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p38 mitogen-activated protein kinase independent SB203580 block of H₂O₂-induced increase in GABAergic mIPSC amplitude

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Abstract

Hydrogen peroxide may serve as a diffusible intermediary messenger under physiological and pathophysiological conditions. H₂O₂ (1mM) increased the amplitude of GABAergic miniature inhibitory postsynaptic currents in substantia gelatinosa of the spinal cord. This effect of H₂O₂ was reversed by the p38 antagonist SB203580, but not by the extracellular signal-regulated kinase 1/2 antagonist PD98059, or the Jun N-terminal kinase antagonist, SP600125. Western blot analysis, however, demonstrated a maximally activated mitogen-activated protein kinase of the slice preparation with no further increase in the phospho-mitogen activated protein kinase by H₂O₂. Confocal microscopy documented no occult neuronal phospho-p38 induction not detected by Western blot. We conclude that SB203580 blocks H₂O₂-induced increase in GABAergic miniature inhibitory postsynaptic amplitude independent of its action on p38 mitogen activated protein kinase.

Keywords

GABAergic; hydrogen peroxide; mitogen-activated protein kinase; mIPSC; SB203580; substantia gelatinosa

Introduction

Activation of microglia cells in the spinal cord and subsequent release of proinflammatory cytokines play a key role in the development of pathological pain. Both extracellular signal-regulated kinase (ERK) 1/2 and p38 mitogen-activated protein kinases (MAPKs) are activated in models of pathological pain and MAPK inhibitors provide relief from painful behaviors in animals (reviewed in Refs [1,2]). Ultimately, the downstream neurophysiological substrate for the development of painful behavior involves alterations in excitability in the periphery (peripheral sensitization) or in the synaptic processing of nociceptive afferent information (central sensitization) [3].

Hydrogen peroxide (H₂O₂), a diffusible reactive oxygen species produced by cells under physiological and pathophysiological conditions, modulates synaptic activity in the brain (reviewed in Refs [4,5]). Sequestration of H₂O₂ decreases painful behavior [6] and as H₂O₂ induces ligand-independent dissociation of heterotrimeric G-protein with subsequent activation of MAPK by the free Gβγ [7], we became interested in the potential role of H₂O₂ as the intermediary messenger between cellular injury and neurophysiological changes leading to painful behavior.

During the course of an investigation of the acute effects of H₂O₂ on GABAergic miniature inhibitory postsynaptic (mIPSC) activity in the substantia gelatinosa (SG) of the spinal cord,

we discovered that H₂O₂ has both a presynaptic effect manifested by an increase in the frequency of mIPSC and a postsynaptic effect observed as an increase in the amplitude of mIPSC. The main presynaptic effect of H₂O₂ in SG neurons is due to a presynaptic release of inositol-trisphosphate receptor-sensitive stored Ca²⁺ [8]. This work reports on an atypical pharmacology exhibited by the p38 MAPK inhibitor SB203580, which blocks the postsynaptic effect of H₂O₂ on the amplitude of mIPSCs.

Methods

Mouse spinal cord slice preparation

All procedures were approved by the Columbia University IUCAC. Young male (C57BL/6) mice (3–5 weeks old) were anesthetized with an intraperitoneal injection of urethane (1.5–2.0 g/kg). After confirming adequate depth of anesthesia, lumbosacral laminectomy was performed and mice were perfused intracardially with ice-cold sucrose-substituted artificial cerebrospinal fluid (sACSF) containing (in mM): 252 sucrose, 2.5 KCl, 2 CaCl₂, 2 MgCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, 10 glucose, 5 kynurenic acid, 340–350 mOsm/kg and pH 7.35. Mice were killed by exsanguination, and the lumbosacral spinal cord (L1–S3) was quickly removed and immediately chilled in an ice-cold sACSF solution. Transverse slices 250-μm thick from the L4–L5 region were cut with a moving blade microtome (Leica Microsystems, Model VT1000S, Wetzlar, Germany) and the slices were kept in normal oxygenated ACSF (126 mM, NaCl instead of sucrose and without kynurenic acid, 300–310 mOsm/kg) at room temperature until used for recording.

Electrophysiology

A spinal-cord slice was transferred to the recording chamber constantly perfused (~3 ml/min) with oxygenated ACSF at 36±1°C. SG neurons and a whole-cell patch-clamp recording were established as described [8]. The solution filling the pipette contained (in mM): 110 CsSO₄, 0.5 CaCl₂, 2 MgCl₂, 5 TEACl, 5 ethylene glycol-bis (*b*-aminoethyl ether), 5 Mg adenosine triphosphatase, 5 *N*-2-hydroxyl piperazine-*N'*-2-ethane sulfonic acid, 270–290 mOsm/kg, pH 7.4. Given the Cl[−] concentrations of ACSF and pipette solutions, the Cl[−] equilibrium potential was −65 mV; thus GABAergic Cl[−] flow produced an outward current when the patched cells were voltage-clamped at 0 mV. GABAergic mIPSCs isolated by adding tetrodotoxin (0.5 μM) and strychnine (2 μM) to ACSF and holding the cells at 0 mV (thereby eliminating the driving force for the excitatory events) were analyzed using the Mini Analysis software (Synaptosoft, Decatur, Georgia, USA) offline. The Kolmogorov–Smirnov statistical test was used for comparison of cumulative distributions of mIPSC parameters and the Mann–Whitney *U*-test for comparison of the mean in the absence and presence of the test drugs with *P*<0.01 being considered significant.

Drugs

All drugs were purchased from Sigma (St Louis, Missouri, USA) except the following: SB203580 and PD98059 (Biomol Research, Plymouth Meeting, Pennsylvania, USA), SP600125 (Stressgen Bioreagents, Victoria, BC, Canada). All drugs were dissolved in dimethylsulfoxide (DMSO) such that the maximal final concentration of the DMSO was less than 0.25%. In control experiments, this DMSO concentration had no effect on mIPSCs. The MAPK inhibitors were used at SB203580 (5 μM), PD98059 (5 μM), and SP600125 (100 nM) in ACSF. Catalase (250 U/ml) was also added to the ACSF and glutathione (GSH, 5 mM) added to the pipette solution.

Western blot

Western blots were carried out by standard methods. Briefly, spinal-cord slices were directly lysed in sodium dodecyl sulfate sample buffer and incubated at 100°C for 10 min before loading on a 10% sodium dodecyl sulfate-polyacrylamide gel. Proteins were separated by electrophoresis, and transferred to a nitrocellulose membrane (BioRad, Hercules, California, USA). The membrane was blocked in 5% milk-TBST, probed with the following primary antibodies (all 1 : 500–1 : 1000 dilution): pan-specific and phospho-specific *MAPK* (Cell Signaling Technology, Beverly, Massachusetts, USA; #9101, #9211, #9252, #9255) all in 3% milk-TBST, and reacted with the horseradish peroxidase-conjugated secondary antibody (1 : 2000) in 3% milk-TBST. After reaction with the Western Lightning chemiluminescence reagent (NEN Life Science Products, Boston, Massachusetts, USA), the images were captured on the EpiChem3 Darkroom System (UVP Inc, Upland, California, USA). Equal loading of the protein was confirmed by probing for Pan-SAPK/Jun N-terminal kinase (JNK) (#9252) antibody.

Immunohistochemistry

Spinal-cord sections were fixed in 4% paraformaldehyde in phosphate buffer (PB; 0.1 M), pH 7.4, for 20 min at 4°C. Nonspecific binding sites were blocked by incubation in PB solution containing 10% normal goat serum for 1 h at 4°C. The antiphospho-p38 antibody was used at 1 : 100. Anti-neuronal nuclei (NeuN) antibody (1 : 200) (Chemicon International Inc., Temecula, California, USA) was used as a neuronal marker and antigial fibrillary acidic protein antibody (1 : 200) (Chemicon International Inc.) to recognize astrocytes. The secondary antibodies were Alexa 488 or 594 coupled to goat antirabbit or antimouse IgG (Invitrogen, Carlsbad, California, USA). Primary and secondary antibodies were diluted in PB solution containing 2% normal goat serum. The slices were incubated in the primary antibody for 48 h at 4°C with gentle shaking, washed three times, and incubated in the secondary antibody for 16 h. The slices were dehydrated through an increasing alcohol series to increase transparency and whole-mounted on a glass slide. Photographs were taken on an Olympus IX50 microscope equipped with a SensiCam cooled CCD camera (Cooke Inc., Auburn, Michigan, USA) or with a Zeiss LSM 510 confocal microscope (Oberkochen, Germany) and were pseudo-colored in Photoshop.

Results

Upward-going GABAergic miniature inhibitory postsynaptic currents were seen in substantia gelatinosa neurons voltage-clamped at 0 mV.

Addition of H₂O₂ (1 mM) resulted in an increase in the observed events (Fig. 1a). Quantification of the event amplitude, frequency, and the monoexponential decay time constant revealed increased amplitude (9.23 ± 0.51 vs. 10.70 ± 0.65 pA, $117 \pm 6\%$ of control, $P < 0.001$) and frequency (0.35 ± 0.55 vs. 4.29 ± 0.86 Hz, $1760 \pm 35\%$ of control, $P < 0.001$) but unchanged decay time constant (6.89 ± 0.30 vs. 7.08 ± 0.42 , 103 ± 4 of control, $P > 0.5$) to 1 mM H₂O₂ ($n = 17$ cells) (Fig. 1b). Lower concentrations of H₂O₂ increased the frequency but without any statistically significant effect on the amplitude [8]. The amplitude increase by 1 mM H₂O₂ was reversed by SB203580, whereas the frequency increase remained unchanged (Fig. 1c and d). Inhibition of ERK1/2 and JNK MAPKs by coapplication of PD98059 and SP600125 did not block the H₂O₂ augmentation of the event amplitude (Fig. 1d). The H₂O₂-induced increase in the frequency was blocked by catalase in the perfusion medium but not GSH inside the patch pipette, suggesting a presynaptic site of action [8]. In contrast, the increase in the amplitude was postsynaptic in origin as either the addition of catalase or the inclusion of GSH had eliminated this H₂O₂ effect (Fig. 1d). Pharmacological evidence upto this point was consistent with a possible novel p38-mediated increase in the postsynaptic GABA_A receptor activity.

We sought biochemical confirmation of the suspected H₂O₂ activation of p38 activation in the spinal-cord slices. Spinal-cord slices, prepared in the identical manner to that in the physiological experiments, were harvested immediately upon slicing (control), at the beginning of the physiological experiment (0 min), and after exposure to H₂O₂ (10 min), and probed for phospho-MAPK by Western blots (Fig. 2a). To our surprise, p38 and ERK1/2 MAPKs appeared fully activated during the rescue incubation of the slices before commencing the physiological recording, but no further increase in phospho-MAPK was observed after addition of H₂O₂. In contrast, JNK remained uninduced. As many cell types exist in a spinal-cord slice, we performed immunohistochemistry in an attempt to determine whether there had been an H₂O₂-induced increase in neuronal phospho-p38 not discernable by the Western blot method.

Consistent with the Western blot results, slices immediately upon cutting demonstrated little phospho-p38 immunoreactivity (Fig. 2b). Slices after rescue incubation corresponding to the 0 time point in the Western blot demonstrated an increase in p38 activation (Fig. 2d), with no apparent further increase after H₂O₂ treatment, corresponding to the 10-min time point in the Western blot (Fig. 2e). Confocal microscopy revealed minimal overlap between the neuronal nuclei marker NeuN and the phospho-p38 signal, suggesting no occult activation of neuronal p38 by the acute administration of H₂O₂ (Fig. 2 D3 and E3).

Discussion

H₂O₂ increased mIPSC peak amplitude and frequency but without change in the event-decay time. The increase in amplitude was blocked by the inclusion of GSH in the recording pipette, indicating that H₂O₂ modulation of the postsynaptic GABA_A receptor was the site of action. Furthermore, the mechanism responsible for the increase in mIPSC frequency was different from that responsible for the increase in the event amplitude as 2-aminoethoxydiphenyl borate, an inhibitor of the inositol-trisphosphate receptor, decreased the mIPSC frequency with no effect on the amplitude [8], whereas SB203580 exhibited the opposite property, having no effect on the frequency but preventing the amplitude increase.

GABAergic synaptic inhibition in SG plays a major role in processing nociceptive information from the primary afferent to the secondary sensory neurons by acting at both presynaptic and postsynaptic sites (reviewed in Ref. [9]). Thus alternations of GABAergic inhibitory synaptic transmission in SG could result in pathological hyperalgesic states. Given the well-described role of MAPK in pathological pain, we were not surprised to see that SB203580, a selective p38 MAPK inhibitor, blocked the enhancement of GABAergic mIPSC by H₂O₂. This observed action of SB203580 was not due to the DMSO carrier and its action as a free-radical scavenger as DMSO alone at the same concentration, or other drugs dissolved in the same carrier, did not block the H₂O₂-induced increase in mIPSC amplitude. Protein kinase A, protein kinase C, Cam-kinase II, and tyrosine-kinase modulation of the GABA_A receptor are well described in the literature (reviewed in Ref. [10]). No reports, however, of MAPK modulation of GABAergic physiology could be found.

As H₂O₂ activates MAPK, we expected that the observed effect of SB203580 on mIPSC was due to the drug antagonism of p38 MAPK. To our surprise, however, Western blot analysis of spinal-cord slices subjected to identical conditions in electrophysiological experiments revealed p38 and ERK1/2 maximally activated before the H₂O₂ challenge. Phosphorylated MAPKs appeared during the postcutting 'rescue incubation' typically used to prepare slice preparations for electrophysiological studies. The cellular stress leading to MAPK activation was most likely the trauma from cutting but it was only manifested during the rescue incubation. Subsequent H₂O₂ challenge demonstrated no further increase in the levels of phospho-MAPK than we would expect if MAPK were previously maximally activated. Immunohistochemistry

and confocal microscopy confirmed that there was no occult activation of neuronal p38 by acute administration of H₂O₂ that was undetected by a Western blot.

The dilemma lies in the observation that the alleged p38-specific antagonist reverses H₂O₂-induced increase in mIPSC amplitude; however, the H₂O₂ challenge did not increase the phospho-p38 level. As SB203580 does not, by itself, affect the mIPSC amplitude or frequency when applied to a slice not challenged by H₂O₂, it supports the idea that acute inhibition of p38 activation has no effect on the postsynaptic GABA_A receptors. SB203580, a substituted imidazole compound, inhibits p38 by competing for the ATP binding site. Despite the very high affinity 48 nM K_d demonstrated for SB203580 binding to p38, the high intracellular concentration of ATP demands low micromolar concentrations of this inhibitor in the context of intact cells (reviewed in Ref. [11]). SB203580 blocks ERK1/2 and downstream p90RSK at concentrations >10 μM [12]. In our study, SB203580 inhibition of the H₂O₂ effect on mIPSC amplitude is not due to such cross inhibition of ERK1/2 or JNK as direct antagonism of ERK1/2 and JNK by their respective selective antagonists did not mimic the effect of SB203580. SB203580 has been reported to activate phospholipase A2 [13] but it is unknown whether this pathway signals to the GABA_A receptor. As long as the mechanism by which SB203580 blocks the H₂O₂-induced increase of the amplitude of GABAergic mIPSC is unknown, work investigating neuronal excitability and pain-related synaptic plasticity in the spinal cord using SB203580 as a selective inhibitor of the p38 MAPK must be interpreted with caution. We also point out the potential danger of using nervous-tissue slice preparations when investigating the MAPK pathways as these stress-activated signaling in neurons appear fully stimulated from the slicing itself, despite the usual care taken in their preparation.

Conclusion

SB203580 reversed the H₂O₂-induced postsynaptic enhancement of GABAergic mIPSC amplitude, most likely been independent of its action as a p38 inhibitor. Further study of H₂O₂-induced increase in GABAergic mIPSC amplitude and its inhibition by SB203580 may reveal a novel mechanism of GABA_A receptor modulation.

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Fig 1. H_2O_2 increases GABAergic mIPSC in spinal cord substantia gelatinosa neurons. (a) GABAergic mIPSC are observed as upward-going spikes on current records obtained from a substantia gelatinosa (SG) neuron voltage-clamped at 0 mV. H_2O_2 evoked a reversible increase in the mIPSC frequency (middle). (b) Cumulative histograms of mIPSC amplitude (left), monoexponential decay time (middle), and inter-event interval (right) for the above events are shown. H_2O_2 caused a reversible shift in the event amplitude and inter-event interval distributions (* $P < 0.05$ or *** $P < 0.001$ by the Kolmogorov–Smirnov test). (c) A cumulative histogram analysis from another neuron treated with SB203580 10 min before the H_2O_2 application is shown. Note the loss of shift in the event amplitude distribution (left) while

maintaining the leftward shift of the inter-event-interval distribution (right). (d) A histogram summary of the effects of the mitogen-activated protein kinase (MAPK) antagonists PD98059/SP600125, SB203580, glutathione (GSH) included inside the patch pipette, and catalase (cat) added to the bath solution on the mIPSC amplitude is shown. Results are graphed as relative amplitude with respect to the predrug application basal event amplitude, which had not been affected by any of the drugs examined. * $P < 0.05$ or ** < 0.01 by Mann–Whitney U -test (number of cells in parentheses).

Fig 2.

Preparation of the spinal-cord slice activates mitogen-activated protein kinase (MAPK) with no further induction of neuronal phospho-p38 by an acute administration of H₂O₂. (a) A representative Western blot shows phospho-extracellular signal-regulated kinase (ERK)1/2, phospho-p38, phospho-Jun N-terminal kinase (JNK), and pan-JNK from spinal-cord slices immediately upon slicing (Cont), after 30 min of rescue incubation (0 min), or after 10 min of exposure to 1mM H₂O₂ (10 min). Immunoreactivity to the respective pan-MAPK (only pan-JNK shown) confirmed equal and unchanged expression of the MAPK proteins. The numbers below the phospho-p38 row are relative intensities based on densitometry (mean±SEM from four independent experiments). (b–e) Photomicrographs of the substantia gelatinosa (SG)

region in spinal-cord slices allow them to be probed for phospho-p38 or neuronal nuclei (NeuN) immunoreactivity for a control slice immediately upon cutting (b), after rescue incubation (d), and H₂O₂ exposure (e). Higher magnification confocal images (D3 and E3) revealed minimal overlap between NeuN and phospho-p38 signals. (c) A control slice immediately after cutting was immunostained for NeuN and glial fibrillary acidic protein (GFAP). The GFAP signal distribution was different from the phospho-p38-positive cells as well. The images are pseudocolored to represent anti-NeuN (green), antiphospho-p38 (red), and anti-GFAP (blue) immunoreactivity. Calibration bar=75 or 10 μ m. SAPK, stress-activated protein kinase.