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ORIGINAL ARTICLES

Glutathione Modulates Ca^{2+} Influx and Oxidative Toxicity Through TRPM2 Channel in Rat Dorsal Root Ganglion Neurons

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Abstract Glutathione (GSH) is the most abundant thiol antioxidant in mammalian cells and maintains thiol redox in the cells. GSH depletion has been implicated in the neurobiology of sensory neurons. Because the mechanisms that lead to melastatin-like transient receptor potential 2 (TRPM2) channel activation/inhibition in response to glutathione depletion and 2-aminoethyldiphenyl borinate (2-APB) administration are not understood, we tested the effects of 2-APB and GSH on oxidative stress and buthionine sulfoximine (BSO)-induced TRPM2 cation channel currents in dorsal root ganglion (DRG) neurons of rats. DRG neurons were freshly isolated from rats and the neurons were incubated for 24 h with BSO. In whole-cell patch clamp experiments, TRPM2 currents in the rat were consistently induced by H_2O_2 or BSO. TRPM2 channels current densities and cytosolic free Ca^{2+} content of the neurons were higher in BSO and H_2O_2 groups than in control. However, the current densities and cytosolic Ca^{2+} release were also higher in the BSO + H_2O_2 group than in the H_2O_2 alone. When intracellular GSH is introduced by pipette TRPM2 channel currents were not activated by BSO, H_2O_2 or rotenone. BSO and H_2O_2-induced Ca^{2+} gates were blocked by the 2-APB. Glutathione peroxidase activity, lipid peroxidation and GSH levels in the DRG neurons were also modulated by GSH and 2-APB inhibition. In conclusion, we observed the protective role of 2-APB and GSH on Ca^{2+} influx through a TRPM2 channel in intracellular GSH depleted DRG neurons. Since cytosolic glutathione depletion is a common feature of neuropathic pain and diseases of sensory neuron, our findings are relevant to the etiology of neuropathology in DRG neurons.

Keywords Buthionine sulfoximine · Glutathione · Oxidative stress · Sensory neurons · TRPM2 channel antagonist

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Neuropathic pain states severely limit the quality of life. There are several types of sensory neurons in dorsal root ganglion (DRG) neurons with responsiveness to different kind of external and internal stimuli. These stimuli such as nociceptive, thermal and mechanical activate different receptors and ion channels that are present in the nerve terminals at the sensory receptive fields. Their expression in selective subsets of DRG neurons determines the response profile of individual neurons to a given stimulus (Staaf et al. 2010). Transient receptor potential (TRP) channels are a group of nonselective cation channels that have important functions in sensory neurons (Nazıroğlu 2011). One subgroup of TRP melastatin is TRP melastatin 2 (TRPM2). The TRPM2 channel protein has two distinct domains with one functioning as an ion channel and the other as an ADP-ribose (ADPR)-specific pyrophosphatase (Fonfria et al. 2005). The TRPM2 channel is also a redox-sensitive Ca^{2+}-permeable cation channel, and the Ca^{2+} influx through TRPM2 induced by H_2O_2 mediates necrotic cell death (Ishii et al. 2006). The channel in neuronal cells such as rat striatal neurons (Fonfria et al. 2005) and organotypic hippocampal culture (Bai and Lipski 2010) can also be gated by oxidative stress.

TRP channels can be indirectly blocked by chemicals. However, for most of TRP channels including TRPM2, the range of pharmacological modulators of TRPM2 is limited. Recently, 2-aminoethyldiphenyl borinate (2-APB) was

described as a TRPM2 channel blocker in addition to its actions as an inositol 1,4,5-triphosphatase ($InsP_3$) receptor antagonist (Togashi et al. 2008). However, reports of the blocking actions of 2-APB on the TRPM2 channel in different neuronal cell systems are conflicting (Nazıroğlu 2011).

Reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2) act as intracellular messengers in such complex processes as mitogenic signal transduction, gene expression, and regulation of cell proliferation when they are generated excessively or antioxidant defense systems are impaired (Nazıroğlu 2007). ROS are able to increase second messengers such as Ca^{2+} (Grupe et al. 2010), and activate signaling kinases such as extracellular signal-regulated kinase (Halliwell 2006). There is also evidence that ROS play an important role in the pathogenesis of many diseases, particularly in neurological diseases because of the susceptibility of neurological cells to oxidative stress (Nazıroğlu 2011). The toxic properties of oxidative stress with regard to the function of TRPM2 channels in DRG, however, still remain unknown.

GSH is the most abundant thiol antioxidant in mammalian cells. Reduced glutathione (GSH) is an endogenous tripeptide that acts both as a nucleophilic scavenger of numerous compounds and as a substrate in the selenium dependent GSH peroxidase (GSH-Px) mediated destruction of hydroperoxides (Nazıroğlu 2009). The intracellular GSH levels are down-regulated by DL-buthionine-(S,R)-sulfoximine (BSO) via inactivation of γ-glutamylcysteine synthetase, the rate limiting enzyme in GSH synthesis (Anderson 1998). BSO is the most specific pharmacological tool known to deplete GSH (Whanger 2001). GSH has been recognized as a highly effective antioxidant blocking the cytotoxic activity of ROS (Anderson 1998). Intracellular derived ROS can also active TRPM2 channels (Nazıroğlu 2007) and GSH protected against neuronal degeneration in DRG neurons after incubation of BSO. Thus, antioxidants may be useful as therapeutic drugs for sensory neuron dependent diseases (Cabungcal et al. 2007). To date, the endogenous protective mechanism against TRPM2 channel-induced DRG neuron apoptosis has not been determined in DRG neurons. Because TRPM2 channels are permeable to Ca^{2+} and have previously been implicated in other neurodegenerative disorders (Nazıroğlu 2011), activation of these channels is a potentially important mechanism that may contribute to the pathogenesis of DRG pain and diseases in DRG neurons.

The molecular mechanism by which oxidative stress and cytosolic GSH depletion lead to gating of TRPM2 channels in DRG neurons remains to be elucidated in detail. To study the role of glutathione and oxidative stress in TRPM2 channels we used an experimental model in which BSO was applied to freshly isolated DRG neurons. Furthermore, the present study was aimed at elucidating the role of 2-APB in modulation of the effects of BSO-induced gate on TRPM2 channels. The study strongly indicates the usefulness of GSH in prevention of sensory neurons neuropathy and pain, as well as targeting secondary mechanisms of regulation of the innate antioxidant response and inhibition of cytosolic Ca^{2+} increase through TRPM2 channels.

Materials and Methods

Preparation of Cell Samples

We used 6 male Wistar rat (aged 12–14 weeks old) in the current study. The study was approved by the Local Experimental Animal Ethical Committee of Süleyman Demirel University (SDU) (protocol number 2010-2-13). The animals were killed by ether asphyxiation and cervical dislocation in accordance with SDU Experimental Animal legislation. Briefly, lumbar dorsal ganglia (DRG, T13–L5) were harvested from the rats. Ganglia were transferred into Dulbecco's modified Eagle's medium (DMEM, Gibco, Istanbul, Turkey) supplemented with 1% penicillin–streptomycin (Sigma, Istanbul, Turkey) in 500 ml of DMEM. The connective tissue was removed and ganglia were treated with collegenase IV (0.28 ml in DMEM), and trypsin (25,000 units/ml in DMEM for 45 min at 37°C and in an atmosphere containing 5% CO_2). After dissociation with a sterile syringe, the cell suspension was centrifuged at 3,500 rpm. 10% of the neurons were seeded on a patch-clamp chamber and electrophysiological studies were carried out 2–3 h after harvesting of the cells. The remaining portions of cells were used for Ca^{2+} signaling experiments and oxidant/antioxidant analyses.

Electrophysiology

Patch clamp techniques have been described in detail elsewhere (Nazıroğlu and Lückhoff 2008a, b). DRG neurons were studied with the patch-clamp technique in the whole-cell mode, using an EPC 10 equipped with a personal computer with Patchmaster software (Heka, Lamprecht, Germany). Pipettes were made of borosilicate glass (Sutter Instrument Borosilicate Glass with filament. O.D.: 1.5 mm., I.D. 0.86 mm and 10 cm in length, Novato, CA, USA). The standard extracellular bath solution contained (in mM): 140 NaCl, 1.2 $MgCl_2$, 1.2 $CaCl_2$, 5 KCl, 10 HEPES, with the pH adjusted with KOH to 7.4. For Na^+ free solutions, Na^+ was replaced by 150 mM NMDG (*N*-methyl-D-glucamine) and the pH was adjusted with HCl. The osmolarity of the solution was 310 mosmol/l. The pipette solution contained in mM: 145 cesium glutamate, 8 NaCl, 10 EGTA, 2 $MgCl_2$

and 10 HEPES (pH 7.2) (adjusted with CsOH). The calcium concentration was adjusted to 1 μM.

Cells were held at a potential of −60 mV, and current–voltage relationships were obtained from voltage ramps from −90 to +60 mV applied over 400 ms. Stock 2-APB and FFA were dissolved in dimethyl sulfoxide and stored at −33°C. Before the experiment, GSH (2 mM) in internal buffer, FFA (0.1 mM), and 2-APB (0.05 mM) in extracellular bath solutions were diluted to reach the final concentrations. For rat studies, 2-APB and FFA were diluted in extracellular solution. All experiments were carried out at room temperature (approximately 20°C). After addition of FFA and 2-APB to standard extracellular bath solution, the pH values of these solutions were adjusted with KOH to 7.4. The FFA and 2-APB were added to the patch chamber (in the bath).

Study Groups for Oxidant/Antioxidant and Cytosolic Ca^{2+} Release Analyses in DRG Neurons

For oxidant/antioxidant and Ca^{2+} release analyses, isolated DRG neurons were obtained from 6 adult Wistar male rats (12–14 weeks). DRG neurons of each animal were divided into four groups as follows: Group I ($n = 6$) was a control group and were incubated (37°C and 5% CO_2) for 24 h with normal medium. Group II ($n = 6$) was a BSO treated group. The DRG neurons were incubated with BSO (0.5 mM) for 24 h. Group III ($n = 6$) was a BSO + GSH group. The DRG neurons were preincubated with BSO (0.5 mM) for 24 h and then they were incubated with GSH (2 mM) for 2 h. Group IV ($n = 6$) was a BSO + 2-APB group. The DRG neurons were preincubated with BSO (0.5 mM) for 24 h and then they were incubated with 2-APB for 1 min.

At the end of the experiments, half of the DRG samples were immediately used for Ca^{2+} signaling analyses. The remaining neurons were washed with phosphate buffer (pH 7.2) and then frozen at −33°C. GSH, GSH-Px and lipid peroxidation analyses were performed within 1 week.

Measurement of Intracellular Free Calcium Concentration ($[Ca^{2+}]_i$)

DRG neurons were loaded with 4 μM fura-2/AM in loading buffer with 1×10^5 cells per ml for 45 min at 37°C in the dark, washed twice with phosphate buffer then incubated for an additional 30 min at 37°C to complete probe de-esterification, and resuspended in loading buffer at a density of 1×10^5 cells per ml according to a procedure published elsewhere (Uğuz et al. 2009). The four groups were exposed to H_2O_2 for stimulation of ($[Ca^{2+}]_i$) release. Fluorescence was recorded from 2 ml aliquots of magnetically stirred cellular suspension at 37°C by using a spectrofluorometer (Carry Eclipse, Varian Inc, Sydney, Australia) with excitation wavelengths of 340 and 380 nm and emission at 505 nm. Changes in $[Ca^{2+}]_i$ were monitored by using the fura-2 340/380 nm fluorescence ratio and were calibrated according to the method of Grynkiewicz et al. (1985).

Ca^{2+} release in the DRG neuron was estimated using the integral of the rise in $[Ca^{2+}]_i$ for 150 s after addition of H_2O_2 (Espino et al. 2009; Uğuz et al. 2009). Ca^{2+} release is expressed in nanomolar quantities taking a sample every second as previously described (Salazar et al. 2008a, b).

Lipid Peroxidation (LP), Reduced Glutathione (GSH), Glutathione Peroxidase (GSH-Px), and Protein Assay

LP levels in the DRG neurons were measured with the thiobarbituric acid reaction by the method of Placer et al. (1966). The values of LP in the neurons were expressed as μmol/g protein.

The GSH content of the DRG neurons was measured at 412 nm using the method of Sedlak and Lindsay (1968) as described in our previous study (Nazıroğlu et al. 2008). GSH-Px activities of the DRG neurons were measured spectrophotometrically (UV-1800, Schimadzu, Kyoto, Japan) at 37°C and 412 nm according to the method of Lawrence and Burk (1976). The protein content in the DRG neurons was measured by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Statistical Analyses

All results were expressed as means ± SD. Significance in three groups was first checked by ANOVA–Kruskal–Wallis test. Then significant values in three groups were assessed with an unpaired Mann–Whitney *U*-test. Data were analyzed by SPSS software, version 9.05 (SPSS, Chicago, IL, USA). *P*-values of less than 0.05 were regarded as significant.

Results

Effects of GSH on TRPM2 Channels Activated by BSO

The TRPM2 channels expressed in rat DRG neurons were studied with the whole-cell patch-clamp technique. The channels were stimulated by H_2O_2 applied to the cytosolic side of the channels by diffusion from the patch chamber because lower concentrations of extracellular H_2O_2 were not able to activate TRPM2 channels in transfected cells

(Nazıroğlu and Lückhoff 2008a, b; Kühn et al. 2009). H_2O_2 activated an inward current in rat DRG neurons. Representative experiments are shown in Figs. 1 and 2. Currents induced by H_2O_2 developed gradually during supplementation of H_2O_2 into the cells and reached amplitudes (at a holding potential of −60 mV) of well above 0.51 nA in the inward direction. These currents were reversibly blocked by 2-APB and NMDG; which abolished the inward and outward component of the currents (Figs. 1b, 2b). No currents were seen in the absence of H_2O_2 (Figs. 1a, 2a). The mean values for the current densities in the absence of H_2O_2 in control rat DRG neurons was 2.5 pA/pF ($n = 12$) (−60 mV holding potential). The mean values of the current densities in H_2O_2, H_2O_2 + GSH groups as pA/pF were 75.6 ($n = 9$) and 5.6 ($n = 6$), respectively. Current densities of the DRG neurons were significantly ($P < 0.001$) higher in the H_2O_2 group than in the control group (Fig. 3).

Effects of Intracellular GSH on TRPM2 Channels Activated by BSO

To corroborate the importance of intracellular GSH decrease for activation of TRPM2 channels, control assays were carried out using BSO, a selective inhibitor of γ-glutamylcysteine synthetase, the rate-limiting enzyme for

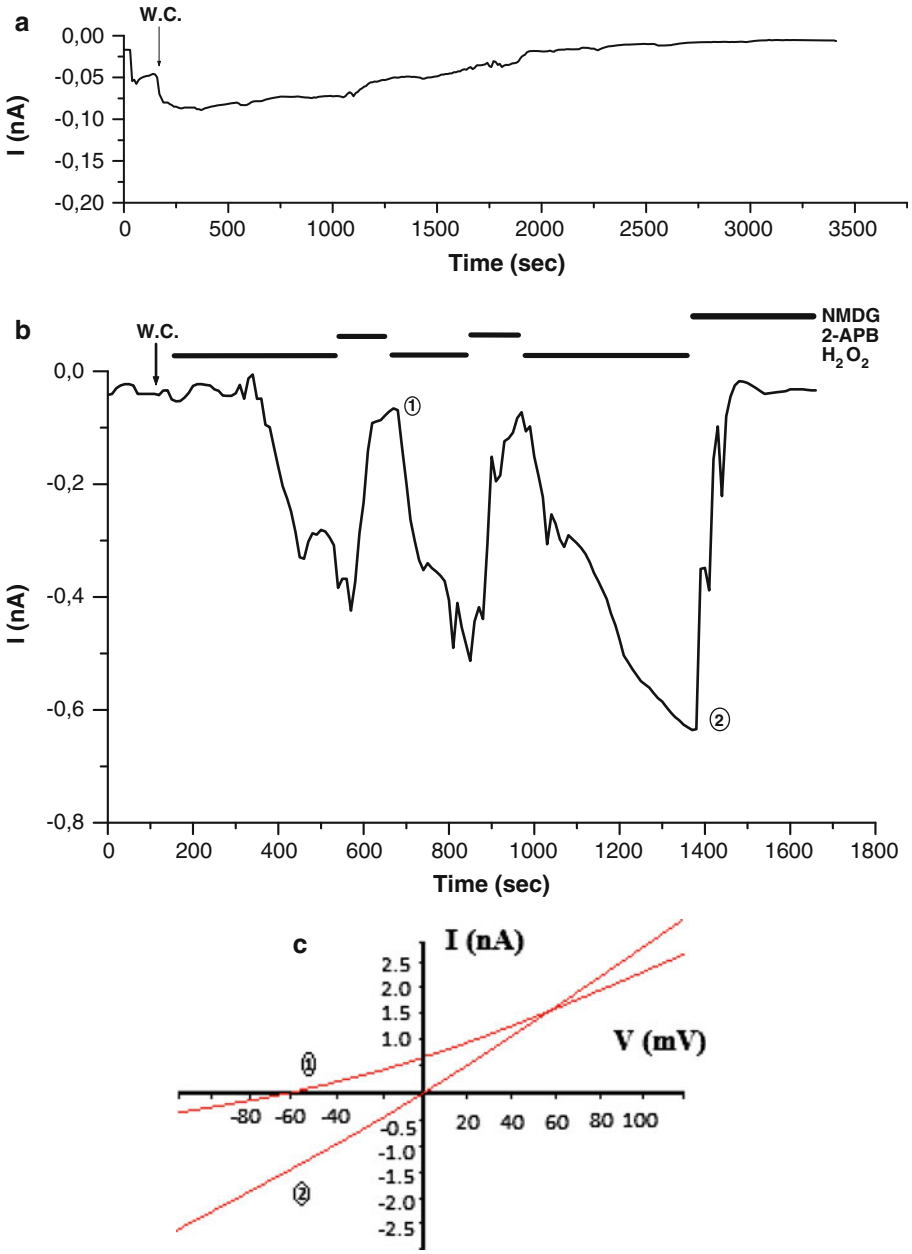

Fig. 1 Effects of extracellular 2-APB on H_2O_2-induced TRPM2 channel activation in DRG neurons. The holding potential was −60 mV. **a** Original recordings from control cell. **b** Cell expressing TRPM2 currents stimulated by H_2O_2 in the bath (chamber) and they were inhibited by 2-APB in bath. **c** Current voltage relationships of 2-APB and ADPR through TRPM2 channel in presence of various extracellular cations (same experiments as in **b**). *W.C.* whole cell

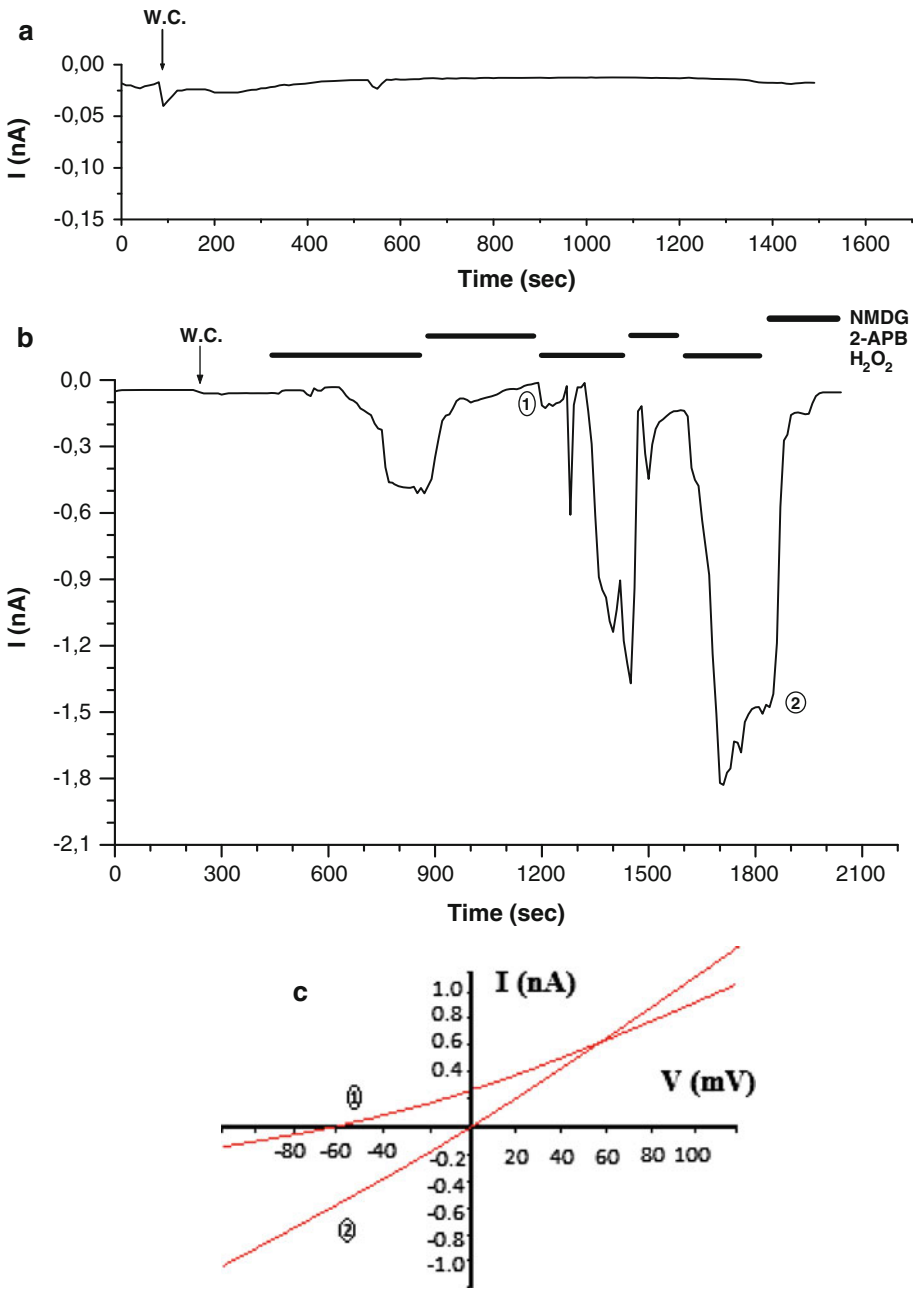

Fig. 2 Effects of 2-APB on H_2O_2 and BSO-induced TRPM2 channel activation in DRG neurons. **a** Original recordings from control cell. **b** Cell expressing TRPM2 currents stimulated by H_2O_2 in the pipette and they were inhibited by 2-APB in bath (chamber). **c** Current voltage relationships of 2-APB and ADPR currents through TRPM2 in presence of various extracellular cations (same experiments as in **b**)

GSH biosynthesis. It was observed that TRPM2 channels in DRG neurons were activated by incubation with BSO via a decrease in intracellular GSH levels and TRPM2 channels seems activate intracellular ROS source mostly (Fig. 2b).

Currents induced by BSO developed gradually during supplementation of H_2O_2 into the cells and reached amplitudes (at a holding potential of −60 mV) of well above 1.2 nA in the inward direction. These currents were reversibly blocked by 2-APB and NMDG; which abolished the inward and outward component of the currents (Fig. 2b). No currents were seen in the absence of BSO (Fig. 2a). The values for the current densities in H_2O_2 (the absence of BSO), BSO + H_2O_2 and BSO + GSH + H_2O_2 groups as pA/pF were 74.5 ($n = 9$), 172.2 ($n = 8$) and 5.6 ($n = 4$) respectively.

There is a protective effect of BSO on gating of the TRPM2 channels in the neuronal cell system and current density in the neurons was significantly ($P < 0.001$) higher in the BSO + H_2O_2 group than in the control. However, the current density was significantly lower in the BSO + H_2O_2 + GSH group than in the BSO + H_2O_2 and H_2O_2 groups (Fig. 3). Control data were obtained on every experimental day using the same animals as those used for studying TRPM2. The data with BSO support the idea that BSO acts by initiating a metabolic cascade resulting in the production of a cytosolic factor such as oxidative stress

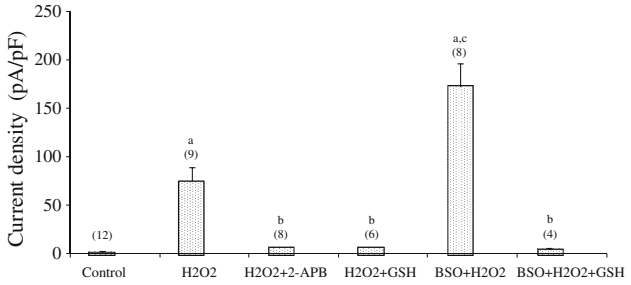

Fig. 3 Effects of glutathione (GSH and in pipette) and 2-APB (in bath) on H_2O_2 (in bath) and BSO (24 h incubation)-induced currents of TRPM2 channel in DRG neuronal cells. For each of the four applications studied in the cells, the initial current density, divided by the cell capacitance (which is a measure of cell size) was recorded as well as maximal current density after administration of H_2O_2 and BSO. The numbers in parentheses indicate numbers of groups. Significant ($^aP < 0.001$ vs. control, $^bP < 0.001$ vs. BSO + H_2O_2 groups, $^cP < 0.001$ vs. H_2O_2) stimulation and inhibition of currents are indicated with an *asterisk* (mean ± SD)

(inhibition of γ-glutamylcysteine synthetase) and that is responsible for the activation of TRPM2 channel activity. Taken together, these results suggest that GSH inhibitors potentiate oxidative stress-provoked Ca^{2+} influx through TRPM2 channel activation by down regulating of intracellular GSH.

Rotenone is a naturally occurring isoflavonoid from the tropical plants *Lonchocarpus* and *Derris*. Rotenone is an intracellular oxidative producer through intracellular ATP depletion and inhibition of mitochondrial complex I enzymes (Bove et al. 2005). It also releases glutamate from presynaptic terminals, leading to an additional increase in ROS production (Freestone et al. 2009). Rotenone gated the TRPM2 channels in the rat DRG neurons (Nazıroğlu et al. 2011). In presence of intracellular GSH, even rotenone as a strong intracellular ROS producer was not able to gate the TRPM2 channels (Fig. 4b).

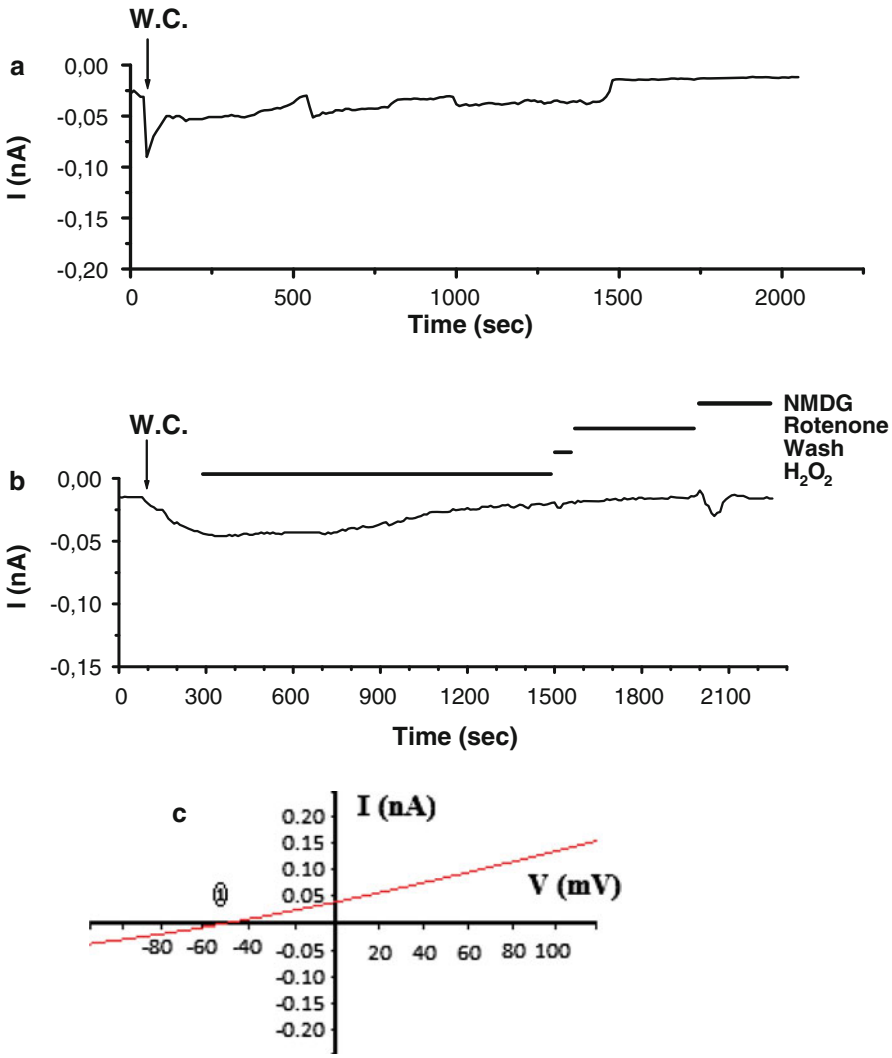

Fig. 4 Protective effects of intracellular GSH on BSO, H_2O_2 or rotenone-induced TRPM2 channel activation in DRG neurons. The holding potential was −60 mV. **a** Original recordings from control cell. **b** Cell expressing TRPM2 currents were not able to stimulate by rotenone and H_2O_2 in bath (chamber) when GSH is present in intracellular buffer (pipette). **c** Same experiments as in (**b**) and current voltage relationships of GSH or H_2O_2 or rotenone are same. *W.C.* whole cell

Effects of TRPM2 Antagonist in DRG Neurons

We next tested whether the extracellular FFA and 2-APB would prevent or attenuate the induction of TRPM2 currents by BSO. First we tested 0.1 and 0.2 mM concentrations of extracellular FFA in the neurons. Unexpectedly, H_2O_2- or BSO-induced Ca^{2+} currents were not inhibited by FFA administration (data not shown).

We attempted to examine the effects of 2-APB (0.05 mM) on the H_2O_2 and BSO-induced TRPM2 currents. The 2-APB brought about an inhibition of H_2O_2, BSO and ADPR-induced TRPM2 currents ($P < 0.001$). The results of the rat experiments are presented in Fig. 4b in which only cells are included. In all cases, the currents were also blocked by substitution of NMDG for Ca^{2+} and Na^+. There were statistically significant differences ($P < 0.001$) between BSO and BSO + 2-APB, H_2O_2 and H_2O_2 + 2-APB (Fig. 3).

The time course of effects in control and BSO groups were 2.66 ± 0.28 and 2.70 ± 0.21 min, respectively. There was no statistical significance on gating time between control and BSO-treated neurons.

Effects of Extracellular GSH and 2-APB on $[Ca^{2+}]_i$ Release in DRG Neurons

Effects of GSH and 2-APB on $[Ca^{2+}]_i$ release in the DRG neurons are shown in Fig. 5. Intracellular $[Ca^{2+}]_i$ release in the neurons was significantly ($P < 0.001$) higher in the BSO + H_2O_2 group than in control. The $[Ca^{2+}]_i$ release in the neurons was significantly ($P < 0.001$) lower in the BSO + H_2O_2 + GSH group than in the BSO + H_2O_2 group. Hence we found that GSH possessed protective effects against oxidative stress-induced $[Ca^{2+}]_i$ release. The $[Ca^{2+}]_i$ release in the DRG neurons was also significantly ($P < 0.001$) lower in the 2-APB group than in the control. Hence we found that TRPM2 channel blocker 2-APB could be protective against H_2O_2-induced $[Ca^{2+}]_i$ release in the BSO + 2-ABP group. However, the $[Ca^{2+}]_i$ release in the neurons was significantly ($P < 0.05$) higher in the BSO + H_2O_2 + 2-APB group than in the BSO + H_2O_2 + GSH group.

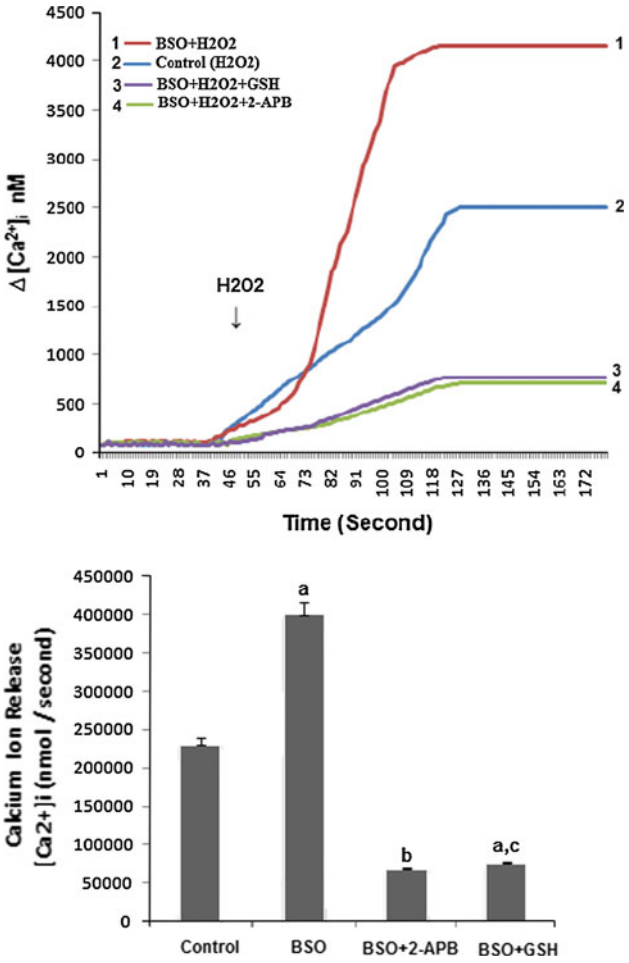

Fig. 5 Effects of BSO and GSH treatments on intracellular Ca^{2+} release in their effects on intracellular Ca^{2+} concentrations in DRG neurons. Extracellular buffer was containing Stimulation was performed by H_2O_2 (100 μM). [a] $P < 0.001$ vs. control; [b] $P < 0.001$ vs. BSO group; [c] $P < 0.05$ vs. BSO + GSH group

Effects of Extracellular GSH and 2-APB on GSH-Px, GSH, and Lipid Peroxidation Values in DRG Neurons

Effects of GSH and 2-APB on GSH-Px, GSH and lipid peroxidation values in DRG neurons are shown in Table 1. Lipid peroxidation levels were significantly ($P < 0.01$)

Table 1 Effects of reduced glutathione (GSH) and 2-APB on rat brain cortex glutathione peroxidase (GSH-Px) activity, GSH, and lipid peroxidation levels in cytosolic GSH-depleted DRG neurons after BSO treatment (mean ± SD)

Parameter	Control ($n = 6$)	BSO ($n = 6$)	BSO + GSH ($n = 6$)	BSO + 2-APB ($n = 6$)
LP (μmol/g protein)	1.73 ± 0.13	2.05 ± 0.39^a	1.74 ± 0.44^c	1.80 ± 0.35^c
GSH (μmol/g protein)	4.69 ± 0.24	3.76 ± 0.64^a	$6.27 \pm 0.49^{b,d}$	$4.81 \pm 0.88^{c,e}$
GSH-Px (IU/g protein)	8.12 ± 0.87	6.68 ± 0.74^b	8.23 ± 0.79^d	$10.10 \pm 0.65^{d,e}$

[a] $P < 0.05$ and [b] $P < 0.001$ vs. control

[c] $P < 0.01$ and [d] $P < 0.001$ vs. BSO group

[e] $P < 0.01$ vs. BSO + GSH group

higher in the BSO group than in controls although Lipid peroxidation levels were significantly ($P < 0.01$) lower in the BSO + GSH and BSO + 2-APB groups than in the BSO group. Hence we found that GSH and 2-APB were protective against oxidative stress-induced lipid peroxidation levels in the neuronal cells.

GSH ($P < 0.05$) level and GSH-Px ($P < 0.01$) activity were significantly lower in the BSO group than in the control. However, GSH and GSH-Px values were increased by GSH incubation and GSH ($P < 0.05$) and GSH-Px ($P < 0.01$) values were significantly higher in the BSO + GSH ($P < 0.001$) and BSO + 2-APB ($P < 0.001$) groups than in the BSO alone groups. Hence, we found that 2-APB and GSH induced antioxidant effects against oxidative stress in the DRG neurons.

Discussion

On a search for biological plausibility of GSH deficit as a vulnerability factor in diseases of sensory neurons, we investigated the consequences on Ca^{2+} influx through TRPM2 channels in the sensory neurons because GSH deficit is observed in the diseases (Cabungcal et al. 2007). For this purpose, we induced a GSH deficit in rat DRG neurons with BSO, a specific inhibitor of rate limiting enzyme for GSH synthesis, γ-glutamyl-cysteine ligase (Anderson 1998). We found that the Ca^{2+} gate through TRPM2 channels was increased in the DRG neurons after BSO incubation. To our knowledge, there is no report on affects of BSO in TRPM2 channels of DRG neurons. In the current study, BSO-induced Ca^{2+} gating via the TRPM2 channel was blocked by both 2-APB and GSH administration. Therefore, the intracellular pathways may be involved in Ca^{2+} gating by the intracellular GSH depletion. We observed that GSH depletion-induced Ca^{2+} entry through TRPM2 is inhibited by 2-APB using a cell system. Therefore, in this study, we believe that cytosolic GSH levels act as a TRPM2 channel regulator in diseases of DRG neurons and sensory neurons.

There has been growing interest in the TRPM2 channel in neuronal damage resulting from oxidative stress (Nazıroğlu 2011). Our results also indicate that Ca^{2+} release through TRPM2 channel activation are increased by ROS during exposure of DRG neurons to BSO although the activation and cytosolic Ca^{2+} release were inhibited by 2-APB. The evidence for this mechanism is based on the following observations: (i) BSO induced a fast rise in free cytosolic Ca^{2+}; and (ii) the rise in free cytosolic Ca^{2+} was significantly reduced by a TRPM2 channel blocker, 2-APB (Togashi et al. 2008; Nazıroğlu et al. 2011).

TRPM2 contains a characteristic structural feature known as a Nudix domain in its C-terminal cytosolic tail (Nazıroğlu 2007). A Nudix domain is a consensus region that is known to be present in a class of pyrophosphatases that degrade nucleoside diphosphates (Clapham 2007). Wehage et al. (2002) reported that oxidative stress (H_2O_2) evokes Ca^{2+} influx by increasing ADPR levels and by subsequent binding of NAD^+ directly to the Nudix motif in the cytosolic C terminal of TRPM2. TRPM2 is also known to respond to intracellular ADPR, a metabolite of NAD^+, via direct binding to the Nudix domain (Hara et al. 2002; Wehage et al. 2002). Since BSO is a strong oxidant, it is possible that intracellular ROS can oxidize NADH to NAD^+ in BSO-treated DRG neurons.

Although the precise mechanism by which GSH protects neurons from cell death is unknown, it is widely accepted to be attributable to its antioxidant properties as well as its elevation of GSH and GSH-Px values. Sulfhydryl-containing reductants such as GSH have the capacity to remove H_2O_2, hydroxyl radicals and superoxide anions by acting as radical scavengers (Whanger 2001). Elimination of these protective agents allows H_2O_2 to exert cytotoxic effects (Shimizu et al. 2002). Reaction of superoxide or H_2O_2 with sulfhydryl compounds results in rapid oxidation of these reducing agents to disulfides. Superoxide oxidizes sulfhydryl groups and is reduced to H_2O_2 in the process, and which then can also oxidize the reductants.

Cytosolic Ca^{2+} has been presented as a key regulator of cell survival but this ion can also induce apoptosis in response to a number of pathological conditions (Nazıroğlu 2009). In addition, the mitochondria act as Ca^{2+} buffers by sequestering excess Ca^{2+} from the cytosol (Hajnóczky et al. 2006). Ca^{2+} mobilizing agonists can effectively produce a rapid, simultaneous and reversible cessation of the movements of both ER and mitochondria, which is strictly dependent on a rise in $[Ca^{2+}]_i$. This inhibition in mitochondrial motility reflects an increased mitochondrial Ca^{2+} uptake and thus enhances the local Ca^{2+} buffering capacities of mitochondria, with important consequences in signal transduction (Bubber et al. 2004). Ca^{2+} overloading of mitochondria can induce an apoptotic program by stimulating the release of apoptosis promoting factors such as cytochrome c, and by generating ROS as a result of respiratory chain damage (Hajnóczky et al. 2006; Salazar et al. 2008b). Furthermore, mitochondria have been found to play a pivotal role in Ca^{2+} signaling (Salazar et al. 2008a; Uğuz et al. 2009). In fact, the release of Ca^{2+} from ER stores by IP3 receptors has been implicated in multiple models of apoptosis by being directly responsible for mitochondrial Ca^{2+} overload (Putney and McKay 1999; Bubber et al. 2004). Stored Ca^{2+} is crucial for a number of cellular functions, including signal transduction cascades that

respond to stress conditions (Nazıroğlu 2007). We provided compelling evidence to support the belief that mitochondrial Ca^{2+} uptake evoked by rises in $[Ca^{2+}]_i$, induces mitochondrial membrane depolarization. Our results indicated that the blockade of both Ca^{2+} uptake into mitochondria with GSH and 2-APB or rises in $[Ca^{2+}]_i$ were able to decrease lipid peroxidation mediated by H_2O_2, which was able to block Ca^{2+} release from intracellular stores.

Bao et al. (2005) described the involvement of TRP channels in rotenone-induced cell membrane depolarization and a decrease in R_m in medium spiny neurons of the striatum. These effects were abolished by FFA. Freestone et al. (2009) reported that TRPM2 or TRPM2-like channels are activated by ROS during exposure of substantia nigra pars compacta neurons to rotenone. They observed also that *N*-(*p*-amylcinnamoyl)anthranilic acid (ACA) and FFA did not change resting $[Ca^{2+}]_i$ concentration or any other measured membrane property. ACA, however, attenuated a rotenone-induced $[Ca^{2+}]_i$ concentration increase while FFA induced a tendency to decrease the $[Ca^{2+}]_i$ concentration increase. We investigated effects of TRPM2 channel antagonists namely 2-APB and FFA on BSO-induced currents in the neuronal cell systems. The 2-APB blocked BSO-induced TRPM2 currents in the DRG neurons. FFA did not block ADPR-induced currents in the cell system.

The results of this present study showed an increase in GSH-Px activity in response to BSO treatment of oxidative stress-exposed DRG neurons. Enhancement of their levels may provide an effective defense from the damaging effects of not only superoxide anion and H_2O_2, but also from the highly reactive and damaging hydroxyl radical (Halliwell 2006). Moreover, the observed restraint stress-induced reductions in GSH levels and activities of GSH-Px scavenge damage to the second line of antioxidant defense. A similar BSO induced-GSH depletion in DRG neurons has previously been reported in rat under stress-induced schizophrenia (Steullet et al. 2006) and human neuroblastoma SK-N-SH cells (Shimizu et al. 2002). 2-APB and GSH are found to reverse effectively this coordinate oxidative stress induced-decline in GSH and GSH-Px by restoring disrupted GSH pathways. It is plausible that TRPM2 channel blockers may exert their observed antioxidant effects via restoration of critical GSH-related processes such as ROS scavenging, detoxification of electrophilic compounds, modulation of cellular redox status and thiol-disulfide status of proteins, and regulation of cell signaling and repair pathways (Whanger 2001). Similarly, it has been demonstrated that total GSH levels were decreased in substantia nigra of patients with Parkinson disease and that the degree of disease severity was correlated with the extent of GSH loss (Sian et al. 1994). Furthermore, in an open-label clinical study, intravenous administration of GSH to a patient with early, untreated Parkinson's disease improved the clinical conditions (Sechi et al. 1996).

In the current study, lipid peroxidation levels were decreased by GSH and 2-APB treatment although GSH and GSH-Px values were increased by GSH and 2-APB treatment. A source of DRG neurons vulnerability that needed to be considered was the low level of GSH available in these cells to scavenge free radicals and reactive intermediates. Depletion of GSH promotes oxidative stress, and increasing GSH levels protects neuronal cell lines or sensory neurons from oxidant-induced injury (Altinkilic et al. 2010; Staaf et al. 2010). However, both increasing intracellular GSH levels and treatment with TRPM2 channel blocker 2-APB ameliorated the effects of BSO on DRG neurons, as assessed by lipid peroxidation, GSH and GSH-Px values.

In conclusion, these results demonstrated that intracellular GSH depletion is capable of activating TRPM2 in the DRG neurons. In addition, the GSH induced protective effects on BSO-induced TRPM2 currents in the neurons. Hence, our results indicated that endogenous GSH levels plays neuronal protective role against BSO-induced Ca^{2+} release through TRPM2 channels in DRG neurons. We suggested that induction of endogenous GSH level, especially by GSH and TRPM2 channels blocker treatments, may provide a potential therapeutic approach for the prevention of neuronal damage induced by products of oxidative stress.

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