

Activation of TRPM2 and TRPV1 Channels in Dorsal Root Ganglion by NADPH Oxidase and Protein Kinase C Molecular Pathways: a Patch Clamp Study

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Received: 21 October 2016 / Accepted: 6 January 2017 / Published online: 17 January 2017
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Abstract Despite considerable research, the mechanisms of neuropathic pain induced by excessive oxidative stress production and overload calcium ion (Ca^{2+}) entry in dorsal root ganglion (DRG) remain substantially unidentified. The transient receptor potential melastatin 2 (TRPM2) and vanilloid 1 (TRPV1) channels are activated with different stimuli including oxidative stress. TRPM2 and TRPV1 have been shown to be involved in induction of neuropathic pain. However, the activation mechanisms of TRPM2 and TRPV1 via NADPH oxidase and protein kinase C (PKC) pathways are poorly understood. In this study, I investigated the roles of NADPH oxidase and PKC on Ca^{2+} entry through TRPM2 and TRPV1 channels in in vitro DRG neurons of rats. Rat DRG neurons were used in whole-cell patch clamp experiments. The H_2O_2 -induced TRPM2 current densities were decreased by *N*-(*p*-amylcinnamoyl)anthranilic acid (ACA), and dose-dependent capsaicin (CAP) and H_2O_2 -induced TRPV1 currents were inhibited by capsazepine (CPZ). The TRPV1 channel is activated in the DRG neurons by 0.01 mM capsaicin but not 0.001 mM or 0.05 mM capsaicin. TRPM2 and TRPV1 currents were increased by the PKC activator, phorbol myristate acetate (PMA), although the currents were decreased by ACA, CPZ, and the PKC inhibitor, bisindolylmaleimide I (BIM). Both channel currents were further increased by PMA + H_2O_2 as compared to H_2O_2 only. In the combined presence of PMA + BIM, no TRPM2 or TRPV1 currents were observed. The CAP

and H_2O_2 -induced TRPM2 current densities were also decreased by the NADPH oxidase inhibitors apocynin and *N*-Acetylcysteine. In conclusion, these results demonstrate a protective role for NADPH oxidase and PKC inhibitors on Ca^{2+} entry through TRPM2 and TRPV1 channels in DRG neurons. Since excessive oxidative stress production and Ca^{2+} entry are implicated in the pathophysiology of neuropathic pain, the findings may be relevant to the etiology and treatment of neuropathology in DRG neurons.

Keywords NADPH oxidase · Protein kinase C · *N* acetyl cysteine · Sensory neurons · TRPM2 channel · TRPV1 channel

Abbreviations

$[\text{Ca}^{2+}]_i$	Intracellular Ca^{2+}
ACA	<i>N</i> -(<i>p</i> -Amylcinnamoyl)anthranilic acid
ADPR	Adenosine diphosphatase ribose
BIM	Bisindolylmaleimide I
CAP	Capsaicin
CPZ	Capsazepine
DRG	Dorsal root ganglion
GSH	Glutathione
NAC	<i>N</i> -Acetylcysteine
NMDG	<i>N</i> -Methyl-D-glucamine
PKC	Protein kinase C
PMA	Phorbol myristate acetate
ROS	Reactive oxygen species
TRP	Transient receptor potential
TRPM2	Melastatin-like transient receptor potential 2
TRPV1	Transient receptor potential vanilloid 1
W.C.	Whole cell

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Introduction

Worldwide, there is high incidence of neuropathic pain among pain types and 2–7% of adults currently suffer from chronic neuropathic pain (Callaghan et al. 2015). The pain rate also increases as a result of disease or certain disease treatments. For example, approximately 20% of people with cancer have cancer-related neuropathic pain due to the cancer itself, or due to the chemotherapy (Kallenborn-Gerhardt et al. 2013; Callaghan et al. 2015). Neuropathic pain is induced by lesions of the nervous system and sensitization of the nociceptive system resulting in allodynia and hyperalgesia, both of which are increased by the lesions (Kallenborn-Gerhardt et al. 2012; Nazırođlu et al. 2012). Oxidative stress stimuli activate cation channels including transient receptor potential (TRP) melastatin 2 (TRPM2) and vanilloid 1 (TRPV1), both of which are highly expressed in the nerve terminals of dorsal root ganglion (DRG) neurons (Caterina et al. 1997; Fonfria et al. 2006; Özgül and Nazırođlu 2012). Accumulating evidence suggests that excessive production of reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), as well as the hot chili pepper component, capsaicin, contributes to the sensitization in persistent pain of DRG neurons (Kallenborn-Gerhardt et al. 2012; Övey and Nazırođlu 2015; Özdemir et al. 2016). Therefore, neuropathic pain associated with diabetes and mechanical injury, resulting from excessive ROS production and overload of Ca^{2+} entry, has been treated in animal models by administration of antioxidants (Sözbir and Nazırođlu 2016; Özdemir et al. 2016; Kahya et al. 2017). However, the sources of ROS production and how ROS contribute to pain sensitization remain poorly understood. With respect to the relevant enzymatic sources of these ROS, the NADPH oxidase enzyme has received considerable attention (Kallenborn-Gerhardt et al. 2012).

The ROS-generating effects of NADPH oxidase in cells are well known. NADPH oxidases may be the primary source of ROS, initiating oxidative stress and converting other enzymes into an ROS-producing state (Wind et al. 2010) to eventually induce pain pathophysiology (Kallenborn-Gerhardt et al. 2013). However, there are some concerns about the specificity of compounds currently used as NADPH oxidase inhibitors. For example, one of the most frequently applied compounds, apocynin, which is a naturally occurring compound isolated from *Picrorhiza kurroa*, also demonstrates NADPH oxidase inhibitory (Kallenborn-Gerhardt et al. 2013) and antioxidant properties (Heumüller et al. 2008). Accumulating evidence indicates that interactions between NADPH oxidase enzymes and TRP channels are important sources of ROS contributing to peripheral pain sensitization. For example, NADPH oxidase activation was reported as a primary source of ROS production in the pain process (Ibi et al. 2008). Protein kinase C (PKC)-mediated phosphorylation of serine residues in TRPV1 resulted in increased sensitivity to hyperalgesia (Chung et al. 2015). Serine protein phosphorylation through modulation of PKC

was markedly decreased in the human glioblastoma cell line by glutathione (GSH) (Lo et al. 2004). Cytosolic GSH depletion-induced oxidative stress activates TRPM2 and TRPV1 channels in DRG neurons (Özgül and Nazırođlu 2012; Övey and Nazırođlu 2015), although in both cases channel activation was blocked by *N*-acetylcysteine (NAC) and GSH treatments (Nazırođlu et al. 2011; Nazırođlu et al. 2013; Sözbir and Nazırođlu 2016). PKC is a family of enzymes involved in numerous important cellular events in neurons, including pain modulation (Hackel et al. 2013) and oxidative stress. PKC induces over-production of ROS through different molecular pathways such as stimulation of NADPH oxidase. Bisindolylmaleimide I (BIM) is a PKC inhibitor, while phorbol myristate acetate (PMA) is an activator of PKC. In pulmonary smooth muscle cells, both PKC and NADPH oxidase pathways were reported to activate L-type voltage-gated Ca^{2+} channels (Chakraborti et al. 2009). Hence, BIM may modulate activation of TRPM2 and TRPV1 channels resulting from PMA-induced oxidative stress due to its PKC modulator effects.

Emerging evidence points to TRPM2 and TRPV1 channels as major pain transducers (Nazırođlu 2007; Nazırođlu et al. 2012). Due to their permeability to Ca^{2+} , they exert a crucial regulatory function, most notably in pain modulation in DRG neurons (Nazırođlu and Özgül 2013). TRPM2 has a special NUDT9 homology domain and enzymatic activity (adenosine 5'-diphosphoribose (ADPR) pyrophosphatase enzyme) located at the C-terminus of the channel. If the domain is activated by ADPR in the presence of extracellular H_2O_2 , the channel is gated (Hara et al. 2002; Nazırođlu and Lückhoff 2008). TRPV1 channels are activated by different stimuli including H_2O_2 and capsaicin while they were inhibited by capsazepine (CPZ) (Caterina et al. 1997; Nazırođlu et al. 2013; Ogawa et al. 2016). It was reported that NADPH oxidase pathways are the major sources of TRPM2- and TRPV1-induced Ca^{2+} entry and superoxide production in cell lines (Hackel et al. 2013; Chen et al. 2013). TRPV1 channels were activated in DRG by oxidative stress induced by NADPH oxidase and the enzyme has also been identified as a source of oxidative stress in DRG (Ibi et al. 2008), but there is no report about the regulation of the TRPM2 channel and oxidative stress through NADPH oxidase and PKC pathways in DRG neurons.

The present study was aimed at elucidating the role of PKC and NADPH oxidase pathways in modulating the effects of TRPM2 and TRPV1 channels in DRG neurons.

Material and Methods

Chemicals

Roswell Park Memorial Institute (RPMI)-1640 medium, NAC, capsaicin (CAP), CPZ, penicillin–streptomycin, H_2O_2 , dimethyl sulfoxide (DMSO), PMA, apocynin, BIM,

and *N*-(*p*-amylcinnamoyl)anthranilic acid (ACA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Collagenase type IV was purchased from Worthington Biochemical Corporation (NJ, USA). Cell culture flasks (50 ml and 25 cm²) were purchased from CELLSTAR (Greiner Bio-One Inc., Istanbul, Turkey). Remaining chemicals and plastic materials were purchased from Santa Cruz Inc. (Istanbul, Turkey).

Animals

Twelve male Wistar albino rats (12 weeks old) were housed in the Experimental Animal Research Center of Süleyman Demirel University (SDU). They were kept under controlled light (12 h day/night cycle) and humidity (60–70%) with free access to water (except in the fasting period) and commercial feed.

Study Groups

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

1. *Control group*. Neuron in the group was not stimulated by H₂O₂ and capsaicin (CAP). The neurons in the control group were incubated (37 °C and 5% CO₂) in the incubator for 2 h with normal medium (DMEM with 5% bovine serum albumin and 1% penicillin–streptomycin).
2. *ACA-treated group*. The DRG neurons were immediately treated with ACA (0.025 mM) in a patch chamber before H₂O₂ stimulation (Nazıroğlu et al. 2013).
3. *CPZ-treated group*. The DRG neurons were immediately treated with CPZ (0.1 mM) in a patch chamber before H₂O₂ stimulation (Nazıroğlu et al. 2013; Övey and Nazıroğlu 2015).
4. *Apocynin-treated group*. The DRG neurons were incubated with apocynin (0.25 mM) for 60 min in the incubator before H₂O₂ (10 mM) and capsaicin (0.01 mM) stimulations (Wind et al. 2010).
5. *NAC-treated group*. The DRG neurons were incubated with NAC (10 mM) before H₂O₂ (10 mM) and capsaicin (0.01 mM) stimulations (Özgül and Nazıroğlu 2012; Nazıroğlu et al. 2013). NAC was added to the cell culture flasks for a 2-h incubation before capsaicin and H₂O₂ stimulations because transfer of the antioxidant into the cell is slow (Nazıroğlu et al. 2013).
6. *BIM-treated group*. The DRG neurons were incubated with BIM (0.01 mM) for 30 min in the incubator before H₂O₂ (10 mM) and capsaicin (0.01 mM) stimulations (Miyano et al. 2010).
7. *PMA-treated group*. The DRG neurons were incubated with PMA (0.001 mM) in the incubator for 30 min before H₂O₂ (10 mM) and capsaicin (0.01 mM) stimulations

(Nazıroğlu and Özgül 2013). The TRPM2 and TRPV1 channels were inhibited by ACA (0.025 mM) and CPZ (0.1 mM), respectively.

8. *PMA plus BIM-treated group*. The DRG neurons were incubated with PMA (0.001 mM) for 30 min in the incubator, and then they were incubated with BIM (0.01 mM) for 30 min before H₂O₂ (10 mM) and capsaicin (0.01 mM) stimulations.

At the end of the treatments and incubations, the DRG samples were immediately used for the patch clamp analyses.

Preparation of DRG Neurons

For preparing DRG neurons, rats were decapitated by cervical dislocation as described previously (Sözbir and Nazıroğlu 2016; Kahya et al. 2017). After decapitation, the skin on the back of the rats is split with fine forceps, and the spinal cord is exposed. Serial sections of the entire L4–L5 DRG neurons from the rats were cut (16 µm), and three sections, at least 100 µm apart, were taken. The DRG neurons are removed and placed into a 15-ml conical tube with a 5-ml cold Dulbecco's modified Eagle's medium (DMEM, Gibco, Istanbul, Turkey) and centrifuged for 5 min at 600×g. After centrifugation, the supernatant is discarded, and the pellet is re-suspended in 5 ml Hank's buffered salt solution (Ca²⁺- and Mg²⁺-free, HBSS) containing 5 mg collagenase D, type IV (0.1%), and 5 mg trypsin, type 1 (0.1%). DRGs are digested for 45 min in a 37 °C water bath with rotation at 1000 g. After digestion, the samples are centrifuged at 500×g for 5 min. The supernatant is discarded, and the pellet is re-suspended in an 8-ml neurobasal medium (NBM) supplemented with B-27, a serum-free supplement (2 mM), L-glutamine (10 µl/ml), and penicillin–streptomycin (Invitrogen). Then, the DRG neurons were transferred to patch chambers for analyses.

Electrophysiology

The neurons were transferred to extracellular buffer [as mM: 40 NaCl, 1.2 MgCl₂, 1.2 CaCl₂, 5 KCl, 10 HEPES, pH 7.4 (KOH)] in a temperature-controlled (22–24 °C) recording chamber (Luigs & Neumann, Ratingen, Germany). In some experiments, Na⁺ was replaced by 150 mM *N*-methyl-D-glucamine (NMDG⁺) and the titration was performed with HCl. Channel activity in DRG neuron was 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) (Özdemir et al. 2016; Kahya et al. 2017). Control records were taken on each experimental day using the same animals as those used for studying TRPM2 and TRPV1. The

neurons 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 200 ms. Bridge balance and access resistance were monitored during recordings, and experiments with $>20\%$ changes were discarded. Whole-cell mode of patch clamp experiments was used in all experiments, using an EPC 10 equipped with a personal computer with Patchmaster software (HEKA, Lamprecht, Germany). The TRPM2 agonist (H_2O_2), TRPV1 agonists (capsaicin and H_2O_2), TRPM2 antagonist (ACA), and TRPV1 antagonist (CPZ) solutions were daily prepared and added to the extracellular buffer in the patch chamber. The inhibition with ACA and CPZ was performed by adding bath solution directly to the bath chamber during measurement. Open tip resistance of borosilicate patch pipettes was between 4 and 7 M Ω . Intracellular solution contained (in mM) 145 cesium glutamate, 8 NaCl, 10 EGTA, 2 MgCl₂, and 10 HEPES (pH 7.2) (adjusted with CsOH). It was reported that TRPM2 gating in neurons is greatly facilitated by elevated concentrations of high intracellular free Ca²⁺ ([Ca²⁺]_i) concentration (McHugh et al. 2003). Hence, the [Ca²⁺]_i in TRPM2 experiments was set to 1 μ M (0.886 mM Ca²⁺, 1 mM Cs-EGTA) instead of the physiologic intracellular Ca²⁺ concentration. The Ca²⁺ concentrations of the intracellular and extracellular solutions were calculated as described previously by using a specific computer program (Özdemir et al. 2016; Kahya et al. 2017).

Preparation of Agonists and Antagonists

Stock solutions (1 mM) of PMA, apocynin, BIM, ACA, capsaicin, and CPZ were dissolved in DMSO and stored at -85 °C. H_2O_2 was freshly prepared each day. Before the experiment, PMA (0.001 mM), apocynin (0.25 mM), BIM (0.01 mM), ACA (0.025 mM), capsaicin (0.001–0.01 mM), and CPZ (0.1 mM) in extracellular bath solutions were diluted to reach the final concentrations. After addition of NAC, PMA, CAP, ACA, and CPZ to standard extracellular bath solution, the pH values of these solutions were adjusted with KOH and to 7.4.

Statistical Analyses

The channel capacitances were compared by one-way ANOVA followed by Tukey's post hoc test. Differences were considered statistically significant when the p value was 0.05. All statistical analyses were performed by SPSS Statistical program (17.0, SPSS Inc., Chicago, Illinois, USA). All the results were expressed as means \pm standard deviation (SD).

Results

Effects of the TRPM2 Antagonist (ACA) on H_2O_2 -Induced TRPM2 Currents in DRG Neurons

TRP channels can be indirectly blocked by various chemicals. However, for most TRP channels including TRPM2, the range of pharmacological modulators is limited. ACA as 0.025–0.040 mM was reported as a potent inhibitor of TRPM2 (Kraft et al. 2006), although reports on the effects of ACA in different cell types are conflicting (Nazıroğlu et al. 2012). Hence, we wanted to further clarify the effects of the channel blocker on TRPM2 channel currents in DRG neurons.

The mean value for the current density (pA/pF) in the control group was 2.21 ($n = 8$) (Fig. 1a), and there was little or no current in control records in the absence of H_2O_2 . In DRG neurons, H_2O_2 -induced currents of TRPM2 channels are reported between 0.52 and 1.2 nA (Özgül and Nazıroğlu 2012; Nazıroğlu et al. 2013; Sözbir and Nazıroğlu 2016; Yazğan and Nazıroğlu 2017). In the current study, we observed 0.62 nA current in the DRG neurons through superfusion of H_2O_2 (10 mM). These currents were blocked by ACA, as well as by substituting NMDG⁺ for Na⁺ (see Fig. 1b). The mean values of the current densities in the H_2O_2 and H_2O_2 + ACA groups as pA/pF were 87.13 ($n = 8$) and 13.00 ($n = 8$), respectively (Fig. 2). The current densities were high in the H_2O_2 and H_2O_2 + ACA groups as compared to the control ($p < 0.001$) although the densities were significantly lower ($p < 0.001$) in the H_2O_2 + ACA group (Figs. 2, 4, and 5). The data with H_2O_2 indicated that H_2O_2 acts by initiating a metabolic cascade resulting in the increase of a cytosolic oxidative stress factor and that this is responsible for the activation of the C-terminal Nudix domain ADPR pyrophosphate enzyme of the TRPM2 channel. H_2O_2 -induced activation of the TRPM2 channels was inhibited by the TRPM2 channel blocker, ACA.

Effects of TRPV1 Antagonist (CPZ) on H_2O_2 -Induced TRPV1 Currents in DRG Neurons

The TRPV1 channels are activated by different stimuli including capsaicin and nitric oxide. Recently, activation of the TRPV1 channel by H_2O_2 was reported in different cell lines (Ogawa et al. 2016). Activation of TRPV1 channels with different stimuli changes in different cell types (Nazıroğlu et al. 2012). To our knowledge, there is no report on H_2O_2 -dependent activation of TRPV1 channels in DRG neurons. Hence, I wanted to clarify the effects of H_2O_2 on TRPV1 channel currents in DRG neurons.

There was little or no current in control records (Figs. 1a) in the absence of H_2O_2 , and the mean value for the current density in control DRG neurons was 3.02 pA/pF ($n = 7$). However, addition of H_2O_2 to the patch chamber activated

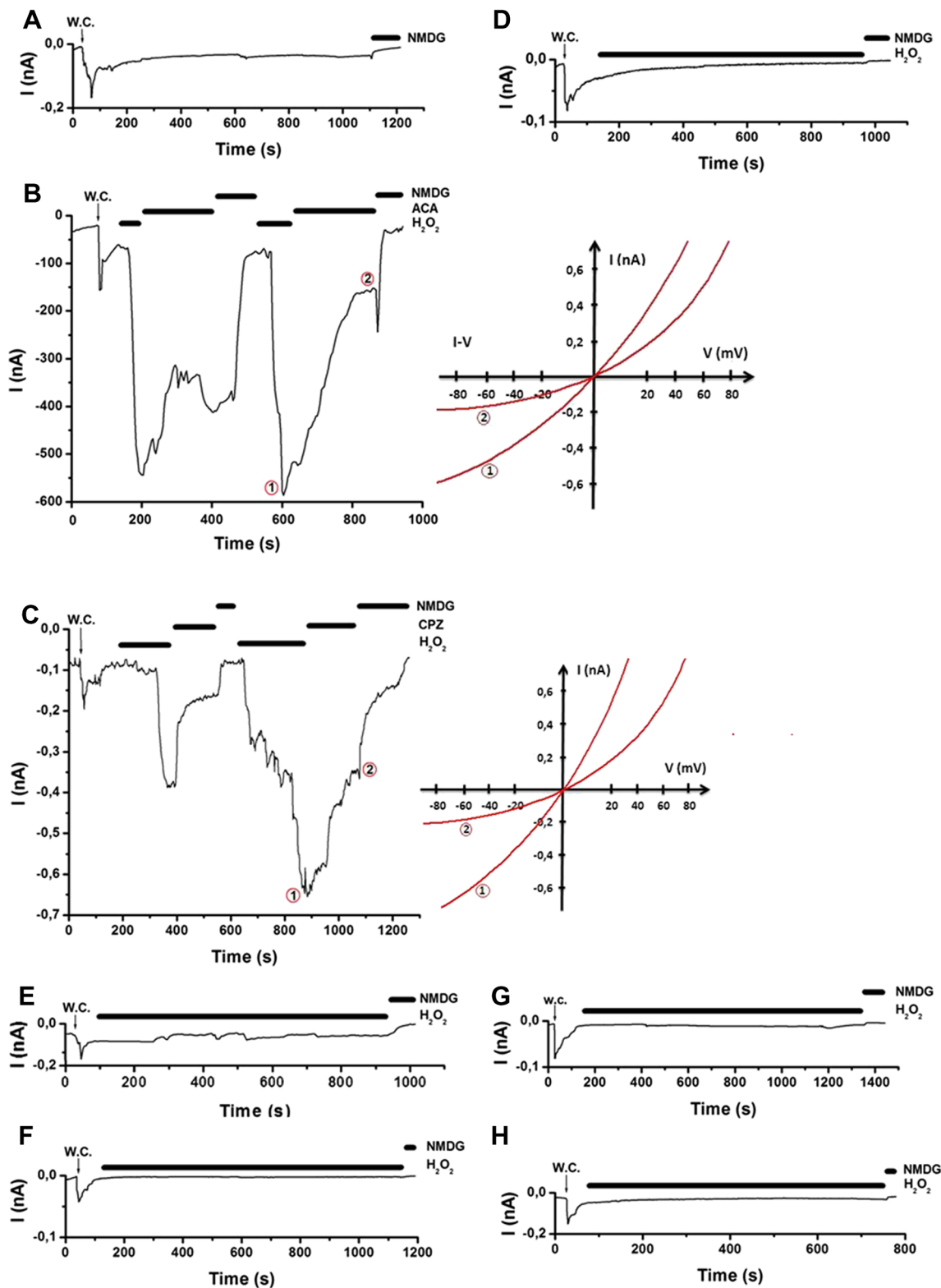


Fig. 1 PKC and NADPH oxidase-dependent activation of the TRPM2 channel in DRG neurons. **a** Original recordings from control neurons at holding potential (-60 mV). **b** TRPM2 currents in DRG neurons were stimulated by H_2O_2 (10 mM) in the bath (chamber), and they were inhibited by ACA (0.025 mM) in the patch chamber. **c** TRPM2 currents in DRG neurons were stimulated by H_2O_2 in the bath (chamber), and they were inhibited by CPZ (0.1 mM) in the patch chamber. **d–h** The DRG

neurons in the **d–h** groups were incubated with extracellular apocynin (0.25 mM for 1 h), NAC (10 mM for 2 h), BIM (0.01 mM for 30 min), PMA (0.001 mM for 1 h), and PMA + BIM (0.01 mM for 1 h and 0.001 mM for 30 min) before H_2O_2 stimulation, respectively. **b, c, g** Current voltage relationships of $NMDG^+$ and H_2O_2 through the TRPM2 channel in the presence of various extracellular cations (same experiments as in panels **b, c**). *W.C.* is used abbreviation of whole cell

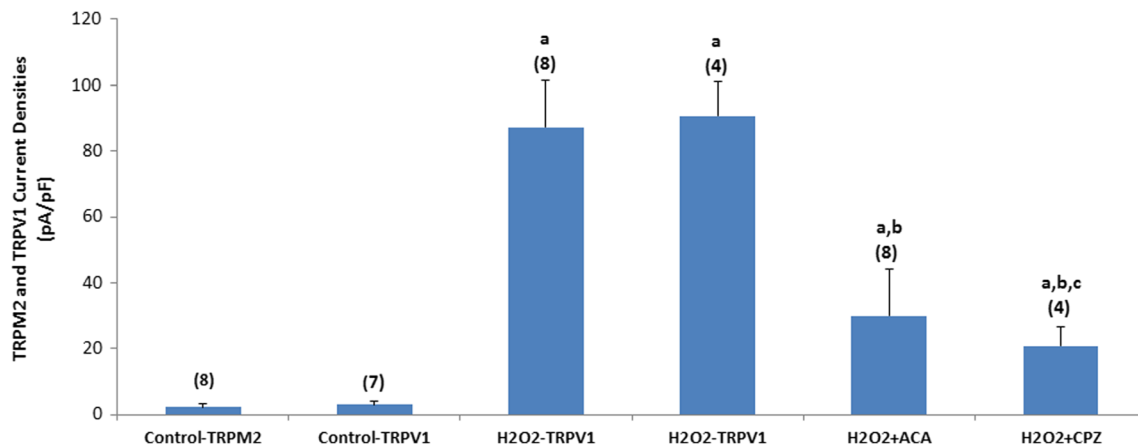


Fig. 2 Effects of TRPM2 (ACA and 0.025 mM) and TRPV1 (CPZ and 0.1 mM) blockers on H₂O₂-induced TRPM2 channel activation in DRG neurons. For each of the four applications studied in the cells, the initial current density (divided by the cell capacitance, a measure of cell size) was determined as well as the maximal current density after

administration of H₂O₂. The numbers in parentheses indicate *n* numbers of the groups. Significant (^a*p* < 0.001 versus controls. ^b*p* < 0.001 versus H₂O₂ groups. ^c*p* < 0.05 versus H₂O₂ + ACA group) (mean ± SD)

TRPV1 channels to well above 0.62 nA. These currents were reversibly and partially blocked by CPZ, as well as by substituting NMDG⁺ for Na⁺ (see Figs. 1c and 3c, f). The mean values of the current densities in the H₂O₂ and H₂O₂ + CPZ groups as pA/pF were 90.67 (*n* = 4) and 20.84 (*n* = 4), respectively. The current densities were high in the H₂O₂ and H₂O₂ + ACA groups as compared to the control (*p* < 0.001), and the densities were comparatively lower (*p* < 0.001) in the H₂O₂ + CPZ group. The current densities were also markedly (*p* < 0.05) lower in the H₂O₂ + CPZ group than in the H₂O₂ + ACA group (Fig. 2). Cysteine residues are main targets of H₂O₂, and a recent report indicated the presence of cysteine

groups in the TRPV1 channel structure (Ogawa et al. 2016). It is possible that cysteines in TRPV1 channels might be the targets of H₂O₂ exposure.

H₂O₂ was reported as an activator of TRPA1 channels in vagal lung C-fibers (Ruan et al. 2014), although the channel is inhibited by AP18 (Yazğan and Nazıroğlu 2017). I tested TRPA1 and TRPM2 currents in response to H₂O₂ in the DRG neuron (data are not shown). The neurons were pre-incubated by addition of ACA (0.025 mM) and AP18 (0.02 mM) combination into the patch chamber before activation of the TRPV1 channel via H₂O₂ (10 mM). The TRPV1 currents were even activated in the presence of AP18 and

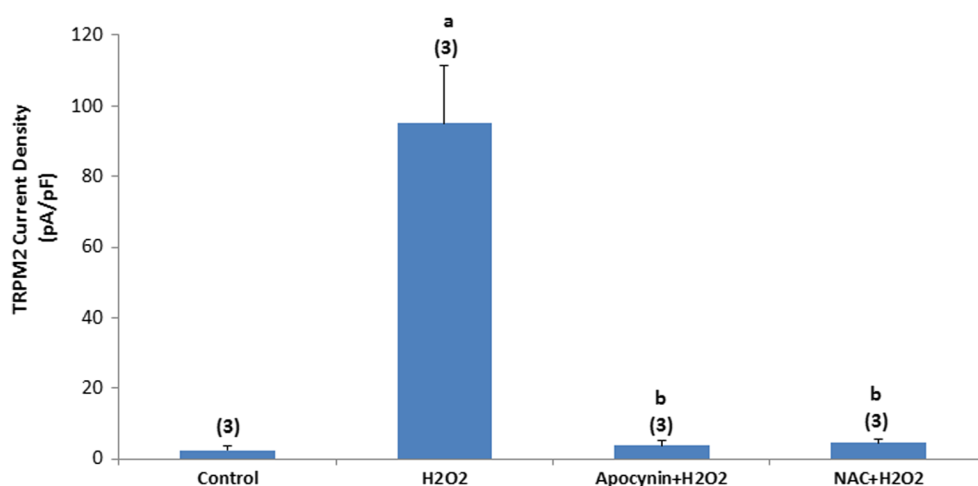


Fig. 4 Protective effects of NADPH oxidase inhibitors (apocynin, 0.25 mM for a 1-h incubation, and NAC, 10 mM for a 2-h incubation) on H₂O₂-induced TRPM2 channel activation in DRG neurons (mean ± SD). The values were expressed as current densities after administration

of H₂O₂. The numbers in parentheses indicate *n* numbers of the groups. (^a*p* < 0.001 versus control. ^b*p* < 0.001 versus the H₂O₂ group. ^c*p* < 0.001 versus H₂O₂ + ACA. ^d*p* < 0.001 versus PMA + H₂O₂. ^e*p* < 0.001 versus PMA + H₂O₂ + ACA)

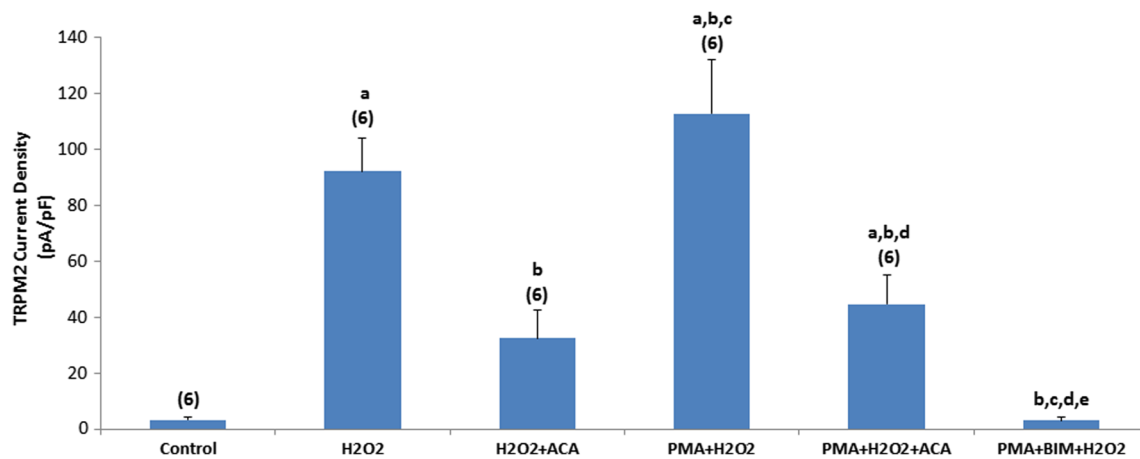


Fig. 5 Effects of the PKC inhibitor (BIM, 0.01 mM for a 30-min incubation) and stimulator (PMA, 0.01 mM for a 30-min incubation) on H₂O₂-induced TRPM2 channel activation in DRG neurons (mean \pm SD). The values were expressed as current densities after administration of

H₂O₂. The numbers in parentheses indicate *n* numbers of the groups. (^a $p < 0.001$ versus control. ^b $p < 0.001$ versus the H₂O₂ group. ^c $p < 0.001$ versus H₂O₂ + ACA. ^d $p < 0.001$ versus PMA + H₂O₂. ^e $p < 0.001$ versus PMA + H₂O₂ + ACA)

ACA combination by H₂O₂. Hence, I decided that the current belongs to the TRPV1 channel.

Effects of TRPV1 Antagonist (CPZ) on Capsaicin-Induced TRPV1 Currents in DRG Neurons

Capsaicin activates TRPV1 channels. Activation of TRPV1 channels with different doses of capsaicin changes in different cell types. For example, dose-dependent (600 nM–0.002 mM) activation of the TRPV1 channel in the intestinal afferent nerve was reported (Miranda-Morales et al. 2010). Hence, I wanted to clarify the dose-dependent effects of capsaicin on TRPV1 channel currents in DRG neurons.

In whole cell chamber bath experiments, superfusion of three doses of capsaicin (0.001, 0.005, and 0.010 mM) evoked a dose-dependent activation of rat DRG neurons (Fig. 3b) as described previously (Miranda-Morales et al. 2010; Deba and Bessac 2015; Yazgan and Naziroglu 2017). In DRG neurons, TRPV1 channel activation doses of capsaicin were reported between 0.001 and 0.015 mM (Deba and Bessac 2015; Yazgan and Naziroglu 2017). The TRPV1 currents in the DRG neurons were activated by treatment of 0.01 mM capsaicin but not 0.001 and 0.005 mM capsaicin and reached amplitudes of well above 0.83 nA (Fig. 3b). These currents were reversibly and partially blocked by CPZ (0.1 mM) (Fig. 3b, e, f). There was little or no current (mean value as pA/pF was 3.71) in control records ($n = 6$) in the absence of capsaicin (Fig. 3a). The mean values of the current densities in the capsaicin and capsaicin + CPZ groups as pA/pF were 106.51 ($n = 6$) and 6.97 ($n = 6$), respectively (Figs. 6 and 7). The current densities were significantly greater ($p < 0.001$) in the capsaicin group as compared to the control, and the densities were significantly lower ($p < 0.001$) in the capsaicin + CPZ group than in the capsaicin group (Fig. 7).

Effects of NADPH Oxidase Modulators (Apocynin and NAC) on H₂O₂, Capsaicin, and PMA-Induced TRPM2 and TRPV1 Channel Activations

Recently, we observed a modulator role of sulfhydryl protective agents such as GSH and NAC on TRPM2 and TRPV1 channel activation in DRG neurons (Özgül and Naziroglu 2012; Naziroglu et al. 2013; Sözbir and Naziroglu 2016). Reports on the effects of antioxidants on the PMA-induced TRPM2 channel activation are conflicting. For example, it was reported that the PMA-induced TRPM2 channel activation was blocked in DRG neurons by vitamin E treatment but not vitamin C or catalase (Naziroglu and Özgül 2013). However, there is no report on the effects of apocynin and NAC on TRPM2 and TRPV1 currents in H₂O₂- and capsaicin-treated DRG neurons. Hence, I examined the potential protective roles of NAC and apocynin on H₂O₂-induced TRPM2 and capsaicin-induced TRPV1 activations in DRG neurons. Exposure of isolated DRG neurons isolated to H₂O₂ and capsaicin significantly ($p < 0.001$) increased the activation of TRPM2 (Figs. 1b and 2) and TRPV1 channels, respectively (Figs. 3b, f and 6). In contrast, H₂O₂- and capsaicin-induced NADPH oxidase (H₂O₂) activation of TRPM2 (Figs. 1d, e and 4) and TRPV1 (Figs. 3c–e and 7) was substantially ($p < 0.001$) reduced in DRG neurons by apocynin and NAC treatments.

Effects of PKC Stimulator (PMA) and Inhibitor (BIM) on TRPM2 and TRPV1 Channel Activations

NADPH oxidase-induced TRPV1 activation is dependent on the PKC pathway (Ibi et al. 2008). Recently, we observed a TRPM2 activator role of PMA via excessive production of ROS in DRG neurons, and this activation was inhibited by vitamin E treatment (Naziroglu and Özgül 2013). To clarify

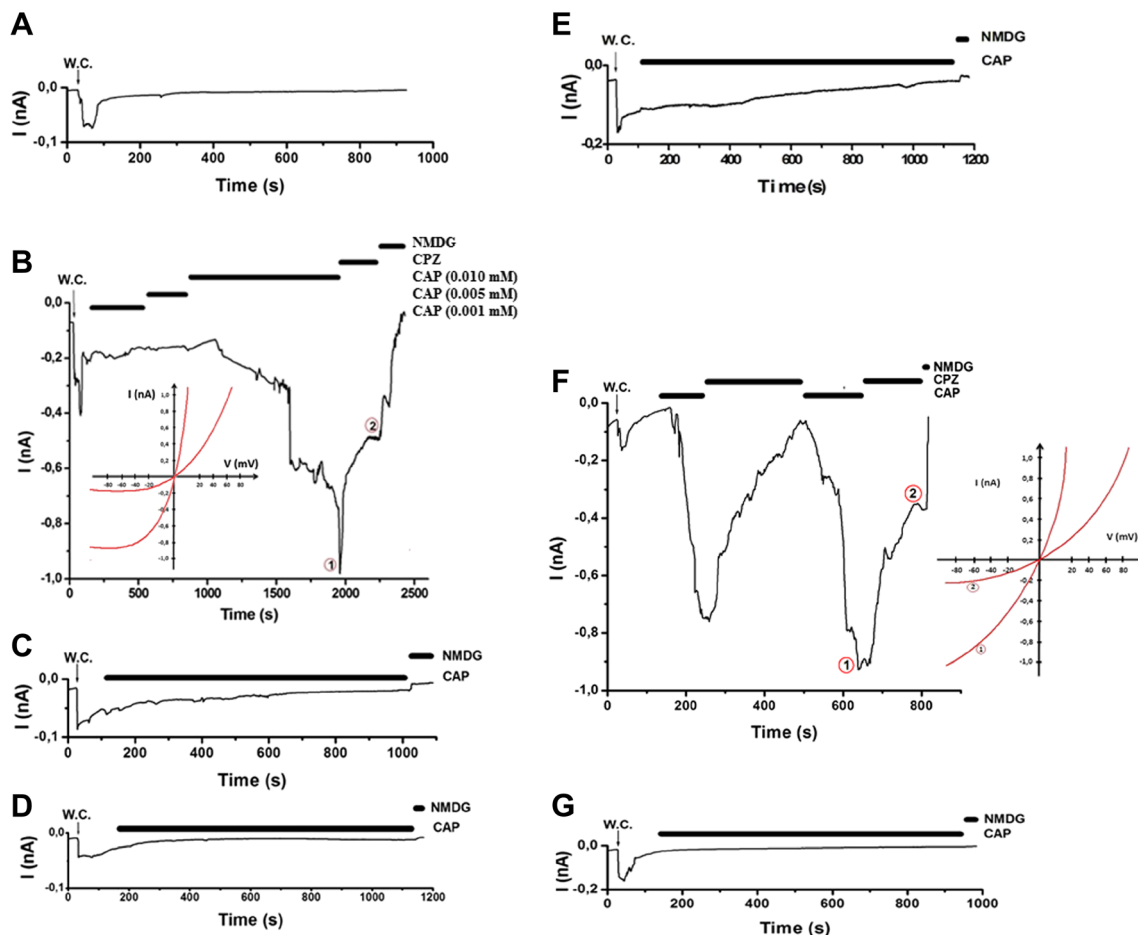


Fig. 3 PKC and NADPH oxidase-dependent activation of the TRPV1 channel in DRG neurons. **a** Original whole cell (W.C.) recordings from control neurons (holding potential was -60 mV). **b** TRPV1 currents in DRG neurons were stimulated by 0.01 mM capsaicin (CAP) but not 0.001 and 0.005 mM CAP in the bath (chamber), and they were inhibited by capsazepine (CPZ and 0.1 mM) in the patch chamber. **c–g** The DRG neurons in the **c–g** groups were incubated with extracellular

apocynin (0.25 mM for 1 h), NAC (10 mM for 2 h), BIM (0.01 mM for 30 min), PMA (0.001 mM for 1 h), and PMA + BIM (0.01 mM for 1 h and 0.001 mM for 30 min) before CAP (0.01 mM) stimulation, respectively. Current voltage relationships of NMDG^+ and H_2O_2 through the TRPV1 channel in the presence of various extracellular cations (same experiments as in panels **b**, **c**, **f**)

the role of NADPH oxidase in the PKC-dependent activation of TRPM2 and TRPV1 channels, I next tested the effects of the PKC inhibitor, BIM, in DRG neurons. During the oxidative burst stimulated by PMA in DRG neurons, TRPM2 and TRPV1 channel activities were determined by measuring the current density of the channels (Fig. 5). The H_2O_2 - and CAP-induced TRPM2 (Figs. 1g and 5) and TRPV1 (Figs. 6f and 7) current densities were markedly ($p < 0.001$) higher in the PMA group than in the control. Both currents were further increased in the PMA group as compared to the H_2O_2 -alone group ($p < 0.001$) (Figs. 5 and 7). The TRPM2 and TRPV1 currents in the PMA group were decreased by ACA and CPZ treatments, respectively ($p < 0.001$) (Figs. 1e, 3d, 5, and 7). The TRPM2 and TRPV1 current densities were significantly ($p < 0.001$) lower in the BIM + H_2O_2 and BIM + capsaicin groups than in the H_2O_2 and PMA + H_2O_2 , capsaicin, and PMA + capsaicin groups (Figs. 1f, 3e, 5, and 7). As compared to the H_2O_2 and capsaicin groups, the TRPM2 and TRPV1

current densities were further increased by H_2O_2 + PMA and capsaicin + PMA exposures. However, the PMA-induced TRPM2 and TRPV1 current densities were totally ($p < 0.001$) inhibited in the PMA + BIM + H_2O_2 and PMA + BIM + capsaicin groups by post-incubation of BIM (Figs. 1h, 3g, 5, and 7) and there were little or no TRPM2 and TRPV1 currents in the PMA + BIM group after H_2O_2 and capsaicin treatments (Figs. 5 and 7).

Discussion

Changes in DRG Ca^{2+} signaling in diabetes contribute to the development of neuropathic pain (Nazıroğlu et al. 2012). Overload Ca^{2+} entry through activation of TRPM2 and TRPV1 channels in the DRG is increased by excessive production of oxidative stress (Nazıroğlu et al. 2011b). PKC and NADPH oxidase pathways are activated by excessive

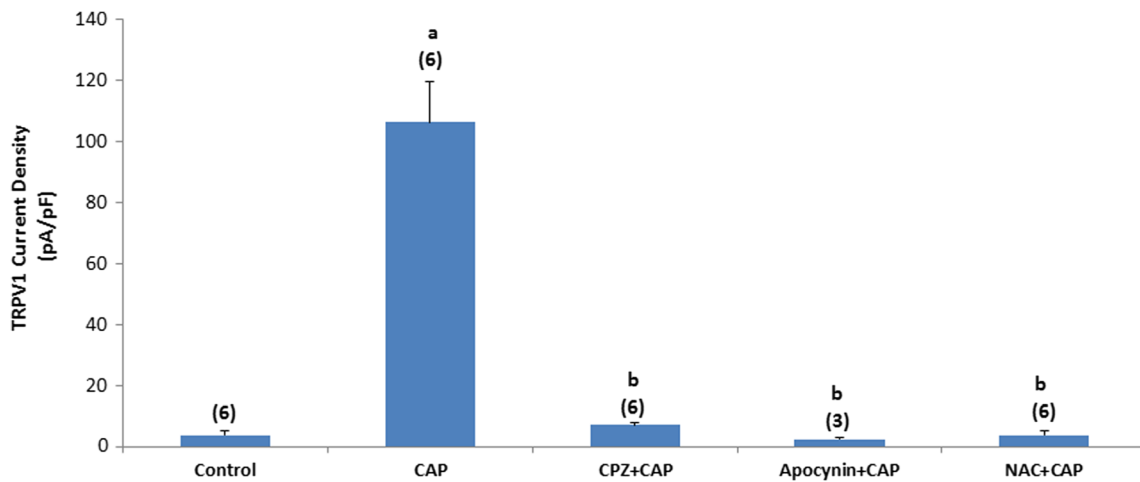


Fig. 6 Protective effects of NADPH oxidase inhibitors (apocynin and NAC) on capsaicin (0.01 mM)-induced TRPV1 channel activation in DRG neurons (mean ± SD). The DRG neurons were incubated with apocynin (0.25 mM) and NAC (10 mM) for 60 min before capsaicin

(CAP, 0.01 mM) stimulation, respectively. The values were expressed as current densities after administration of CAP. The numbers in parentheses indicate n numbers of the groups. ^a*p* < 0.001 versus control. ^b*p* < 0.001 versus the CAP group)

intracellular free Ca²⁺ entry via TRPM2 and TRPV1 channels (Ibi et al. 2008; Nazırođlu and Özgöl 2013). In experimental models, pain was blocked by inhibition of these pathways (Kallenborn-Gerhardt et al. 2012; Kallenborn-Gerhardt et al. 2013). The TRPM2 and TRPV1 channels are mainly expressed in DRG neurons (Caterina et al. 1997; Fonfria et al. 2006; Özgöl and Nazırođlu 2012). Therefore, inhibition of NADPH oxidase and PKC pathway-induced excessive oxidative stress and overload Ca²⁺ entry via inhibition of TRPM2 and TRPV1 channels in DRG neurons may represent important therapeutic targets in the treatment of peripheral neuropathic pain. I observed that Ca²⁺ entry via TRPM2 and TRPV1 channels was increased in DRG neurons in response to PMA, H₂O₂, and dose-dependent capsaicin. Furthermore, PMA-, H₂O₂-, and capsaicin-induced Ca²⁺ entry through the TRPM2 and TRPV1 channels was decreased to control levels

by NADPH oxidase inhibitors (apocynin and NAC) and the PKC inhibitor, BIM. To the best of my knowledge (Ibi et al. 2008; Nazırođlu and Özgöl 2013), this study represents the first examination of the effects of apocynin and BIM on PKC and NADPH oxidase-dependent gating of TRPM2 and TRPV1 channels in DRG neurons. Therefore, as a result of the findings in this study, I believe that inhibitors of PKC and NADPH oxidase have the potential to serve as TRPM2 and TRPV1 channel modulators in diseases involving DRG neurons.

The C-terminal domain of TRPM2 contains the enzyme ADPR pyrophosphatase, and this enzyme is sensitive to ROS and reactive nitrogen species (Nazırođlu 2007). TRPM2 channels are gated by extracellular and intracellular ROS, possibly by interacting with the ADPR pyrophosphatase enzyme in the tail of the C domain of the protein (Fonfria et al.

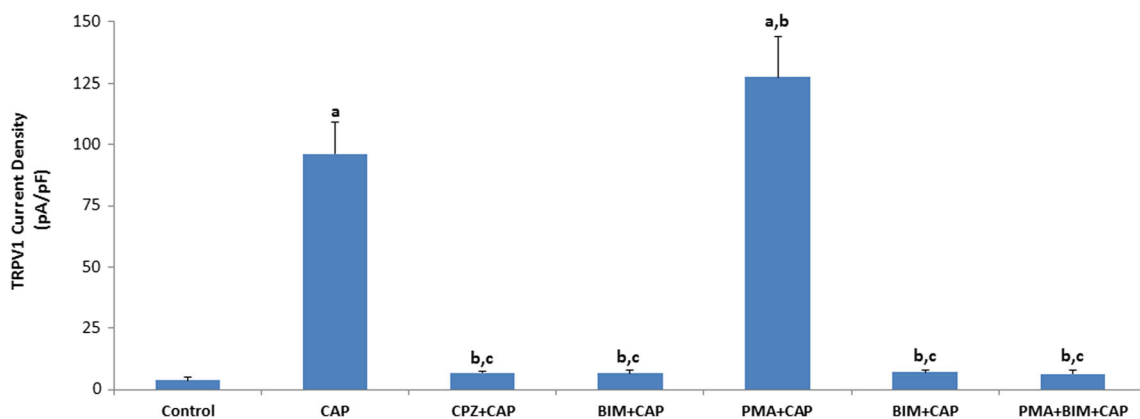


Fig. 7 Effects of PKC inhibitor (BIM) and stimulator (PMA) on capsaicin-induced TRPV1 channel activation in DRG neurons (mean ± SD and n = 3). The DRG neurons PMA and BIM groups were incubated with PMA (0.001 mM) and BIM (0.01 mM) for 30 min before capsaicin (CAP, 0.01 mM) stimulation, respectively. In the PMA + BIM group, the

neurons were pre-incubated with PMA for 30 min and then they were further incubated with BIM for 30 min before CAP (0.01 mM) stimulation. The values were expressed as current densities after administration of CAP (^a*p* < 0.001 versus control. ^b*p* < 0.001 versus the CAP group. ^c*p* < 0.001 versus the PMA + CAP group)

2006; Nazırođlu and Lückhoff 2008; Nazırođlu and Özgöl 2013). It is well known that excessive ROS production results in excessive production of H₂O₂ and thereby contributes to sensitization in persistent pain of DRG neurons (Kallenborn-Gerhardt et al. 2012; Özdemir et al. 2016). In the current study, increased current densities and sensitization in TRPM2 channel activation via stimulation of H₂O₂, NADPH oxidase, and PKC productions were observed in the DRG neurons. The data with H₂O₂ support the previous reports (Hara et al. 2002; Nazırođlu and Lückhoff 2008) that H₂O₂ acts by initiating a metabolic cascade resulting in the increase of NADPH oxidase and PKC enzymes, and those H₂O₂ production pathways are responsible for the activation of ADPR pyrophosphatase in the C domain of the TRPM2 channel.

The activation threshold and sensitivity of TRPV1 channels are changed by different mechanisms such as mechanical injury and high temperature. The sensitivity of TRPV1 is reduced by PKC-mediated phosphorylation of serine residues (Chung et al. 2015). An obligate role of cysteine residues in the pore region of TRPM2 and TRPV1 channels has been reported (Mei et al. 2006). If oxidation of cysteine residues of TRPM2 and TRPV1 occurs, the channel structure may be modified such that overload Ca²⁺ entry will occur through over-activation of TRPM2 and TRPV1 channels. High amounts of intracellular free Ca²⁺ might then induce excessive reactive oxygen species (ROS) production via mitochondrial membrane depolarization, resulting in even further activation of the channels by mitochondrial ROS production (Özdemir et al. 2016; Kahya et al. 2017). NAC is a sulfhydryl group containing antioxidant and is an important member of the glutathione redox cycle. NAC has an important role in scavenging ROS production in mitochondria (Nazırođlu et al. 2013). For this reason, NAC is believed to play critical roles in protecting cells from hazardous mitochondrial ROS. In the current study, I observed a modulator role of apocynin and NAC in TRPM2 and TRPV1 channels likely due to interaction with cysteine residues and protection against excessive ROS production.

NADPH oxidase activation is regulated by Ca²⁺ and diacylglycerol (DAG) signaling pathways. In addition to oxidative stress, PKC isoenzymes are also activated by Ca²⁺ and DAG signaling pathways. Recent studies suggest that NADPH oxidase pathways in the DRG and neuroblastoma SH-SY5Y cell lines may be more important than ADPR as the major sources of TRPM2-induced Ca²⁺ entry and superoxide production (Hackel et al. 2013; Chen et al. 2013). NADPH oxidase activation was reported as a primary source of ROS production in DRG neurons (Ibi et al. 2008). Both TRPM2 and TRPV1 channels are activated in hippocampal and DRG neurons by cytosolic glutathione depletion-induced oxidative stress (Özgöl and Nazırođlu 2012; Övey and Nazırođlu 2015). Recently, we also observed a modulator role

of sulfhydryl antioxidant agents such as GSH and NAC on TRPM2 and TRPV1 channel activation in DRG neurons (Nazırođlu et al. 2011; Nazırođlu et al. 2013; Sözbir and Nazırođlu 2016). Vitamin E treatment reduced the PMA-induced TRPM2 channel activation in DRG neurons. However, there is no report on PMA-induced TRPM2 and TRPV1 activations in NAC-treated DRG neurons. In the current study, treatment of isolated DRG neurons with PMA and H₂O₂ significantly (*p* < 0.001) increased the activation of TRPM2 and TRPV1 channels and the TRPM2 and TRPV1 activations were decreased in DRG neurons by NAC treatment. Similarly, Ibi et al. (2008) reported an increase of PMA-induced PKC isoforms in DRG neurons by NAC pretreatment. These findings suggest that the activation of TRPM2 and TRPV1 channels by ROS derived from NADPH oxidase is decreased in DRG neurons by pretreatment with NAC and subsequent inhibition of PKC.

In conclusion, the main finding of the current study is that NADPH oxidase blockers apocynin and NAC decreased the TRPM2 and TRPV1 channel-dependent Ca²⁺ influx in DRG neurons. I clearly observed that the channels are modulated by H₂O₂- and dose-dependent capsaicin-induced oxidative stress in the neurons by a PKC-dependent pathway. Hence, our results indicate that NADPH oxidase and PKC enzyme inhibition acts as a neuronal modulator against oxidative stress-induced Ca²⁺ mobilization through TRPM2 and TRPV1 channels in DRG neurons. This finding is of particular significance and may provide an explanation for the neuropathic and peripheral pain properties of NADPH oxidase and PKC enzymes, in keeping with the proposed role of excessive Ca²⁺ entry in the pathology of neuropathic pain.

Acknowledgements The authors wish to thank Prof. Dr. James W. Putney (NIEHS, NC, USA) for helpful discussions on the manuscript.

Compliance with Ethical Standards The study was approved by the Local Experimental Animal Ethical Committee of SDU (protocol number 2010-13).

Financial Disclosure There is no financial disclosure and support for the current study.

Conflict of Interest The author declares that he has no conflict of interest.

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