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Aminoethoxydiphenyl Borate and Flufenamic Acid Inhibit Ca^{2+} Influx Through TRPM2 Channels in Rat Dorsal Root Ganglion Neurons Activated by ADP-Ribose and Rotenone

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Abstract Exposure to oxidative stress causes health problems, including sensory neuron neuropathy and pain. Rotenone is a toxin used to generate intracellular oxidative stress in neurons. However, the mechanism of toxicity in dorsal root ganglion (DRG) neurons has not been characterized. Melastatin-like transient receptor potential 2 (TRPM2) channel activation and inhibition in response to oxidative stress, ADP-ribose (ADPR), flufenamic acid (FFA) and 2-aminoethoxydiphenyl borate (2-APB) in DRG neurons are also not clear. We tested the effects of FFA and 2-APB on ADPR and rotenone-induced TRPM2 cation channel activation in DRG neurons of rats. DRG neurons were freshly isolated from rats and studied with the conventional whole-cell patch-clamp technique. Rotenone, FFA and 2-APB were extracellularly added through the patch chamber, and ADPR was applied intracellularly through the patch pipette. TRPM2 cation currents were consistently induced by ADPR and rotenone. Current densities of the neurons were higher in the ADPR and rotenone groups than in control. The time courses (gating times) in the neurons were longer in the rotenone than in the ADPR group. ADPR and rotenone-induced TRPM2 currents were totally blocked by 2-APB and partially blocked by FFA. In conclusion, TRPM2 channels were constitutively activated by ADPR and rotenone, and 2-APB and FFA induced an inhibitory effect on TRPM2 cation channel currents in rat DRG neurons. Since oxidative stress is a common feature of neuropathic pain and diseases of sensory neurons, the present findings have broad application to the etiology of neuropathic pain and diseases of DRG neurons.

Keywords TRPM2 channel · Antagonists · ADP-ribose · Oxidative stress · Dorsal root ganglion neuron

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Introduction

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 kinds of external and internal stimuli. These stimuli, i.e., 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 stimuli (Staaf et al. 2010). Transient receptor potential (TRP) channels are a group of nonselective cation channels that play important functions in sensory neurons (Nazıroğlu 2011). One subgroup 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. The primary gating mechanism of TRPM2 is through the binding of intracellular ADPR (Perraud et al. 2001). 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).

TRP channels have been blocked by nonspecific chemicals. However, in most TRP channels, including TRPM2, the range of pharmacological modulators is scarce. Flufenamic acid (FFA) is a nonsteroidal anti-inflammatory drug described as a TRPM2 blocker (Hill et al. 2004;

Nazıroğlu et al. 2007). Recently, 2-aminoethoxydiphenyl borate (2-APB) was also described as a TRPM2 channel blocker in addition to its actions as an inositol 1,4,5-triphosphatase ($InsP_3$) receptor antogonist (Xu et al. 2005; Togashi et al. 2008). However, reports on 2-APB and FFA as TRPM2 channel blockers in different neuronal cell systems are conflicting (Nazıroğlu 2011).

Mitochondrial dysfunction is widely accepted to contribute to degeneration processes in neurodegenerative diseases, and neurotoxin impairment of oxidative phosphorylation is used to create cellular and animal models for these disorders (Salazar et al. 2008). Rotenone is a naturally occurring isoflavonoid from the tropical plants *Lonchocarpus* and *Derris* (Bove et al. 2005). Rotenone decreases intracellular ATP levels and increases the production of reactive oxygen species (ROS) including superoxide anion, hydrogen peroxide (H_2O_2) and singlet oxygen (Cormier et al. 2003). It also releases glutamate presynaptic terminals, leading to an additional increase in ROS production (Freestone et al. 2009). Mitochondria-derived ROS can also activate TRPM2 channels (Nazıroğlu 2007). As 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 neuron-dependent pain and disorders.

The molecular mechanism by which oxidative stress and ADPR lead to gating of TRPM2 channels in DRG neurons (Staaf et al. 2010) remains to be elucidated in detail. To study the role of ADPR and oxidative stress in TRPM2 channels, we used an experimental model in which ADPR and rotenone were applied to freshly isolated DRG neurons. Furthermore, the present study was aimed at elucidating the role of 2-APB and FFA on modulation of the effects of ADPR and rotenone-induced gating in TRPM2 channels.

Materials and Methods

Preparation of Cell Samples

We used male Wistar rats (12–14 weeks old) in the current study. The study was approved by the Experimental Animal Ethical Committee of Suleyman Demirel University (SDU, protocol 2010-20-01). Animals were killed by ether asphyxiation and cervical dislocation in accordance with SDU experimental animal protocols. Briefly, lumbar dorsal ganglia (T13–L5) were harvested from the rats. Ganglia were transferred into Dulbecco's modified eagle medium (DMEM; GIBCO, Grand Island, NY) supplemented with 1% penicillin–streptomycin (Sigma, Istanbul, Turkey) in 500 ml DMEM; the connective tissue was removed and ganglia were treated with collegenase IV (Worthington, Lakewood, NJ; 0.28 ml in DMEM, 45 min at 37°C and 5% CO_2) and trypsin (25,000 units/ml in DMEM). After dissociation with a sterile insulin syringe, the cell suspension was centrifugated at 3,500 rpm. Cells were seeded in a patch-clamp chamber, and electrophysiological studies were carried out within 2 h.

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, Lambrecht, Germany). Patch-clamp pipettes were made of borosilicate glass (Sutter Instrument, Novato, CA; flament OD 1.5 mm, OD 0.86 mm, 10 cm length). The standard extracellular bath solution contained (in mM) 140 NaCl, 1.2 $MgCl_2$, 1.2 $CaCl_2$, 5 KCl and 10 HEPES, pH adjusted with KOH to 7.4. For Na^+-free solutions, Na^+ was replaced by 150 mM *N*-methyl-D-glucamine (NMDG) and the pH was adjusted with HCl. Osmolarity of the solution was 310 mOsm/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.

Neurons were held at a potential of −60 mV, and current–voltage (*I–V*) relations were obtained from voltage ramps from −90 to +60 mV applied over 400 ms. Stock (500 mM) ADPR, 2-APB and FFA were dissolved in dimethyl sulfoxide and stored at −33°C. Before the experiment, ADPR (1 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. All experiments were carried out at room temperature. After addition of FFA and 2-APB to standard extracellular bath solution, the pH of these solutions was adjusted with KOH to 7.4. FFA and 2-APB were added to the patch chamber extracellularly (in the bath).

Statistical Analysis

All results were expressed as means ± SD. Significance in three groups was firstly checked by ANOVA-Kruskal Wallis test. Then, significant values in three groups were assessed with an unpaired Mann–Whitney *U*-test. Data were analyzed using the SPSS statistical program (version 9.05 software; SPSS, Inc., Chicago, IL). $P < 0.05$ was regarded as significant.

Results

Effects of ADPR

The TRPM2 channels expressed in rat DRG neurons were studied with the whole-cell patch-clamp technique. The channels were stimulated by ADPR (1 mM) applied to the cytosolic side of the channels by diffusion from the patch pipette. ADPR (0.3–1 mM) and high Ca^{2+} (1 μM) have been used in transfected cell systems for gating the TRPM2 channel (Buelow et al. 2008; Nazıroğlu and Lückhoff, 2008a, b; Kühn et al. 2009). First, 0.3 mM ADPR was unable to activate the TRPM2 channels (data not shown). Hence, we selected 1 mM ADPR in the current study. ADPR (1 mM) activated an inward current in rat DRG neurons. Representative experiments are shown in Figs. 1 and 2. Currents induced by ADPR developed gradually during infusion of ADPR into the cells and reached amplitudes (at a holding potential of 60 mV) of well above 0.75 nA in the inward direction. These currents were reversibly blocked by replacement of external Na^+ with the large impermeable cation NMDG; this abolished the inward and outward components of the currents (Fig. 2b). No currents were seen in the absence of ADPR (Figs. 1a, 2a). The mean value for the current densities in the absence of ADPR in control DRG neurons was 0.39 ± 0.99 pA/pF ($n = 8$). The value for the current densities of ADPR in the

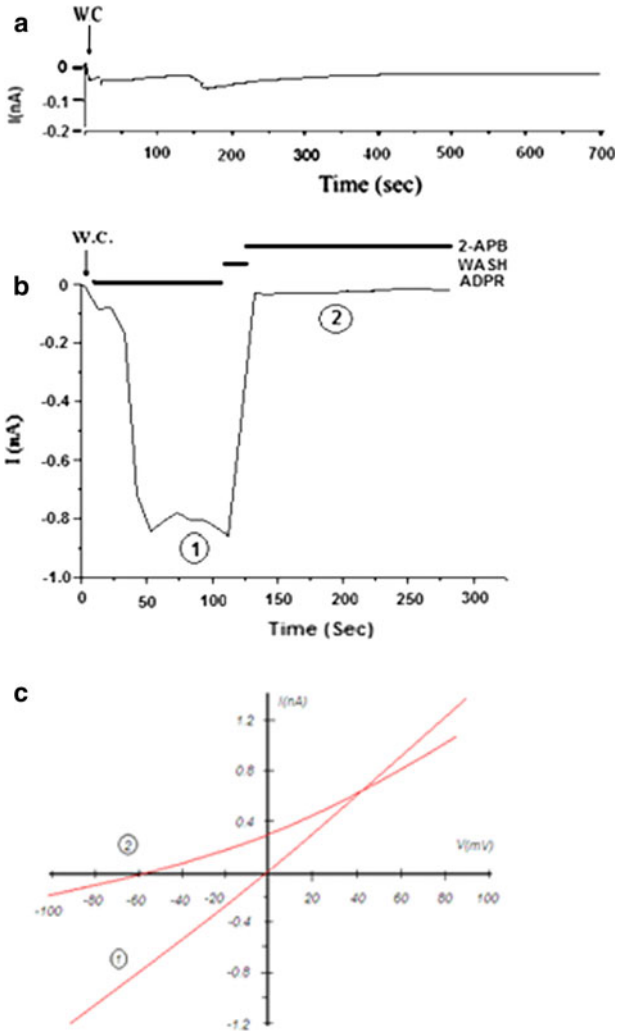

Fig. 1 Effects of extracellular 2-APB on ADPR-induced TRPM2 channel activation in DRG neurons. Holding potential was −60 mV. **a** Original recordings from control cell. **b** Cell expressing TRPM2 currents stimulated by ADPR (1 mM) in the pipette and they inhibited by 2-APB (0.05 mM) in bath (chamber). **c** Current–voltage relationships of 2-APB and ADPR currents through TRPM2 in presence of various extracellular cations (same experiments as in **b**). *W.C.* whole cell

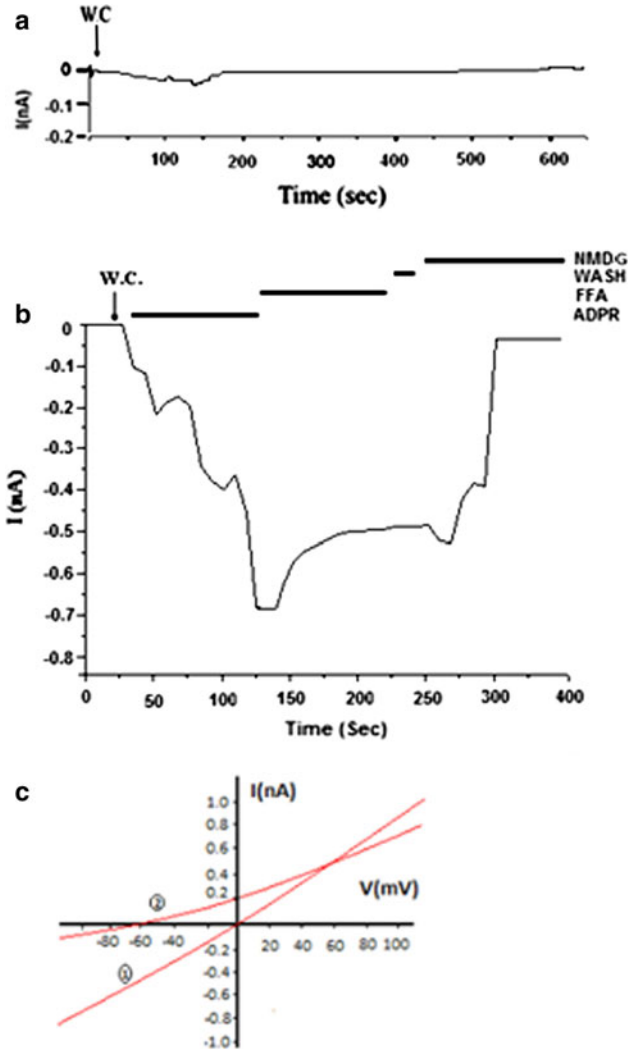

Fig. 2 Effects of extracellular FFA on ADPR-induced TRPM2 channel activation in DRG neurons. Holding potential was −60 mV. **a** Original recordings from control cell. **b** Cell expressing TRPM2 currents stimulated by ADPR (1 mM) in the pipette and inhibited by FFA (0.1 mM) in bath (chamber). **c** Current–voltage relationship of NMDG and rotenone currents through TRPM2 in presence of various extracellular cations (same experiments as in **b**). *W.C.* whole cell

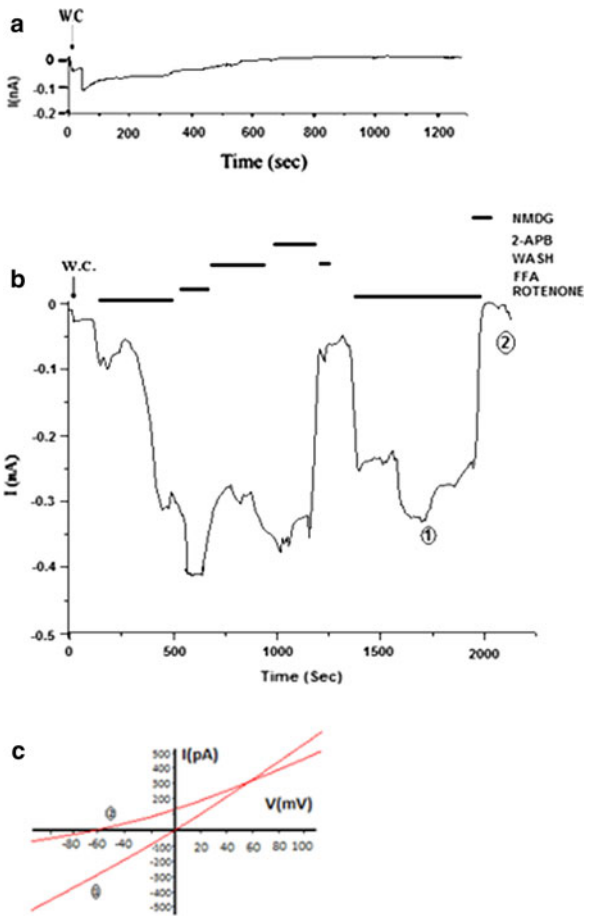

Fig. 3 Effects of extracellular FFA on rotenone-induced TRPM2 channel activation in DRG neurons. Holding potential was −60 mV. **a** Original recordings from control cell. **b** Cell expressing TRPM2 currents stimulated by rotenone (0.1 mM) in bath (chamber) and inhibited by FFA (0.1 mM) in bath (chamber). **c** Current–voltage relationships of FFA and rotenone currents through TRPM2 in presence of various extracellular cations (same experiments as in **b**). *W.C.* whole cell

DRG neurons was 125.5 ± 23.0 pA/pF ($n = 8$). Current densities were significantly ($P < 0.001$) higher in the ADPR group than in control (Fig. 3).

Effects of Rotenone

Mitochondria are the most likely source of ROS in DRG neurons, and rotenone inhibited mitochondrial complex I (NADH dehydrogenase) (Betarbet et al. 2000). Previous reports in transfected cells (Nazıroğlu and Lückhoff 2008a, b) indicated that TRPM2 channels were gated by high levels of H_2O_2 (10 mM), and TRPM2 channels seem to activate intracellular ROS mostly. In the present experiments, we chose the intracellular mitochondrial ROS producer rotenone in the rat DRG neuron system. We were able to evoke TRPM2 currents consistently with extracellular rotenone (Figs. 3, 5). There was a significant ($P < 0.001$) effect of rotenone on gating the TRPM2 channels in the cell system (Fig. 6). Control data were obtained on every experimental day of studying TRPM2. The positive data with rotenone would support the idea that rotenone acts by initiating a metabolic cascade, resulting in the production of a cytosolic factor such as oxidative stress and ADPR that is responsible for the activation of TRPM2 channel activity.

Effects of TRPM2 Antagonist in DRG Neurons

We next tested whether FFA and 2-APB would prevent or attenuate the induction of TRPM2 currents by ADPR and rotenone. First, we tested 0.1 mM concentrations of extracellular FFA in the cells. Unexpectedly, FFA exerted slight ($P < 0.05$) inhibition even its highest available concentration (Figs. 4, 6).

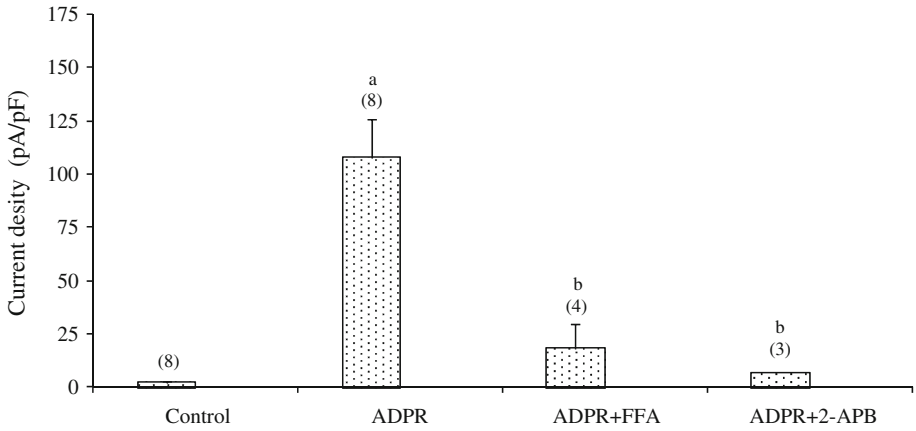

Fig. 4 Effects of FFA (0.1 mM in bath) and 2-APB (0.05 mM in bath) on ADPR (1 mM in pipette)-induced currents of TRPM2 channel in DRG neuronal cells. For each of the two applications studied, the initial current density was divided by the cell capacitance, a measure of cell size as well as maximal current density after dialysis with ADPR. *Numbers in parentheses* indicate numbers of groups. Significant ($^aP < 0.001$ vs. control and $^bP < 0.001$ vs. ADPR) stimulation and inhibition of currents are indicated (mean ± SD)

Next, we examined the effects of 2-APB (0.05 mM) on the ADPR- and rotenone-induced TRPM2 currents. The 2-APB induced inhibition of ADPR-induced TRPM2 currents ($P < 0.001$). The values for the current densities of the control, ADPR, ADPR + FFA and ADPR + 2-APB groups in the DRG neurons (pA/pF) were 2.3 ± 0.3 ($n = 8$), 107.8 ± 17.9 ($n = 8$), 18.2 ± 11.5 ($n = 4$) and 6.5 ± 0.3 ($n = 3$) (−60 mV holding potential), respectively. The values for the current densities of the control, rotenone, rotenone + FFA and rotenone + 2-APB groups in the DRG neurons (pA/pF) were 1.4 ± 0.4 ($n = 4$), 140.9 ± 28.3 ($n = 8$), 111.8 ± 20.5 ($n = 4$) and 3.3 ± 0.9 ($n = 4$) (−60 mV holding potential), respectively. The results of rat experiments are presented in Figs. 4b and 5b, in which only cells are included. In all cases, however, the currents were also blocked by NMDG for checking cell membrane viability. The current densities were significantly lower in rotenone + 2-APB ($P < 0.001$) and rotenone + FFA ($P < 0.05$) than in the rotenone group (Fig. 6).

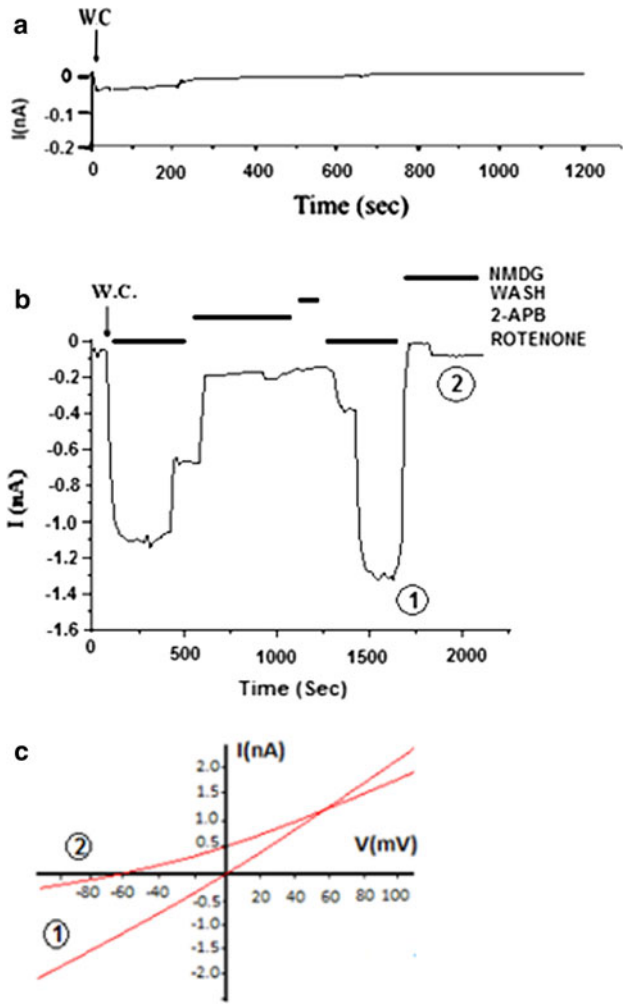

Fig. 5 Effects of extracellular 2-APB on rotenone-induced TRPM2 channel activation in DRG neurons. Holding potential was –60 mV. **a** Original recordings from control cell. **b** Cell expressing TRPM2 currents stimulated by rotenone (0.1 mM) in bath (chamber) and inhibited by 2-APB (0.05 mM) in bath (chamber). **c** Current–voltage relationships of 2-APB and rotenone currents through TRPM2 in presence of various extracellular cations (same experiments as in **b**). *W.C.* whole cell

Gating Time of TRPM2 Channels by ADPR and Rotenone

The time course (gating time) effects of ADPR and rotenone in rat DRG neuronal cells were 2.09 ± 0.38 and 4.26 ± 0.63 min, respectively. Gating time of rotenone was significantly ($P < 0.001$) longer than in ADPR.

Discussion

We found that ADPR and rotenone stimulated TRPM2 currents in DRG neurons. To our knowledge, there is no report on the effects of ADPR and rotenone in TRPM2 channels of rat DRG neurons. In the current study, intracellular ADPR- or rotenone-induced TRPM2 gating was blocked by either 2-APB or FFA. Therefore, the intracellular pathways may be involved in Ca^{2+} gating by intracellularly added ADPR. In addition, 2-APB is known to affect many proteins including TRP channels as well as IP_3 receptors (Xu et al. 2005). We observed that ADPR-induced Ca^{2+} entry through TRPM2 is inhibited by 2-APB using neuronal cells. Therefore, in this study, we believe that 2-APB acted as a TRPM2 channel inhibitor in DRG neurons.

There has been growing interest in the TRPM2 channel in neuronal damage resulting from oxidative stress (Nazıroğlu 2011). Our results also indicate that TRPM2 channels are activated by ROS during exposure of DRG neurons to rotenone. The evidence for this mechanism is based on the following observations: (1) rotenone induced a fast rise in free cytosolic Ca^{2+} and (2) the rise in free cytosolic Ca^{2+} was reduced by a TRPM2 channel blocker, 2-APB (Togashi et al. 2008).

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 evokes Ca^{2+} influx by increasing intracellular ADPR levels and by subsequent binding of NAD^+ directly to the Nudix motif in the cytosolic C terminus 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 rotenone is a strong oxidant, it is possible that intracellular ROS can oxidize NADH to NAD^+ in rotenone-treated cells.

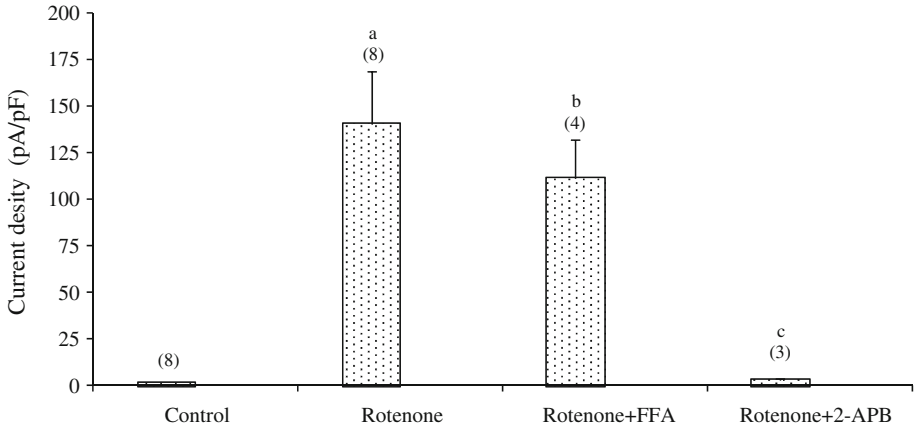

Fig. 6 Effects of FFA (0.1 mM in bath) and 2-APB (0.05 mM in bath) on rotenone (0.1 mM in bath)-induced currents of TRPM2 channel in DRG neuronal cells. For each of the two applications studied, the initial current density was divided by the cell capacitance, a measure of cell size as well as maximal current density after dialysis with rotenone. *Numbers in parentheses* indicate numbers of groups. Significant ($^{a}P < 0.001$ vs. control, $^{b}P < 0.05$ and $^{c}P < 0.001$ vs. rotenone) stimulation and inhibition of currents are indicated (mean ± SD)

In the present study we examined the effects of rotenone and ADPR on activation of TRPM2 channel in DRG neurons. The present study found that nonselective cation channel currents were induced by ADPR and rotenone. The ADPR- and rotenone-induced activation of TRPM2 channels is separately characterized by increased current levels. Activation of TRPM2 by oxidative stress has been reported from two approaches. One group suggested that it gates the channel independently of ADPR (Perraud et al. 2005), and the activation of TRPM2 by oxidative stress is probably linked to the activity of the poly(ADPR) polymerase (PARP-1), an enzyme that transfers multiple ADPR groups to proteins. Evidence for this intracellular pathway resulting in TRPM2 activation has been confirmed by the use of inhibitors of PARP-1, which were able to interfere with the H_2O_2-induced TRPM2 activation (Perraud et al. 2005; Buelow et al. 2008). However, although evidence has been presented to indicate that PARP-1 is present in mitochondria, oxidative stress has also been suggested to have direct effects on mitochondria (Guse 2005; Nazıroğlu 2007), such that the interpretation of its capacity to induce DNA damage and activate PARP-1 may not be justified. In addition, PARP-1 inhibitors are known to be promiscuous within the PARP family because of their structural mimicry of nicotinamide (Guse 2005), and the concentrations required to inhibit oxidant-mediated TRPM2 gating are well above those required to inhibit PARP-1 in vitro, precluding an unambiguous interpretation of the in vitro or in vivo targets of these compounds. Finally, it is not obvious, at least to these authors, why PARP-1 activation should be connected to activation of a plasma membrane channel (Perraud et al. 2005). Other groups have shown ADPR- and oxidative stress-induced opening of TRPM2 channels both extracellularly and intracellularly (Wehage et al. 2002; Nazıroğlu and Lückhoff 2008a, b). Indeed, currents through ADPR and rotenone as well as an increase in free Ca^{2+} were consistently observed. In the DRG neurons, our current responses in whole-cell configuration also raise the possibility of a direct and intracellular effect of ADPR and rotenone on TRPM2 channels. The results of the current study support strongly the hypothesis of intracellular gating mechanisms in TRPM2 channels of the DRG neurons by ADPR and oxidative stress.

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, caused a decrease in rotenone-induced $[Ca^{2+}]_i$ concentration, while FFA induced a tendency to decrease the $[Ca^{2+}]_i$ concentration. We investigated the effects of TRPM2 channel antagonists 2-APB and FFA on ADPR- and rotenone-induced currents in the cell systems. In DRG neurons 2-APB blocked totally ADPR- and rotenone-induced TRPM2 currents. FFA also blocked ADPR-induced currents in the cell system, although it induced a slight block on rotenone-induced TRPM2 currents.

In conclusion, these results demonstrate that intracellular ADPR and extracellular rotenone are capable of activating TRPM2 in DRG neurons. Hence, our results indicate that endogenous oxidative stress has a neurodegenerative effect in DRG neurons. In addition, TRPM2 channel antagonists 2-APB and FFA induced effects on ADPR and

rotenone-induced currents. TRPM2 is a widely expressed channel that is involved in both health and disease of neurological cells (Clapham 2007; Nazıroğlu 2011); the novel regulatory mechanism of TRPM2 channel by oxidative stress (rotenone) and ADPR in DRG neurons may have physiological and clinical relevance.

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Conflict of interest None.

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