency while statistically not significant (136.9±17.0% of the forskolin condition, p=0.232, n=6). In addition, in the presence of forskolin, superfusion of H_2O_2 did not potentiate the mIPSC amplitude (96.0±6.7% of the forskolin control, p=0.780, n=6). The results indicate that activation of adenylate cyclase might inhibit the potentiating effect of H_2O_2 on the frequency of presynaptic GABA release and on the amplitude of postsynaptic GABA-activated Cl^- current.

Effect of PKA inhibition on the potentiating effect of H_2O_2 on mIPSCs

It is well known that in the CNS a change in intracellular cAMP concentration affects a variety of cellular signaling via PKA. To address the possible involvement of PKA in the potentiating effect of H_2O_2 on presynaptic GABA release, we examined the effects of H-89, a cAMP-dependent PKA inhibitor, on the H_2O_2 potentiation of GABAergic mIPSCs. Superfusion of H-89 (100 nM) did not alter the frequency and amplitude of GABAergic mIPSC (108.9± 20.8% of the control, p=0.692, n=5 for the frequency; 110.6± 5.3% of the control, p=0.166, n=5 for the amplitude; Fig. 6). In the presence of H-89, superfusion of H_2O_2 slightly increase the mIPSC frequency while statistically not significant (167.2±34.3% of the H-89 control frequency, p=0.184, n=5). In addition, in the presence of H-89, superfusion of H_2O_2 did not potentiate the mIPSC amplitude (95.1±3.7% of the H-89 control, p=0.657, n=5). The results indicate that inhibition of PKA pathway might inhibit the potentiating effect of H_2O_2 on the frequency of presynaptic GABA release and on the amplitude of postsynaptic GABA-activated Cl^- current.

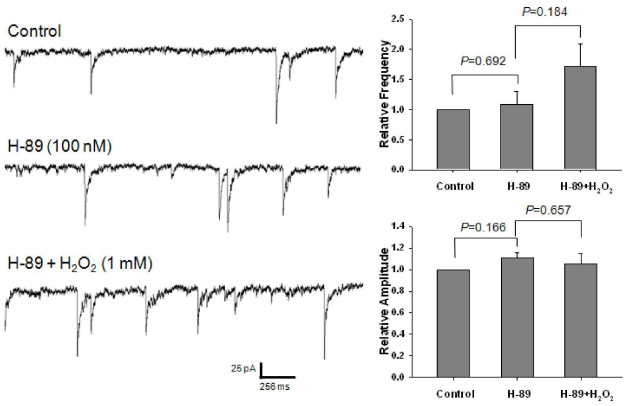

Fig. 6. Effect of H−89 on the potentiating of H_2O_2 on GABAergic mIPSCs. Each recording trace shows the control current trace, the current trace recorded during the superfusion of 100 nM H−89, and the current trace recorded during the superfusion of 1 mM H_2O_2 in the presence of H−89. Bar histograms show the mean±S.E.M. of the relative frequency and amplitude of mIPSCs recorded in the above conditions. H−89 did not significantly alter the frequency and amplitude of mIPSCs. In the presence of H−89, H_2O_2 did not significantly alter the frequency and amplitude of mIPSCs.

DISCUSSION

The present study demonstrates that oxidative stress caused by H_2O_2 potentiates the frequency and amplitude of GABAergic mIPSCs in mechanically isolated single neurons retaining intact synaptic boutons of the rat cerebral cortex. This potentiating effect of H_2O_2 on GABAergic synaptic transmission might be mediated by activation of cAMP−dependent PKA pathway. The results suggest that some conditions resulting in oxidative stress result in the inhibition of the cerebral cortex neuronal activity.

The potentiating effect of H_2O_2 on the activity of rat sympathetic preganglionic neurons was blocked by the pretreatment with catalase [27], which breaks down H_2O_2 into water and molecular oxygen [1] and by the pretreatment with N−acetyl−cysteine, which increases cellular pools of free radical scavengers [27]. In the present study, the potentiating effect of H_2O_2 on GABAergic mIPSC frequency was also completely blocked by catalase and NAC. This finding indicates that oxygen free radicals might directly mediate the potentiating effect of H_2O_2 on the presynaptic GABA release in the rat cerebral cortical neurons.

Oxidative stress induced by ROS might induce the alteration of the neuronal activity through the modulation of inhibitory or excitatory synaptic input strength. H_2O_2 and O_2^- inhibited the uptake of glutamate and enhanced the release of glutamate, resulting in NMDA receptor overstimulation [28,29]. In addition, H_2O_2 stimulated glutamate release, elevation of $[Ca^{2+}]_c$ and ROS generation, which in turn led to neuronal cell death in cultured cortical cells [30]. A recent report demonstrated that H_2O_2 increased GABAergic mIPSCs through presynaptic release of Ca^{2+} from inositol−1,4,5−trisphosphate receptor−sensitive Ca^{2+} stores in the spinal cord substantia gelatinosa neurons [22]. In addition, several reports have demonstrated that cAMP−dependent PKA has an important role in certain conditions showing an increase in presynaptic GABA release in many neuronal preparations [31−33]. However, it is not clear whether cAMP−dependent PKA is involved in the effect of H_2O_2 on presynaptic GABA release. In the present study, activation of PKA with forskolin increased presynaptic GABA release and occluded the potentiating effect of H_2O_2 on the frequency of GABAergic mIPSCs (Fig. 5). Inhibition of PKA with H−89 occluded the potentiating effect of H_2O_2 on the frequency of GABAergic mIPSCs (Fig. 6). These results indicate that the frequency of presynaptic GABA release might be potentiated by H_2O_2 through activation of PKA. On the other hand, forskolin did not alter the amplitude of GABAergic mIPSCs, while the potentiating effect of H_2O_2 on the amplitude of GABAergic mIPSCs was blocked by the pretreatment with forskolin. H−89 also blocked the potentiating effect of H_2O_2 on the amplitude of GABAergic mIPSCs. The results indicate that the amount of GABA released from presynaptic terminals might be increased by H_2O_2, not through activation or inhibition of PKA, but through other mechanisms. From the results of a recent report [22], it can be suggested that the potentiating effect of H_2O_2 on the amount of GABA released from presynaptic terminals might be through IP3−induced presynaptic Ca^{2+} increase.

From the present study, it can be suggested that increased intracellular ROS of the neuronal nerve terminals of cerebral cortical neurons may increase the frequency and amount of GABA release from the presynaptic terminals and then inhibits the postsynaptic neuronal activity via increased GABAergic inhibitory synaptic transmission. This result is consistent with the previous result that H_2O_2 inhibits the neuronal excitability via hyperpolarization of the membrane potential (unpublished data). The sensitivity of $GABA_A$ receptor function to ROS may be an important factor in the development of neuronal damage associated with cerebral ischemia and other neurodegenerative conditions. These findings provide a rationale for testing the efficacy of combined antioxidant pharmacotherapy with drugs that enhance GABAergic neurotransmission [34].

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