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Hydrogen peroxide increases GABAergic mIPSC through presynaptic release of calcium from IP₃ receptor-sensitive stores in spinal cord substantia gelatinosa neurons

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Abstract

GABAergic interneurons of the spinal cord substantia gelatinosa regulate the transmission of nociceptive information. Hydrogen peroxide (H₂O₂) is likely a diffusible messenger contributing to the development of long-lasting pathological pain states after nerve injury. In this study, we examined the presynaptic effects of H₂O₂ on the inhibitory interneurons of mouse substantia gelatinosa (SG) using whole-cell patch-clamp recordings from spinal cord slices. H₂O₂ increased the frequency of GABAergic miniature inhibitory postsynaptic current (mIPSC) in a concentration-dependent (10–1000 μM) manner. The profound increase in mIPSC frequency was diminished by thapsigargin or cyclopiazonic acid suggesting that the intracellular stored pool was the source of presynaptic calcium. Further examination revealed the 2-aminoethoxydiphenyl borate blockable inositol-(1,4,5) trisphosphate receptor (IP₃R) regulated pool of stored calcium as the likely source. The phospholipase C (PLC) blocker, 1-(6-[[17β]-3-methoxyestra-1,3,5[10]-trien-17-yl]-amino]hexyl)-1H-pyrrole-2,5-dione (U73122), did not block the frequency increase, which suggested that the site of action of H₂O₂ lies downstream in the IP₃ signalling pathway, and nifedipine-sensitivity of the frequency increase indicated a possible role of calcium-induced calcium-release. However, a direct examination of L-type voltage-gated calcium channels (VGCC) demonstrated that H₂O₂ did not increase the calcium influx through these channels. The H₂O₂ effect on mIPSC frequency was markedly reduced in the opisthotonus (*Opt*) mutant mice with a known deletion in the IP₃R1 gene. We demonstrated that H₂O₂ increased presynaptic activity in the GABAergic interneurons by the release of calcium from the IP₃R-regulated intracellular pool. The presynaptic IP₃R could emerge as a novel target for preventing H₂O₂-induced synaptic plasticity in substantia gelatinosa leading to pathological pain states.

Keywords

GABA; IP₃R; mouse; presynaptic modulation

Introduction

Alterations in the spinal cord nociceptive pathway resulting in the development of pathological pain after injury are likely to involve use-dependent changes in presynaptic and postsynaptic mechanisms (Ji *et al.*, 2003). Activity-dependent elevation in the postsynaptic intracellular calcium through influx from outside the neuron as well as release from the intracellular stored calcium pools are well established to play a key role in postsynaptic plasticity in the hippocampus and other well studied circuits in the brain (reviewed in Rose & Konnerth,

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2001;Bardo *et al.*, 2006). The role of presynaptic intracellular calcium in synaptic plasticity is less clear (reviewed in Collin *et al.*, 2005) although a recent report suggests that presynaptic release from ryanodine receptor (RyR)-sensitive stored calcium pool underlies long-term depression of the hippocampal CA3–CA3 synapse (Unni *et al.*, 2004). Whether the inositol-(1,4,5) trisphosphate receptor (IP₃R) regulated pool of stored calcium also plays a role in presynaptic physiology is largely unexplored.

Hydrogen peroxide (H₂O₂) is an endogenous diffusible reactive oxygen species (ROS) produced mainly in the mitochondria and also secondary to the activation of NMDA and AMPA receptors (Mailly *et al.*, 1999;Avshalumov & Rice, 2002;Avshalumov *et al.*, 2003). H₂O₂ is present in low micromolar concentrations in the normal hippocampus (Lei *et al.*, 1998) increasing to 100s of micromolar upon reperfusion of the ischemic brain (Hyslop *et al.*, 1995). In the hippocampus, H₂O₂ has a concentration-dependent biphasic effect on long-term potentiation – enhancement at low micromolar and inhibition at higher concentrations (reviewed in Kamsler & Segal, 2004).

We became interested in how H₂O₂ affects the spinal cord physiology because of its implication as a soluble mediator positioned between cellular damage and spinal cord synaptic plasticity thought to be the neurophysiological substrate of pathological pain. Spinal cord injury results in the increase in H₂O₂ (Liu *et al.*, 1999) and several observations support the role of H₂O₂ as a signalling molecule in central sensitization. Hyperalgesia after chronic constriction injury of the sciatic nerve was reversed by dietary supplementation of *N*-acetyl-cysteine most likely through increased cellular glutathione implicating ROS in the pathogenesis (Wagner *et al.*, 1998). In a more direct demonstration, systemic injection of phenyl-*N*-tert-butyl nitron, a ROS scavenger, was shown to relieve the spinal nerve ligation-induced mechanical allodynia (Kim *et al.*, 2004). In another model, ROS produced in the spinal trigeminal nucleus was necessary for the behavioural manifestation of persistent facial pain (Viggiano *et al.*, 2005). Despite evidence for a role of H₂O₂ in mediating pathological pain at the behavioural level, there is little known about the effects of H₂O₂ on synaptic physiology of the spinal cord nociceptive circuitry.

In this paper, we describe the effects of H₂O₂ on the presynaptic activity of GABAergic interneurons in the spinal cord substantia gelatinosa (SG) using miniature inhibitory postsynaptic current (mIPSC) as our assay. Our main finding was that H₂O₂ increased the GABAergic mIPSC frequency by releasing presynaptic calcium from an IP₃R-sensitive pool of stored calcium. Our study revealed spinal cord IP₃Rs in the GABAergic interneurons as critical transducers of the presynaptic modulatory action of H₂O₂.

Materials and methods

Mouse spinal cord slice preparation

All procedures were approved by the Columbia University IUCAC. Young male C57BL6 mice (3–5-weeks old) were anaesthetized with an intraperitoneal injection of urethane (1.5–2.0 g/kg). After confirming adequate depth of anaesthesia, lumbosacral laminectomy was performed, the 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, killed by exsanguination, and the lumbosacral spinal cord (L1–S3) 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.

A heterozygous opisthotonus (*Opt*)-mutant (*Itpr1^{Opt}*) breeding pair was obtained from Jackson Laboratory (Bar Harbor, ME, USA) and genotype of the offspring confirmed by PCR of ear clippings using the primer pairs (F1, GGAGGTCCTGGATCTAGTT; R1, TGTTTGCCTCCTTCCAGAAG; wild-type specific, 178 bp) and (F2, AGCTTGAACCAAGTCCACC; R2, GAAATGAGGATCTGGAGCC; mutant specific, 400 bp) (Street *et al.*, 1997). For RT-PCR confirmation of the expression of the expected wild-type and mutant mRNA, 500 ng of total RNA (Absolutely RNA Miniprep, Stratagene, La Jolla, CA, USA) isolated from the mouse spinal cord was subjected to a single-tube reaction (Superscript One-Step RT-PCR, Invitrogen, Carlsbad, CA, USA) with reverse transcription at 50 °C for 30 min followed by heat inactivation of the reverse-transcriptase enzyme at 94 °C for 5 min, denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min for 35 cycles with a final extension for 10 min. The primers used were Exon 1-Fwd (AGCTTGAACCAAGTCCACC) and Exon D-Rev (CCTTGAAGAACTTCTCTGATTTC), which amplified both the wild-type (410 bp) and *Opt* (100 bp) mRNA.

Opt(−/−) developed seizures 10–14 days postnatal but all died soon after precluding electrophysiological experiments on spinal cord slices. In contrast, *Opt*(+/-) showed no obvious signs of abnormality exhibiting normal breeding as well. The slice-patch experiments on this mutant-line were conducted on *Opt*(+/-) and their wild-type littermates at 3–4 weeks of age.

Electrophysiology

A single spinal cord slice was transferred to the recording chamber constantly perfused (~3mL/min) with oxygenated ACSF at 36 ± 1 °C. The slice was viewed under an upright microscope (BX51WI, Olympus Co., Tokyo, Japan) equipped with a 40× water immersion objective and a contrast enhanced video scope system (C2741, Hamamatsu Photonics, Hamamatsu, Japan). Recording electrodes with resistance of 4–8 MΩ were pulled from borosilicate glass capillaries (1.5 mm OD) using a P87 electrode puller (Sutter Instrument Co, Novato, CA, USA). The spinal cord SG region was visually identified by its relatively translucent appearance due to lack of myelination (Yoshimura & Nishi, 1993) and a whole-cell patch-clamp recording established using an Axon 200B amplifier (Molecular Devices, Sunnyvale, CA, USA). The neurons of SG are heterogeneous with distinct morphology, electrical properties, chemosensitivity, and afferent terminations (Baba *et al.*, 1999; Heinke *et al.*, 2004); we did not characterize the identity of the neurons recorded, therefore the results are derived from a mixed population of SG neurons. Access resistance and input capacitance were electronically compensated by approximately 60–70% and monitored throughout the experiment to confirm the stability of the recording. The solution filling the pipette contained (in mM); 110 CsSO₄, 0.5 CaCl₂, 2 MgCl₂, 5 TEACl, 5 EGTA, 5 Mg ATP, 5 HEPES, 270–290 mOsm/kg, pH 7.4. Given the Cl[−] concentrations of ACSF and pipette solutions, the Cl[−] equilibrium potential was approximately −65 mV, thus GABAergic Cl[−] flow produced an outward current when the patched cells were voltage-clamped at 0 mV. GABAergic mIPSCs were isolated by adding tetrodotoxin (0.5 μM) to block the voltage-gated sodium channels and strychnine (2 μM) to block the glycine receptors to the ACSF. Glutamatergic miniature excitatory post synaptic currents (mEPSCs) were eliminated by holding the cells at 0 mV thereby eliminating the driving force for the excitatory events. Preliminary experiments with addition of CNQX and DL-[3H] 2-amino-5-phosphonovaleate to the perfusion medium to pharmacologically block the glutamatergic excitatory synaptic transmission, offered no apparent advantage in isolating IPSCs therefore neither was included in the perfusion medium. Signals filtered at 2 kHz were digitized at 10 kHz through a Digidata1200B interface controlled by the pClamp9 software (Molecular Devices).

GABAergic mIPSCs were analysed using the Mini Analysis software (Synaptosoft, Decatur, GA, USA) off-line. The threshold for detection of mIPSC was set at three times RMS for the background noise and each automatically identified event was visually confirmed to be free of artifacts. More than 50 events were collected and cumulative distributions of peak amplitude, monoexponential decay time constant, and frequency of events obtained. In all instances, mIPSCs were recorded for several minutes to establish baseline values. Without altering the perfusion rate and temperature, the perfusion medium was then changed to one containing the indicated concentrations of the drug for 10 min prior to data collection.

Data are presented as mean $\pm$ SEM unless otherwise indicated. The Mann–Whitney nonparametric test was used to determine the statistical significance between means and the Kolmogorov–Smirnov test was used to compare cumulative distributions of mIPSC parameters in the absence and presence of the test drugs. $P < 0.05$ was considered significant for both tests.

The L-type high-threshold voltage-gated calcium current (VGCC) was isolated by replacing the external Ca^{2+} with 10 mM Ba^{2+} ; HEPES replaced phosphate buffer to prevent precipitation of the Ba^{2+} -supplemented solution. Phosphocreatine (10 mM) was added to the internal solution to minimize run-down of the L-type VGCC. Cells were held at -50 mV and jumped to various activating voltages to obtain the current–voltage relationship or to a fixed -20 mV every 20 s when examining for a drug effect.

Drugs

All drugs were purchased from Sigma (St. Louis, MO, USA) except the following: cyclosporin A and 1-(6-[[[17 β]-3-methoxyestra-1,3,5[10]-trien-17-yl]-amino]hexyl)-1H-pyrrole-2,5-dione (U73122; Biomol, Plymouth Meeting, PA, USA); cyclopiazonic acid and thapsigargin (EMD Biosciences, Darmstadt, Germany); ryanodine (Wako, Tokyo, Japan). H_2O_2 was freshly dissolved into ACSF immediately before the experiments. Thapsigargin (2 μM) and cyclopiazonic acid (10 μM) were used to deplete Ca^{2+} intracellular stores by inhibiting the smooth endoplasmic reticulum Ca^{2+} -ATPase (Reyes & Stanton, 1996). Ryanodine (10 μM) was used to block the release of Ca^{2+} from intracellular store. Cyclosporin A (10 μM) was used to inhibit the mitochondrial permeability transition pore opening (Crompton *et al.*, 1988). 2-Aminoethoxydiphenyl borate (2-APB; 200 μM) was used as an antagonist of the IP_3R (Maruyama *et al.*, 1997). U-73122 (10 μM) was used as an inhibitor of phospholipase C (PLC; Baskys *et al.*, 2005). Further support for the drug concentrations used can be found in the reference cited in an excellent review by Koulen & Thrower (2001). All drugs were dissolved in dimethylsulphoxide (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.

Immunohistochemistry

Spinal cord sections were fixed in 4% paraformaldehyde in PB (0.1 M), pH 7.4, for 20 min at 4 °C. Nonspecific binding sites were blocked by incubation in PBS containing 10% normal goat serum for 1 h at 4 °C. A commercially available antibody for IP_3R type 1 (Calbiochem, San Diego, CA, USA) was used at a dilution of 1 : 100. Neuronal nuclei (NeuN) antibody (1 : 200; Chemicon International Inc., Temecula, CA, USA) was used as a neuronal marker and glial fibrillary acidic protein (GFAP) antibody (1 : 200; Chemicon International Inc) to recognize astrocytes. The secondary antibodies were Alexa 488 or 594 coupled to goat anti-rabbit or anti-mouse IgG (Molecular Probes, Invitrogen, Carlsbad, CA, USA). Primary and secondary antibodies were diluted in PBS 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 a Zeiss LSM 510 NLO confocal microscope (Oberkochen, Germany) and psuedo-coloured in Photoshop.

Results

Patch-clamped SG neurons exhibited both spontaneous mEPSCs and mIPSCs. When neurons were held at 0 mV to minimize the driving force for the mEPSCs, the outward going mIPSCs were seen as upward going events with a basal frequency of 0.24–0.45 Hz, mean amplitude of 8.35 ± 0.32 pA, and a monoexponential decay time constant of 6.71 ± 0.26 ms ($n = 38$ neurons). Application of H_2O_2 resulted in a reversible and concentration-dependent increase in the mIPSC frequency (Fig. 1A and B). Analyses of mIPSC demonstrated an increase in the event amplitude as well as a profound increase in the frequency (plotted as an interevent interval), however, with no effect on the monoexponential decay time constant (Fig. 1C). A statistically significant increase in mIPSC frequency was observed at a H_2O_2 concentration of $100 \mu\text{M}$ [0.48 ± 0.13 (control) vs. 0.78 ± 0.2 (H_2O_2) Hz, $n = 6$, $P < 0.01$ by Mann–Whitney test) with the maximum, near tenfold increase in the frequency but reversible effect, observed at 1 mM [0.35 ± 0.05 (control) vs. 4.29 ± 0.86 (H_2O_2), Hz, $n = 17$, $P < 0.001$ by Mann–Whitney test; Fig. 1D]. At higher H_2O_2 concentrations, the frequency of mIPSC deteriorated below the baseline after drug washout. Addition of the catalase, which converts H_2O_2 to water in the ACSF, abolished the H_2O_2 -induced increase in mIPSC frequency while inclusion of a reactive oxygen species scavenger, glutathione, inside the patch pipette had no effect on the frequency. The baseline mIPSC frequency with only extracellular catalase or intracellular glutathione before the addition of H_2O_2 was unchanged at 0.29 ± 0.13 ($n = 4$) and 0.28 ± 0.03 ($n = 8$) Hz, respectively. These observations establish the H_2O_2 -induced increase in mIPSC frequency as a presynaptic phenomenon. In contrast, the increase in the mIPSC amplitude observed at 1 mM H_2O_2 (Fig. 1C) was abolished by intracellular glutathione (Fig. 1D) suggesting this may be a manifestation of a postsynaptic modulation of the GABA_A receptors by H_2O_2 . This postsynaptic effect of H_2O_2 will be examined in another paper and will not be considered further here.

The frequency of spontaneous miniature synaptic events correlates with the presynaptic Ca^{2+} concentration (Augustine *et al.*, 2003) therefore, we investigated the source of presynaptic Ca^{2+} . In general, presynaptic Ca^{2+} can arise from stored Ca^{2+} within the neuron and/or from influx through VGCC although unlike action potential-dependent evoked release, miniature synaptic events depend less on Ca^{2+} influx. The pools of stored Ca^{2+} consist of those within the mitochondria or within the endoplasmic reticulum further separated into the IP_3R - or the RyR-sensitive components (Fig. 2A). We first explored the role of stored Ca^{2+} using well-known inhibitors of the intracellular pools.

That the H_2O_2 -induced increase in mIPSC frequency resulted from the release of stored- Ca^{2+} was suggested by the observation that thapsigargin and cyclopiazonic acid, both drugs that reduce the amount of stored- Ca^{2+} , reduced the H_2O_2 -effect on frequency. Replacing Ca^{2+} in ACSF with Mg^{2+} and EGTA (2 mM ; nominal Ca^{2+} -free solution) decreased the mIPSC frequency at the baseline and upon H_2O_2 challenge but the deterioration in the mIPSC frequency was not reversible even after returning the perfusion medium to normal ACSF (data not shown). Cyclosporin A ($10 \mu\text{M}$), an inhibitor of the opening of the mitochondrial transition pore, or ryanodine ($10 \mu\text{M}$), an inhibitor of the RyR at this concentration, had no significant effect on the baseline or on the H_2O_2 -induced increase in mIPSC frequency. In contrast, 2-APB ($200 \mu\text{M}$), a nonselective antagonist of the IP_3R , essentially eliminated the H_2O_2 -induced increase in the mIPSC frequency [0.45 ± 0.05 (control) vs. 0.76 ± 0.23 (2-APB alone) vs. 0.81 ± 0.29 (2-APB and H_2O_2) Hz, $n = 8$]. These pharmacological experiments suggested that the increase in presynaptic Ca^{2+} arose from a H_2O_2 -induced release from the IP_3R -sensitive pool of stored- Ca^{2+} (Fig. 2B).

IP₃, the natural ligand for the IP₃R, arises from the enzymatic activity of PLC-β. PLC-β, in turn, is activated by a variety of upstream mechanisms including activation of G-protein coupled receptors and dissociation of the heterotrimeric G-protein (Rhee & Bae, 1997). As H₂O₂ induces a ligand-independent dissociation of the heterotrimeric G-protein (Nishida *et al.*, 2000), we examined for a potential involvement of upstream signalling in the apparent IP₃R-dependent increase in presynaptic Ca²⁺. If the H₂O₂-effect on frequency involves upstream signalling, inhibition of PLC-β should block the downstream manifestation on mIPSC. The PLC antagonist, U73122, did not prevent the H₂O₂-induced mIPSC frequency increase (Fig. 2C) pointing to a H₂O₂ site-of-action downstream from PLC-β.

Next, we examined for a potential role of Ca²⁺-influx through the VGCC as a source of presynaptic Ca²⁺. The postsynaptic neuron is voltage-clamped therefore the voltage is controlled. However, the membrane potential in the GABAergic interneurons, which are presynaptic to the patch clamped neuron, could fluctuate triggering opening of the VGCC as tetrodotoxin resistant sodium channels exist in the spinal cord (Shah *et al.*, 2000; Matayoshi *et al.*, 2005) and the dorsal root ganglion (DRG). In addition, H₂O₂-enhancement of Ca²⁺-flux through the L-type VGCC has been reported (Akaishi *et al.*, 2004; Hudasek *et al.*, 2004) leading to the possibility that VGCC activity could elevate presynaptic Ca²⁺ causing a direct increase in the vesicular release or an indirect increase in the Ca²⁺ release from the IP₃R (Bezprozvanny *et al.*, 1991).

Nifedipine (10 μM), a dihydropyridine antagonist of L-type VGCC, blocked the H₂O₂-induced increase in mIPSC frequency (Fig. 2C and D) while having no effect on the basal mIPSC frequency. This inhibition of H₂O₂-effect was reversible and as effective as 2-APB demonstrated above. Ni (50 μM), more selective for the T-type VGCC, had little effect on the H₂O₂-induced frequency increase (data not shown). Given this surprising nifedipine blockade of H₂O₂ effect, we further explored the possible role of L-type VGCC in regulating mIPSC frequency.

It is technically hard, if not impossible, to examine directly the effects of H₂O₂ on presynaptic terminals of the SG neurons electrophysiologically. Therefore, we examined the somatic Ca²⁺ current elicited by voltage steps under whole-cell patch-clamp. Step voltage jumps from -50 mV to depolarized potentials elicited inward going currents consistent with activation of the L-type VGCC. It should be noted that significant current activation was observed even at -40 mV with the peak current observed near -15 mV, both significantly shifted leftward on the voltage-axis for a typical high voltage-activated L-type VGCC, but consistent with the characteristics of L-type VGCC in other spinal cord neurons (Voisin & Nagy, 2001). Addition of BayK 8644 increased the current magnitude with no effect on the voltage dependence of activation while nifedipine eliminated the current (Fig. 3A). Acute administration of H₂O₂ (500 μM), a concentration resulting in a profound increase in the mIPSC frequency, did not increase Ca²⁺ influx through the L-type VGCC (Fig. 3B).

We wanted to see whether enhanced Ca²⁺ influx through the L-type VGCC could increase the mIPSC frequency. BayK 8644 (5 μM), which produced an approximate 50% increase in the magnitude of the L-type VGCC activity at -20 mV, did increase the mIPSC frequency with no significant effect on the event amplitude or decay time (Fig. 3C and D), confirming that L-type VGCC could play a role in presynaptic Ca²⁺ regulation. However, the BayK 8644-induced increase in mIPSC frequency was not inhibited by 2-APB (Fig. 3E) suggesting that the L-type VGCC-mediated and H₂O₂-mediated mechanisms are different. The fact that 2-APB did not block the BayK 8644-induced increase in mIPSC frequency also suggested that 2-APB is not a nonspecific inhibitor of the presynaptic release mechanism. Thus despite the effective pharmacological inhibition by nifedipine, the H₂O₂-induced increase of mIPSC frequency is

not secondary to a BayK 8644-like increase in Ca^{2+} -influx through the presynaptic L-type VGCC.

Three isoforms of IP_3R have been described with all isoforms expressed in the brain and $\text{IP}_3\text{R}2$ exclusively expressed in the astrocytes (Sharp *et al.*, 1999). However, the expression of IP_3R in the spinal cord has not been confirmed. Given the functional evidence of the presence of IP_3R in the spinal cord, which we have obtained, we sought to confirm the presence of IP_3R in the spinal cord neurons by immunohistochemistry. We examined the distribution of $\text{IP}_3\text{R}1$ in the spinal cord compared to the distribution in the cerebellum.

In frozen sections of the cerebellum, the anti- $\text{IP}_3\text{R}1$ immunoreactivity was clearly observed in the Purkinje cell body and neurites (Fig. 4A) as previously reported (Sharp *et al.*, 1999). The same experiment was repeated in 250- μm -thick sections of the lumbar spinal cord with coimmunostaining for neuronal nuclei with anti-NeuN or for astrocytes with anti-GFAP (Fig. 4B), respectively. Imaging with a confocal microscope (Fig. 4C) revealed a neuritic and perisomatic staining pattern that only partly overlapped with the NeuN signal suggesting that the $\text{IP}_3\text{R}1$ isoform is likely to be present at neuritic terminals appropriately positioned to play a functional role in presynaptic modulation of neurotransmitter release.

Lastly, we sought confirmation of the role of IP_3R in H_2O_2 -induced increase in presynaptic mIPSC frequency using a mouse genetic model. Null-mutants of all three individual IP_3Rs have been created with only $\text{IP}_3\text{R}1$ knock out showing neurological symptoms (Matsumoto *et al.*, 1996; Futatsugi *et al.*, 2005). In addition, the *Opt* mutant-mouse with a known deletion in two exons of the $\text{IP}_3\text{R}1$ gene showing identical symptoms to the knock out has been described (Street *et al.*, 1997). We investigated the effects of H_2O_2 on GABAergic mIPSC in the *Opt*-mutant because this line was available from a commercial vendor. In our hands, the homozygote *Opt*($-/-$) died within 2 weeks thus limiting our electrophysiological study to heterozygote *Opt*($+/-$) with normal survival despite the much reduced expression of both the wild-type and mutant $\text{IP}_3\text{R}1$ proteins (Street *et al.*, 1997). RT-PCR of the spinal cord demonstrated the expected expression of the mutant and wild-type $\text{IP}_3\text{R}1$ mRNA consistent with the genotype (Fig. 5A). Baseline properties of GABAergic mIPSC from *Opt*($+/-$) were no different from the wild-type ($+/+$) littermates (amplitude; 7.40 ± 0.59 vs. 8.17 ± 0.62 pA; decay time constant: 7.32 ± 0.30 vs. 7.16 ± 0.95 ms; frequency; 0.32 ± 0.10 vs. 0.29 ± 0.08 Hz; $P > 0.5$ for all comparisons by Mann–Whitney test). H_2O_2 (500 μM) elicited only a minimal increase in the mIPSC frequency in *Opt*($+/-$) in contrast to the wild-type littermate *Opt*($+/+$) control, $140 \pm 54\%$ ($n = 7$) vs. $1110 \pm 50\%$ ($n = 8$), respectively ($P < 0.001$ by Mann–Whitney test). The H_2O_2 response wild-type *Opt*($+/+$) control was indistinguishable from the C57BL6, $1110 \pm 50\%$ ($n = 8$) vs. $1250 \pm 220\%$ ($n = 10$), respectively ($P > 0.5$ by Mann–Whitney test) used elsewhere in this study indicating little genetic drift between the C57BL6 parental and the *Opt* mice lines with respect to the pharmacological response to H_2O_2 despite long periods of separate inbreeding (Fig. 5B and C). These results from *Opt* mice augment our conclusion largely based on pharmacological experiments that presynaptic IP_3Rs , specifically the $\text{IP}_3\text{R}1$ subtype, most likely mediates the increase in mIPSC by H_2O_2 .

Discussion

Acute administration of H_2O_2 increases mIPSC frequency

We demonstrated that an acute administration of H_2O_2 resulted in a marked increase in the frequency of mIPSC. As inclusion of ROS scavenger in the recording electrode had minimal effect on the frequency, whereas catalase in the perfusion medium abolished the increase in the frequency, we concluded that H_2O_2 acted on the presynaptic neuron to increase the mIPSC frequency. A pharmacological dissection of this phenomenon and the use of *Opt*-mutant mice lead us to the conclusion that presynaptic IP_3Rs play a role as the frequency increase was

blocked by the IP₃R antagonist 2-APB and absent in the *Opt*(+/-)-mutant. To our knowledge, our present work is the first to document a role of presynaptic IP₃R in GABA release from spinal cord substantia gelatinosa interneurons and its modulation by a pathophysiologically relevant molecule, H₂O₂.

The role of IP₃R in the regulation of postsynaptic Ca²⁺-mediated synaptic plasticity is well established (Bardo *et al.*, 2006). Likewise, RyR in presynaptic Ca²⁺ dynamics has also been well documented (Bouchard *et al.*, 2003). However, reports of the role of IP₃R in presynaptic physiology are few. Han *et al.* (2001) took advantage of an autaptically innervated single rat retinal ganglion cells that allowed drugs included inside the recording patch pipette to affect the presynaptic physiology. Inclusion of heparin to block the IP₃R or an antagonist of the PLC- β pathway decreased mEPSC frequency documenting a role of presynaptic IP₃R in excitatory neurotransmitter release. A recent report documented an effect of menthol on synaptic events recorded from a DRG-dorsal horn coculture model (Tsuzuki *et al.*, 2004) remarkably similar to H₂O₂. Menthol, a selective activator of the transient receptor potential cation channel (TRP), subfamily M, member 8 (TRPM8) cation channel increased mEPSC frequency blocked by 2-APB. The authors concluded that Ca²⁺ influx through the cation permeable TRPM8 channels resulted in a calcium-induced calcium release from the presynaptic IP₃R-sensitive Ca²⁺ store. In agreement with this work, Northern blot hybridization demonstrated predominant expression of the TRPM8 mRNA in adult DRG and trigeminal ganglia (Peier *et al.*, 2002). Further *in situ* hybridization localized the signal to the mostly small diameter DRG. But TRPM8 was not detected in the spinal cord thus it is unlikely that the TRPM8 channels mediate the H₂O₂ effects on substantia gelatinosa inhibitory interneurons. However, we cannot rule out the presence of other H₂O₂-sensitive multifunctional TRP channels such as TRPM2 and TRPM7 (Harteneck, 2005) in the spinal cord. In fact, both TRPM2 and TRPM7 are activated by H₂O₂ (reviewed in Miller, 2006). Nevertheless, our work is consistent with these earlier reports and extends the role of presynaptic IP₃R in regulating synaptic release to the GABAergic interneurons of SG.

Although 2-APB has been used extensively as a selective inhibitor of the IP₃R since the introduction of this compound by Maruyama *et al.* (1997), recent reports indicate that this drug blocks store-operated channels, volume-regulated anion channels, Ca²⁺-ATPase, gap junctions, cationic and TRPC5 (TRP, subfamily C) channels (Bootman *et al.*, 2002; Harks *et al.*, 2003; Peppiatt *et al.*, 2003; Xu *et al.*, 2005). Although not statistically significant ($P = 0.062$, Mann-Whitney test), APB alone appeared to somewhat increase the basal mIPSC frequency (Fig. 2B) perhaps reflecting some of the non-IP₃R-related effect of this drug. The modest increase in the basal frequency is not nearly enough to occlude the H₂O₂ effect that increases the frequency by almost 1000% (tenfold over control) and does not alter the conclusion that APB inhibits the H₂O₂-induced frequency increase. Nevertheless, reliance on this drug alone to implicate IP₃R in the H₂O₂ effect on mIPSC frequency is dangerous. Our conclusion that the IP₃R-sensitive pool of stored Ca²⁺ mediates the H₂O₂ effect was also based on the negative results with several RyR modulators. In addition, the loss of H₂O₂-induced increase in mIPSC frequency in the *Opt*(+/-) mutant supports the role of IP₃R1 in this process. The dramatic loss of the H₂O₂ response even in *Opt*(+/-) heterozygotes suggests that the presence of even a single mutant-protein in the tetrameric functional IP₃R may render the receptor nonresponsive. However, confirmation of this speculation will require further studies.

Where does H₂O₂ work along the IP₃ cascade?

The nonspecific PLC blocker, U73122, had no effect on the H₂O₂-induced mIPSC frequency increase suggesting that H₂O₂ was working downstream of PLC. PLC- γ usually associated with phosphorylation and activation by receptor tyrosine kinase was directly activated by H₂O₂ (Bai *et al.*, 2002) and could contribute towards IP₃ generation. As U73122 is a

nonspecific PLC inhibitor blocking the PLC- γ isoform as well, the increase in mIPSC frequency secondary to H_2O_2 activation of PLC is unlikely.

If H_2O_2 works downstream of PLC, it can act at the level of the ligand–receptor interaction or on the Ca^{2+} release from the IP_3R itself. The possibility of H_2O_2 acting on the IP_3 – IP_3R interaction, particularly on the Ca^{2+} regulation of this interaction, is discussed below in the context of nifedipine sensitivity. Can H_2O_2 directly act on the IP_3R to alter the Ca^{2+} release process? Oxidation of critical cysteine residues increases RyR1 sensitivity to Ca^{2+} while decreasing the inhibitory effect of high Ca^{2+} concentrations (reviewed in Hidalgo, 2005). Although it is not known whether oxidation of IP_3R affects its function in a similar manner, multiple oxidizable cysteines are present in this large protein of approximately 2700 amino acids. A confirmation of direct oxidation of the IP_3R protein and its functional consequence must await further site-directed mutagenesis studies.

Nifedipine-sensitivity of an IP_3R -mediated process

One surprising result from this study was that nifedipine, a dihydropyridine VGCC blocker, inhibited the H_2O_2 -induced increase in mIPSC. In fact, in our experiments, nifedipine was as effective an inhibitor as the IP_3R antagonist 2-APB. Nifedipine blocks RyR-sensitive release of stored Ca^{2+} by inhibiting the initial calcium flux through the VGCC required for calcium-induced calcium-release and also blocks the depolarization-evoked Ca^{2+} release via the direct mechanical transduction between L-type VGCC and RyR1. To our knowledge, there is no report of a nifedipine blockade of an IP_3R -mediated process. How can we reconcile this nifedipine-sensitivity with our pharmacological data pointing to the IP_3R -sensitive pool as the source of presynaptic Ca^{2+} ?

Although our whole-cell patch-clamp experiments documented that H_2O_2 did not increase the Ba^{2+} -current through L-type VGCC in our preparation, calcium-induced calcium-release is essentially a local microdomain phenomenon where only Ca^{2+} influx in close proximity to the Ca^{2+} release channel is critical (reviewed in Bouchard *et al.*, 2003). Electrophysiologically undetectable increase in Ca^{2+} influx due to a small depolarization of the membrane potential significantly increased transmitter release (Awatramani *et al.*, 2005). Therefore, a possibility remains that H_2O_2 is increasing Ca^{2+} flux through nifedipine-sensitive VGCC in the vicinity of the IP_3R manifesting as the nifedipine inhibition of the increase in mIPSC. If the effect of H_2O_2 is solely on L-type VGCC in localized microdomain or elsewhere, BayK 8644 should accurately recapitulate the phenomenon. BayK 8644 did increase the mIPSC frequency but this frequency increase was not blocked by 2-APB. This insensitivity of BayK 8644-induced frequency increase to 2-APB is expected as the direct effect of increased presynaptic Ca^{2+} directly acting on the releasable vesicles should not involve the IP_3R .

A direct nifedipine inhibition of the IP_3R must be entertained. The IP_3R consists of an N-terminal ligand binding domain and a C-terminal Ca^{2+} release domain connected by a modulatory domain (reviewed in Joseph, 1996). Hydrophobicity analysis of the C-terminal domain identified 6-transmembrane segments with a hair-pin P-loop structure between transmembrane domains 5 and 6 consistent with the canonical topology of voltage-gated ion channels, including the L-type VGCC. In RyR1, which also demonstrates a structural similarity with VGCC, BayK 8644 can increase the channel open probability but nifedipine did not block the channel (Sagawa *et al.*, 2001). To the best of our knowledge, dihydropyridine sensitivity of the IP_3R at the single channel level has not been explored directly, but given the structural homology between the channel domain of the IP_3R and the VGCC, a direct blocking effect of nifedipine on the IP_3R is a distinct possibility.

Lastly, indirect nifedipine effect at the circuitry-mediated pathways beyond the cell/synapse level directly monitored in this study must be entertained. Spontaneous release of glutamate,

acetylcholine, and other transmitters acting on their respective Gq-coupled metabotropic receptors could regulate the cellular PLC activity and the level of IP₃ (Rhee & Bae, 1997). Nifedipine decrease of the basal IP₃-tone through inhibition of such spontaneous transmitter release within the complex architecture of the slice could indirectly manifest as the observed inhibition of the H₂O₂-induced frequency increase. However, the observation that PLC activity is not required for the H₂O₂-effect, and that nifedipine in itself does not decrease the spontaneous mIPSC frequency argues against the indirect-action of nifedipine via modulation of endogenous transmitter release.

Although the question of how nifedipine inhibits the H₂O₂-induced increase in presynaptic activity remains unresolved, our observations shed some light on the reports that L-type VGCC antagonists inhibited behavioural manifestation of pain in several well-established inflammatory models of chronic pain (Venegas & Schaible, 2000; Gupta *et al.*, 2003) most-likely to be mediated by ROS.

Physiological significance of H₂O₂ release of Ca²⁺ from the IP₃R-sensitive pool

Enhanced probability of presynaptic release of neurotransmitter quanta, as demonstrated by increase in the mIPSC frequency, increases the likelihood of multiquantal release resulting in giant mIPSCs and a larger magnitude of action potential-evoked IPSCs (Llano *et al.*, 2000). In fact, menthol, through activation of presynaptic TRPM8 increased mEPSC frequency in a cocultured DRG-dorsal horn cell synapse and also enhanced action potential dependent excitatory synaptic transmission (Tsuzuki *et al.*, 2004). Therefore, the direct consequence of increased presynaptic activity should manifest itself as an initial enhancement of synaptic transmission. On the other hand, sustained release of the neurotransmitter should result in a presynaptic depletion of releasable vesicles and a long-term decrease in synaptic transmission. Indeed, RyR-sensitive presynaptic Ca²⁺ release was necessary for long-term depression in the hippocampus (Unni *et al.*, 2004).

Prolonged activation of the IP₃Rs could accelerate the establishment of GABAergic inhibitory synaptic depression with a long-term enhancement of the overall excitability. Such a biphasic enhancement at low and depression at high concentrations of H₂O₂ has been observed in widely different synaptic transmissions in the neuromuscular junction (Giniatullin & Giniatullin, 2003) and the hippocampus (Kamsler & Segal, 2003). Whether the presynaptic Ca²⁺ from any stored-pools is coupled to evoked neurotransmitter release under physiological conditions has not been settled (reviewed in Collin *et al.*, 2005), however, our observation that H₂O₂ increases presynaptic activity provides an additional mechanistic framework for the role of this diffusible ROS as a retrograde messenger (Kamsler & Segal, 2004).

In this study, we focused on the effects of H₂O₂ on the presynaptic physiology of GABAergic inhibitory synapses. GABAergic inhibition in the SG originates mostly from the interneurons (Islet cells; Lu & Perl, 2003) providing inhibition to the central cells receiving excitatory inputs from the sensory afferents and to itself via recurrent contacts. Furthermore, the heterogeneity of SG neurons and the actual site of increase in GABAergic inhibition within the local circuit (e.g. inhibition of an inhibitory interneurons and a net dysinhibition could enhance overall excitation) could influence the overall pharmacological effect of H₂O₂. In addition, excitatory synapses in the DRG-spinal cord coculture also demonstrate IP₃R-dependent regulation of TTX-resistant spontaneous events (Tsuzuki *et al.*, 2004) and H₂O₂ is likely to enhance mEPSC in the SG neurons as well. The net effect of enhanced presynaptic release probability at both inhibitory and excitatory synapses on the overall nociceptive transmission in the spinal cord is undoubtedly complex and requires further investigation. Nevertheless, a targeted pharmacotherapy aimed at preventing H₂O₂-induced presynaptic release of stored Ca²⁺ from IP₃R-sensitive pool could preclude the development of intense activity-dependent synaptic plasticity leading to chronic pain states.

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Abbreviations

2-APB, 2-aminoethoxydiphenyl borate; DRG, dorsal root ganglion; GFAP, glial fibrillary acidic protein; H₂O₂, hydrogen peroxide; IP₃R, inositol-(1,4,5) trisphosphate receptor; mEPSC, miniature excitatory post synaptic current; mIPSC, miniature inhibitory postsynaptic current; NeuN, neuronal nuclei; Opt, opisthotonus; PLC, phospholipase C; ROS, reactive oxygen species; RyR, ryanodine receptor; SG, substantia gelatinosa; TRP, transient receptor potential cation channel; TRPC5, transient receptor potential cation channel, subfamily C; TRPM8, transient receptor potential cation channel, subfamily M, member 8; U73122, 1-(6-[[[17β]-3-methoxyestra-1,3,5[10]-trien-17-yl]-amino]hexyl)-1H-pyrrole-2,5-dione; VGCC, voltage-gated calcium current.

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Fig1. H_2O_2 increases the frequency and amplitude of GABAergic mIPSC. (A) Pharmacologically isolated spontaneous mIPSC events obtained from a patch-clamped SG neuron held at 0 mV are seen as upward going spikes. Administration of H_2O_2 (bar, 1 mM) resulted in an increase in the frequency of events. (B) Instantaneous event-frequency plotted as the inverse of the interevent interval for the above trace. Note the large reversible increase in the mIPSC frequency. (C) Cumulative histograms of event amplitude, decay time, and frequency for the cell shown above. Reversible and significant shifts in the distributions of amplitude and frequency were observed. Solid line (control), dashed line (during H_2O_2), and dotted line (wash out) for all panels. *P*-values from Kolmogorov–Smirnov test. (D) A summary histogram

showing H_2O_2 concentration-dependent effect on event amplitude, decay time, and frequency. Addition of extracellular catalase (250 U/mL) abolished the H_2O_2 -induced increase in the event amplitude and frequency but the inclusion of glutathione (GSH; 5 mM) inside the patch pipette only inhibited the H_2O_2 effect on amplitude. The ordinate is fold change from control so 0 represents no change from control. Number of cells is denoted in parentheses and the *P*-value from Mann–Whitney test.

Fig 2. Release of IP_3 sensitive stored- Ca^{2+} mediates the H_2O_2 -induced increase in event frequency. (A) A summary diagram of the potential sites of action of H_2O_2 leading to the increase in mIPSC frequency. $G\alpha$ (G-protein α -subunit), $G\beta\gamma$ (G-protein $\beta\gamma$ -subunits), $PLC\beta$ (phospholipase C- β), PIP_2 (phosphoinositide bisphosphate), IP_3 (inositol trisphosphate), DAG (diacylglycerol), VGCC (voltage gated calcium channel), IP_3R (IP_3 receptor), Mito (mitochondria), RyR (ryanodine receptor). (B) A summary bar plot of the effect of the indicated drugs on the H_2O_2 (500 μM)-induced increase in mIPSC. Thap (thapsigargin, 2 μM), CPA (cyclopiazonic acid, 10 μM), CSA (cyclosporin A, 10 μM), Rya (ryanodine, 10 μM), 2-APB (2-aminoethoxydiphenyl borate, 200 μM). The baseline mIPSC frequency for this series was 0.14

–0.48 Hz ($n = 37$ neurons) with the modulator drug pretreatment alone having no statistically significant effect except for thapsigargin where the basal frequency increased from a control of 0.15 ± 0.05 to 0.29 ± 0.09 Hz ($P < 0.05$ by Mann–Whitney test). (C) Same experiment but with the PLC inhibitor, U73122 ($10 \mu\text{M}$), or the L-type VGCC inhibitor, nifedipine ($10 \mu\text{M}$). (D) A summary bar plot for U73122 and nifedipine. Note that the nifedipine inhibition of the H_2O_2 effect was reversible as denoted by the nifedipine wash bar. Neither U73122 nor nifedipine had a significant effect ($P > 0.5$ for both drugs) on the basal frequency (0.23 ± 0.08 (control) to 0.28 ± 0.10 (with U73122) Hz, $n = 6$; 0.19 ± 0.09 (control) to 0.21 ± 0.08 (with nifedipine) Hz, $n = 10$). Number of cells indicated in parentheses and * denotes statistical significance at $P < 0.05$ by Mann–Whitney test.

Fig 3.

Acute administration of H₂O₂ does not increase Ba²⁺ influx through the L-type Ca²⁺ channel. (A) Traces of Ba²⁺ current from a SG neuron voltage clamped at -50 mV and stepping to various depolarized potentials by 10 mV increments. After recording in the control 10 mM Ba²⁺ ACSF solution, the same cell was sequentially exposed to Bay K8644 (5 μM) and nifedipine (10 μM). The resulting current-voltage relationship is plotted to the right. (B) The same condition except the currents were evoked every 20 s by a -50 to -10 mV step. BayK 8644 increased the peak current to $145.5 \pm 8.7\%$ of control ($n = 3$, $P < 0.01$, Mann-Whitney test) while H₂O₂ had no effect $101.8 \pm 3.5\%$ of control ($n = 7$, $P > 0.5$, Mann-Whitney test). (C) A raw GABAergic mIPSC current trace obtained as in Fig. 1 but during BayK 8644

application. (D) Analysis of the above trace presented as cumulative fraction for the event amplitude, decay time, and interevent interval for control (solid), BayK 8644 (dashed), and recovery (dotted) lines. (E) A bar plot summary of the effect of 2-APB (200 μM) on BayK 8644 (5 μM)-induced changes in mIPSC parameters ($n = 4$ for both).

Fig4.

IP₃R expression in the spinal cord. Phase contrast, with arrows pointing to the Purkinje cells (A1), NeuN (A2), and IP₃R1 (A3) views of a 10 μm cerebellar frozen section. Note the absence of NeuN immunoreactivity in the Purkinje cells. (Scale bar, 20 μm.) (B1) A low magnification phase contrast view of a 250-μm-thick spinal cord section. An arrow pointing to the small box on the dorsal horn outlines the approximate area viewed under the confocal microscope (B2) (Scale bar, 9.5 μm). The section was coimmunostained with either anti-NeuN (green) or anti-GFAP (blue) as neuronal or astrocytic markers, respectively. (C). Spinal cord section immunostained with NeuN (green) and IP₃R1 (red). The images are confocal low magnification view (1) (Scale bar, 60 μm), a selected high magnification view (2, 3), and an overlap of the two channels (4) (Scale bar, 12 μm.)

Fig5. H₂O₂-induced increase in mIPSC is abolished in the heterozygous opisthotonus mutant mice. (A, top) PCR genotyping of a representative litter [wild-type (lanes 1, 3 and 7), heterozygote (lanes 2, 4, 5 and 8), and homozygote (lane 6)] demonstrating the *Opt*-specific 400-bp product and a smaller 178-bp product common to both the wild-type and *Opt*-allele. (Bottom) An RT-PCR confirmation of the expected expression of IP₃R1 mRNA in the spinal cord of *Opt*(-/-), *Opt*(+/-) and wild-type (+/+) littermate control. The RT(-) control was on the wild-type (+/+) spinal cord mRNA. (B) mIPSC frequency vs. time during application of 500 μ M H₂O₂ in *Opt*(+/-) (left) and wild-type (+/+) littermate control (right). (C) A bar plot summary of the mIPSC properties during 500 μ M H₂O₂ application. * denotes $P < 0.01$ by Mann-Whitney test.