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TRPM2 channel protective properties of N-acetylcysteine on cytosolic glutathione depletion dependent oxidative stress and Ca^{2+} influx in rat dorsal root ganglion

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ABSTRACT

N-acetylcysteine (NAC) is a thiol-containing (sulphydryl donor) antioxidant, which contributes to regeneration of glutathione (GSH) and also acts through a direct reaction with free radicals. Thiol depletion has been implicated in the neurobiology of sensory neurons and pain. We reported recently an activator role of intracellular GSH depletion on calcium influx through transient receptor potential melastatin-like 2 (TRPM2) channels in rat dorsal root ganglion (DRG). NAC may have a protective role on calcium influx through regulation of TRPM2 channels in the neurons. Therefore, we tested the effects of NAC on TRPM2 channel currents in cytosolic GSH depleted DRG in rats.

DRG neurons were freshly isolated from rats and the neurons were incubated for 24 h with buthionine sulfoximine (BSO). In whole-cell patch clamp experiments, TRPM2 currents in the DRG incubated with BSO were gated by H_2O_2. TRPM2 channels current densities, cytosolic free Ca^{2+} content, and lipid peroxidation values in the neurons were higher in H_2O_2 and BSO + H_2O_2 group than in controls; however GSH and GSH peroxidase (GSH-Px) values were decreased. BSO + H_2O_2-induced TRPM2 channel gating was totally inhibited by extracellular NAC and partially inhibited by 2-aminoethyl diphenylborinate. GSH-Px activity, lipid peroxidation and GSH levels in the DRG neurons were also modulated by NAC.

In conclusion, we observed a modulator role of NAC on Ca^{2+} influx through a TRPM2 channel in intracellular GSH depleted DRG neurons. NAC incubation before BSO exposure appears to be more protective than NAC incubation after BSO exposure. Since cytosolic thiol group depletion is a common feature of neuropathic pain, our findings are relevant to the etiology and treatment of pain neuropathology in DRG neurons.

1. Introduction

Transient receptor potential (TRP) channels are non-selective cation channels that have important functions in sensory neurons [1]. One member of the subgroup of TRP melastatin (TRPM) is TRP melastatin 2 (TRPM2). The channel protein has two distinct domains with one functioning as an ion channel and the other as an ADP-ribose (ADPR)-specific pyrophosphatase [2]. The TRPM2 channel is also a redox-sensitive Ca^{2+}-permeable cation channel, and Ca^{2+} influx through TRPM2 induced by H_2O_2 mediates necrotic cell death [3]. The channel is expressed in neuronal cells, such as rat striatal neurons [2], organotypic hippocampal culture [4] and DRG neurons [5–7], where it can also be gated by oxidative stress.

Increased neuronal activity induces increased production of reactive oxygen species (ROS) and neuronal degeneration [8] because of the susceptibility of neurological cells to oxidative stress [9]. Numerous reports indicate that ROS at physiological concentrations act as requisite signaling molecules in processes underlying sensory pain formation [5–7,10]. ROS are not only major contributors to a variety of neurological diseases but also act as intracellular second messengers in several cellular signal transduction pathways [10]. ROS products such as hydrogen peroxide (H_2O_2) are produced in response to receptor stimulation, which affects the function of various proteins, including TRPM2 channels, through oxidation of cysteine residues [11]. These damaging effects of ROS are kept under control by endogenous antioxidants, which include reduced glutathione (GSH), ascorbic acid and antioxidant enzymes such as glutathione peroxidase (GSH-Px) and catalase [12]. Oxidative stress occurs when antioxidant systems are overwhelmed by ROS, and the resultant oxidative damage leads to cell Ca^{2+} influx through TRPM2 channels. GSH is synthesized in two sequential enzymatic reactions catalyzed by γ-glutamylcysteine synthetase and GSH synthetase [13]. The

Abbreviations: 2-APB, 2-aminoethyl diphenylborinate; ADPR, adenosine diphosphatase ribose; DRG, dorsal root ganglion; LP, lipid peroxidation; $NMDG^+$, N-methyl-D-glucamine; ROS, reactive oxygen species; TRP, transient receptor potential; TRPM2, melastatin-like transient receptor potential 2; W.C., whole cell; $[Ca^{2+}]_i$, intracellular Ca^{2+}.

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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 [14].

N-Acetylcysteine (NAC) is a sulphydryl donor, which contributes to regeneration of GSH and which also acts through a direct reaction with free ROS [15]. Its free thiol group is capable of interacting with electrophilic groups of ROS [16]. It is protective for a range cell types, including neuronal death in DRG neurons induced by oxidative stress [17]. The antioxidant action of NAC is proposed to originate from its ability to stimulate GSH synthesis, therefore maintaining intracellular GSH levels that scavenge ROS [16]. Thus, NAC can restore the imbalance between pro-oxidant/antioxidant systems during oxidative stress.

Due to its potent ROS scavenging capacities, NAC has been largely used in the research of various neurological diseases, and has proved to be effective as predicted. Intracellularly-derived ROS can also directly active TRPM2 channels [18] and NAC protected against neuronal degeneration in DRG neurons after incubation with BSO. Thus antioxidants may be useful as therapeutic drugs for sensory neuron dependent diseases [19]. To date, the endogenous protective mechanism against TRPM2 channel-induced DRG neuron Ca^{2+} influx through TRPM2 channels has not been determined in DRG neurons. As TRPM2 channels are permeable to Ca^{2+} and have previously been implicated in other neurodegenerative disorders [9], activation of these channels is a potentially important mechanism that may contribute to the pathogenesis of DRG pain and diseases in DRG neurons.

Recently, we observed that intracellular GSH but not extracellular GSH inhibited completely the oxidative stress-induced TRPM2 cation channel functions and Ca^{2+} influx in DRG neurons [6]. The molecular mechanism by which oxidative stress and cytosolic thiol groups depletion lead to gating of TRPM2 channels in DRG neurons remains to be elucidated in detail. To study the role of NAC and oxidative stress in TRPM2 channels we used an experimental model in which BSO was applied to freshly isolated DRG neurons. Our findings strongly indicate the usefulness of NAC in prevention of sensory neurons neuropathy and pain, and also reveal secondary mechanisms of regulation of the innate antioxidant response and inhibition of cytosolic Ca^{2+} increase through TRPM2 channels.

2. Material and methods

2.1. Chemicals

Fura 2-AM/AM was obtained from Callbiochem (Darmstadt, Germany) and other materials were purchased form Sigma-Aldrich (St.Louis, MO, USA) if a specific company name didn't express.

2.2. Preparation of cell samples

We used 7 male Wistar rats (12–14 weeks old) in the current study. The study was approved by the Local Experimental Animal Ethical Committee of Suleyman Demirel University (SDU) (Protocol number: 18.01.2011-05). The animals were anesthetized by ether asphyxiation in accordance with SDU Experimental Animal legislation. DRG neurons (DRG, T13–L5) were carefully dissected from peripheral nerve roots. The neurons were incubated in Dulbecco's modified Eagle's medium (DMEM, Gibco, Istanbul, Turkey) 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 (Worthington, USA, 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 1500 *g* and the neurons were removed for patch-clamp, Ca^{2+} signaling and oxidative stress analysis.

2.3. Study groups

DRG neurons of each animal were divided into seven groups as follows:

Group I (n = 7) was the control group and were incubated (37 °C and 5% CO_2) for 24 h with normal medium.
Group II (n = 7) was the NAC group and were incubated with NAC for 3 h with normal medium.
Group III (n = 7) was the BSO treated group. The DRG neurons were incubated with BSO (0.5 mM) for 24 h [6].
Group IV (n = 7) was the BSO + NAC group. The DRG neurons were pre-incubated with BSO (0.5 mM) for 24 h and then they were incubated with NAC (2 mM) for 3 h.
Group V (n = 7) was the BSO + NAC + 2-APB group. The DRG neurons were pre-incubated with BSO (0.5 mM) for 24 h and then they were post-treated with 2-APB for 1 min.
Group VI (n = 7) was the NAC + BSO group. The DRG neurons were incubated with NAC (2 mM) for 3 h and then they were post-incubated with BSO (0.5 mM) for 24 h.
Group VII (n = 7) was the NAC + BSO + 2-APB group. The DRG neurons were pre-incubated with NAC (2 mM) for 3 h and then they were post-incubated with BSO (0.5 mM) for 24 h and 2-APB for 1 min.

At the end of the experiments, half of the DRG samples were immediately used for patch-clamp and 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 one week.

2.4. Electrophysiology

Patch clamp techniques have been described in detail elsewhere [5–7]. 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): 145 NaCl, 1 $MgCl_2$, 1 $CaCl_2$, 5 KCl, 10 HEPES, 10 D-glucose with the pH adjusted with NaOH 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, 8 EDTA, 1 $MgCl_2$, 1 $CaCl_2$ 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 (I–V) relationships were obtained from voltage ramps from −90 to +60 mV applied over 400 s. Stock 2-APB was dissolved in dimethyl sulfoxide and stored at −33 °C. Before the experiment, NAC (2 mM) and 2-APB (0.05–0.10 mM) in extracellular bath solutions were diluted to reach the final concentrations. All experiments were carried out at room temperature (approx 23 °C). After addition of 2-APB to standard extracellular bath solution, the pH values of these solutions were adjusted with NaOH to 7.4. The 2-APB was added to the patch-chamber (in the bath).

2.5. Measurement of intracellular free calcium concentration ($[Ca^{2+}]_i$)

Cells were trypsin-digested, allowed to sediment, resuspended in Hepes-buffered medium, consisting of 20 mM Hepes (7.4), 103 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM MgSO4, 0.5 mM CaCl2, 25 mM NaHCO3, 15 mM glucose and 0.1% bovine serum albumin (fatty acid free), and then incubated for 45 min with 5 μM fura