J Membrane Biol (2011) 241:51–57
DOI 10.1007/s00232-011-9356-8

ORIGINAL ARTICLES

TRPM2 Channel Membrane Currents in Primary Rat Megakaryocytes Were Activated by the Agonist ADP-Ribose but Not Oxidative Stress

Mustafa Nazıroğlu

Received: 24 January 2011 / Accepted: 7 March 2011 / Published online: 22 April 2011

Abstract Melastatin-like transient receptor potential 2 (TRPM2) channel activation/inhibition mechanisms in response to ADP-ribose (ADPR), oxidative stress, flufenamic acid (FFA) and 2-aminoethoxydiphenyl borate (2-APB) are not clear. We tested the effects of FFA and 2-APB on ADPR-induced TRPM2 cation channel currents in rat native bone marrow megakaryocytes. Megakaryocyte cells were freshly isolated from rat bone marrow and studied with the conventional whole-cell patch-clamp technique. Extracellular H_2O_2, FFA and 2-APB were added through the patch chamber, while intracellular ADPR was applied through the pipette. Nonselective cation currents were consistently induced by ADPR but not H_2O_2. Current density of ADPR in the cells was significantly ($P < 0.001$) higher than in control. The time courses of ADPR effects in the megakaryocytes were characterized by a delay of 2.24 ± 0.73. The ADPR-induced Ca^{2+} gate was not blocked by either the IP_3 receptor inhibitor 2-APB or the PLC inhibitor FFA. In conclusion, TRPM2 channels were constitutively activated by intracellular ADPR, although the channel currents in rat native megakaryocytes were not affected by extracellular H_2O_2, 2-APB or FFA. Activation of TRPM2 channels in megakaryocytes seems to be intracellular and ADPR-dependent.

Keywords TRPM2 channel · Channel antagonist · ADP-ribose · Oxidative stress · Megakaryocyte

M. Nazıroğlu (✉)
Department of Biophysics, Medical Faculty, Süleyman Demirel University, Morfoloji Binasi, 32260 Isparta, Turkey
e-mail: mnaziroglu@med.sdu.edu.tr; mn131@le.ac.uk

Introduction

Reactive oxygen species (ROS) including superoxide anion, hydrogen peroxide (H_2O_2) and singlet oxygen 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, 2011). ROS increase second messengers such as Ca^{2+} (Grupe et al. 2010) and activate signaling kinases such as extracellular signal–regulated kinase (Guyton et al. 1996). There is also evidence that ROS play an important role in the pathogenesis of many diseases, particularly in blood diseases due to susceptibility of platelets to oxidative stress (Förstermann 2010). For example, Rosado et al. (2004) reported the involvement of H_2O_2 in capacitive calcium entry in human platelets. Additionally, Pariente et al. (2001) showed that Ca^{2+} was released from intracellular mitochondrial and nonmitochondrial Ca^{2+} stores in mouse pancreatic acinar cells by H_2O_2. However, the toxic properties of oxidative stress with regard to the function of transient receptor potential (TRP) melastatin 2 (TRPM2) channels in platelets and megakaryocytes are as yet unknown.

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). The channel in native blood cells such as neutrophils (Heiner et al. 2006) and mouse megakaryocytes (Carter et al. 2006; Nazıroğlu 2010) can also be gated by ADPR. TRP channels are blocked by nonspecific chemicals. However, as in most

TRP channels, the range of pharmacological modulators of TRPM2 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 antagonist (Xu et al. 2005; Togashi et al. 2008). However, reports of 2-APB and FFA as TRPM2 channel blockers in different cell systems are conflicting.

The ion channel phenotype of the mammalian platelet has proven difficult to study using direct electrophysiological techniques due to its small and fragile nature (Mahaut-Smith 2004). Several groups have recognized that mature, primary megakaryocytes express most, if not all, platelet proteins and that these megakaryocyte proteins are able to participate in major platelet functional responses (Tolhurst et al. 2008). In contrast to platelets, direct electrophysiological studies of primary megakaryocytes have been reported by multiple laboratories (reviewed in Mahaut-Smith 2004). Indeed, patch-clamp studies of megakaryocytes were critical in the identification of TRPM2 cation channel-induced, Ca^{2+}-permeable channels in murine megakaryocytes (Tolhurst et al. 2008) and Kv1.3-deficient mouse megakaryocytes (Nazıroğlu 2010). There are no reports of the ability of ADPR and oxidative stress to influence cation channels in the rat megakaryocyte of the presence of TRPM2 in this well-studied surrogate.

The molecular mechanism by which ADPR leads to gating of TRPM2 channels in native megakaryocytes 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 H_2O_2 were applied to freshly isolated rat native megakaryocytes. Furthermore, the present study was aimed at elucidating the role of 2-APB and FFA in modulating the effects of ADPR-induced gating on TRPM2 channels.

Materials and Methods

Preparation of Cell Samples

We used male Wistar rats (aged 12–14 weeks) in the current study. The animals were killed by CO_2 asphyxiation and cervical dislocation in accordance with Leicester University Experimental Animal legislation. Marrow samples were gently flushed from the lumen of femur and tibia bones using standard extracellular bath solution (see Electrophysiology). Bone marrow samples were slowly cycled in Eppendorf tubes for 2–3 h. Cells were seeded in a patch-clamp chamber, and electrophysiological studies were carried out within 1 h.

Electrophysiology

Patch-clamp techniques have been described in detail elsewhere (Nazıroğlu 2010). Cells were studied with the patch-clamp technique in the whole-cell mode, using an Axopatch 200B equipped with a personal computer with Clampfit v9.0 (Axon Instruments, Union City, CA). Pipettes were made of borosilicate glass. Membrane currents during voltage ramps were filtered at 1 kHz and an acquired rate of 5 kHz, using a Digidata 1200 A/D converter and Pclamp 6 (Axon CNC; Molecular Devices, Sunnyvale, CA). Membrane currents were also digitized at a slower rate (60 Hz, filtered at 30 Hz) using a Cairn Research (Faversham, Kent) acquision system to provide continuous recording of holding current. The standard extracellular bath solution contained (in mM) 145 NaCl, 1.0 $MgCl_2$, 1.0 $CaCl_2$, 5 KCl, 10 HEPES and 10 glucose, pH adjusted with NaOH to 7.35. Na^+ was replaced by 150 mM *N*-methyl-D-glucamine (NMDG), and the pH was adjusted with HCl and NMDG-Cl. In some electrophysiological recordings, saline was used to eliminate K^+-selective membrane currents; thus, KCl in the external saline was replaced with an equal concentration of NaCl. Hence, K^+ free extracellular solution contained (in mM) 150 NaCl, 1.0 $MgCl_2$, 10 HEPES and 10 glucose, pH adjusted with NaOH to 7.35. The pipette solution contained (in mM) 150 cesium chloride, 0.1 EGTA, 2 $MgCl_2$ and 10 HEPES (pH 7.2, adjusted with CsOH). The calcium concentration was adjusted to 1 μM (0.886 mM $CaCl_2$).

Cells were held at a potential of −70 mV, and current–voltage (*I–V*) relations were obtained from voltage ramps from −80 to +80 mV applied over 300 ms. H_2O_2 was daily prepared in extracellular bath solution. Stock (500 mM) ADPR, 2-APB and FFA were dissolved in dimethyl sulfoxide (DMSO) and stored at −20°C. Before the experiment, ADPR (0.3 and 1 mM) in internal buffer, FFA (0.1–0.2 mM) and 2-APB (0.05 mM) in extracellular bath solutions were diluted to reach the final concentrations. 2-APB and FFA were diluted in K^+-free extracellular solution. All experiments were carried out at room temperature.

Data are expressed as mean ± SD. Statistical significance between groups was assessed with the Mann–Whitney *U*-test. A value of $P < 0.05$ was considered significant.

Results

Effects of ADPR

TRPM2 channels expressed in rat megakaryocytes were studied with the patch-clamp technique in conventional whole-cell mode. Channels were stimulated by ADPR (0.3–1 mM) applied to the cytosolic side by diffusion from the patch pipette. ADPR (0.3 mM) and high Ca^{2+} (1 μM

Ca^{2+} with EGTA) have been used in transfected cell systems for gating the TRPM2 channel (Nazıroğlu and Lückhoff 2008a, b; Kühn et al. 2009). However, I was unable to obtain substantial inward currents with 0.3 mM ADPR ($n = 5$, data not shown). In contrast, 1 mM ADPR activated an inward current in rat megakaryocytes, and the activation was induced temporarily by ADPR. Representative experiments are shown in Fig. 1. Currents induced by ADPR developed gradually during infusion of ADPR into the cells and reached amplitudes (at a holding potential of −70 mV) of well above 0.6 nA in the inward direction. These currents were reversibly blocked by replacement of external Na^+ with the large impermeable cation NMDG; this was abolished by the inward and outward components of the currents (see Fig. 1b). No currents were seen in the absence of ADPR (Fig. 1a). The value for the current densities in the absence of ADPR in control rat

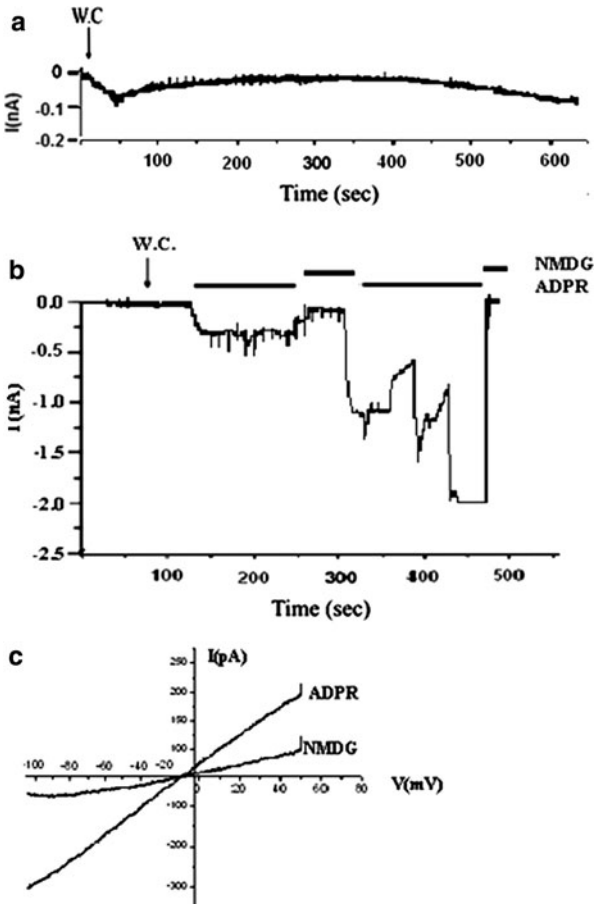

Fig. 1 Effects of intracellular ADPR on TRPM2 channels in native rat megakaryocytes. Normal bath solution (145 mM Na^+) was changed to a solution with NMDG as the main cation (150 mM, no Ca^{2+} present). Holding potential was −70 mV. **a** Original recordings from control cell. **b** Cell expressing TRPM2 currents stimulated with ADPR (1 mM) in the pipette. **c** Current–voltage relationships of ADPR currents through TRPM2 in the presence of various extracellular cations (same experiments as in **b**). *W.C.*, whole cell

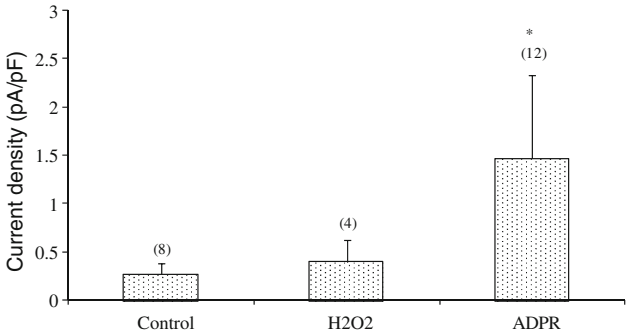

Fig. 2 Effects of H_2O_2 (10 mM in bath) and ADPR (1 mM in pipette) on currents of TRPM2 channels in native rat megakaryocytes. For each of the two applications studied in the cells, the initial current density divided by the cell capacitance, a measure of cell size, as well as maximal current density after dialysis with ADPR and H_2O_2 was administered. Numbers in parentheses indicate the number of cells studied. *Significant ($P < 0.001$) stimulation of currents vs. control (without ADPR) (mean ± SD)

megakaryocytes was 0.40 ± 0.22 pA/pF ($n = 8$) (−70 mV holding potential). The value for the current densities of ADPR in megakaryocytes was 1.47 ± 0.86 pA/pF ($n = 12$). Current densities of the megakaryocytes were significantly ($P < 0.001$) higher in the ADPR-applied group than in control (Fig. 2).

Effects of H_2O_2

H_2O_2, a ROS, is often used in experimental models of oxidative stress, although it may also function as an important signaling molecule in diverse cellular physiological functions such as proliferation and development (Veal et al. 2007). Previous reports on transfected cells (Nazıroğlu and Lückhoff 2008a, b) indicated that TRPM2 channels were gated by high levels of H_2O_2 (10 mM). In the present experiments, I chose the same amount of H_2O_2 in the rat megakaryocyte cell system. I was not able again to evoke TRPM2 currents consistently with extracellular H_2O_2 (Fig. 3). Control data were obtained on every experimental day of studying TRPM2. Values for the current densities of the control and H_2O_2 groups in megakaryocytes were 0.27 ± 0.11 pA/pF ($n = 8$) and 0.40 ± 0.22 pA/pF ($n = 4$) (−70 mV holding potential), respectively. The negative data with H_2O_2 would not support the idea that H_2O_2 acts by initiating a metabolic cascade resulting in the production of a cytosolic factor such as ADPR that is responsible for the activation of TRPM2 channel activity.

Effects of TRPM2 Antagonists in Megakaryocytes

I next tested whether antagonists (FFA and 2-APB) would prevent or attenuate the induction of TRPM2 currents by ADPR. First, I tested two concentrations (0.1 and 0.2 mM)

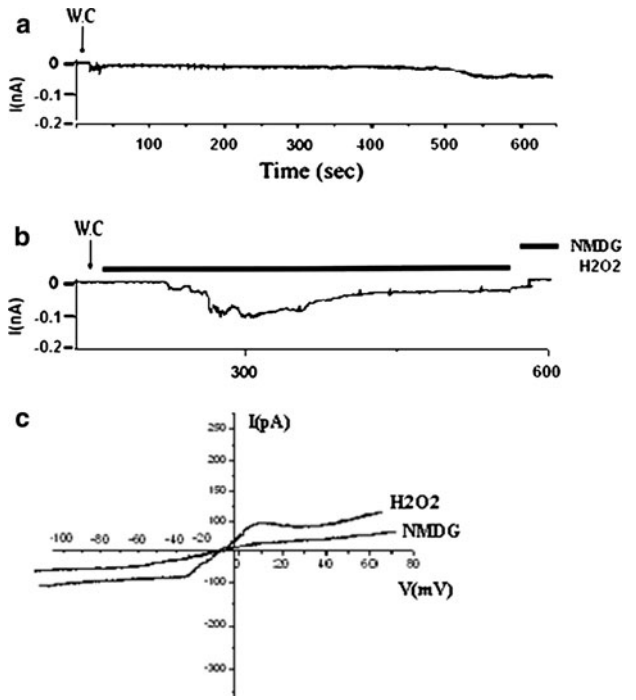

Fig. 3 Effects of extracellular H_2O_2 on TRPM2 channels in native rat megakaryocytes. Normal bath solution (145 mM Na^+) was changed to a solution with NMDG as the main cation (150 mM, no Ca^{2+} present). Holding potential was −70 mV. **a** Original recordings from control cell. **b** Cell expressing TRPM2 currents stimulated with H_2O_2 (10 mM) in the bath chamber. **c** Current–voltage relationships of ADPR currents through TRPM2 in the presence of various extracellular cations (same experiments as in **b**). *W.C.*, whole cell

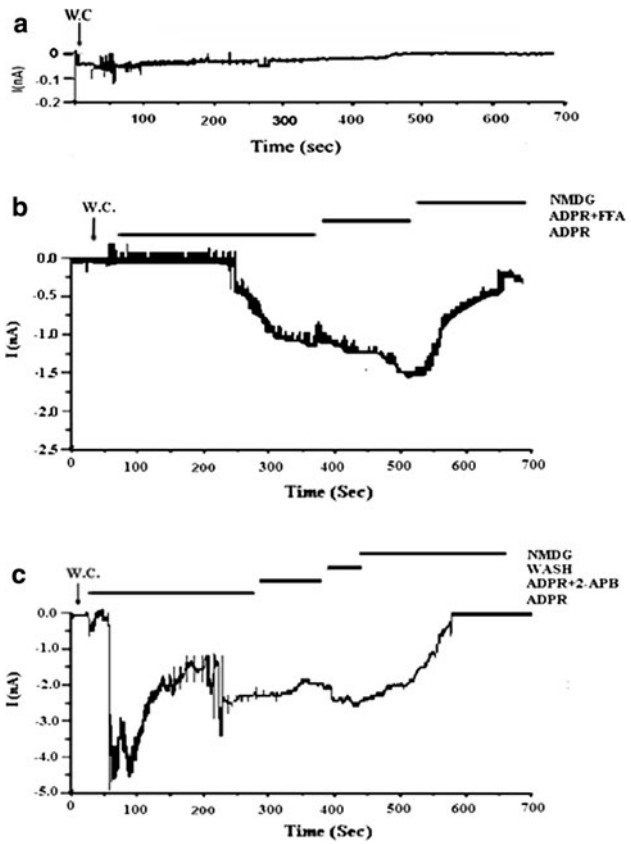

Fig. 4 There was no inhibitor role of extracellular 2-APB and FFA on ADPR-induced currents of the TRPM2 channel in native rat megakaryocytes. When the whole-cell K^+-free bath solution (145 mM Na^+) was changed to a solution with NMDG as the main cation (150 mM, no Ca^{2+} present), 2-APB (0.05 mM in K^+-free bath solution) or FFA (0.2 mM in K^+-free bath solution). Holding potential was −70 mV. **a** Original recordings from control cell. **b** Ca^{2+} selectivity of TRPM2. A cell expressing TRPM2 was stimulated with ADPR (1 mM in pipette), and the channel was not inhibited by FFA. **c** Ca^{2+} selectivity of TRPM2. A cell expressing TRPM2 was stimulated with ADPR, and the channel was not inhibited by 2-APB. *W.C.*, whole cell

of extracellular FFA in the cells. FFA did not exert inhibition even at the highest available concentration (Fig. 4). I also attempted to examine the effects of 2-APB (0.05 mM) on the ADPR-induced TRPM2 currents. Unexpectedly, 2-APB did not also lead to inhibition of ADPR-induced TRPM2 currents, which suggests that the noninhibiton was mediated by $InsP_3$ receptors. The results of rat experiments are presented in Fig. 4b, c, in which only cells are included. In all cases, however, the currents were blocked by substitution of NMDG for Na^+. There was no statically significant difference among ADPR, ADPR + FFA and ADPR + 2-APB (Fig. 5).

Discussion

I found that ADPR stimulated Ca^{2+} gating in megakaryocyte cells, although H_2O_2 did not induce an effect on TRPM2 channel current in the cells. To our knowledge, this is the first report on the effects of ADPR and H_2O_2 in TRPM2 channels of rat megakaryocytes. In the current study, ADPR-induced Ca^{2+} gating was not blocked by either the IP_3 receptor inhibitor 2-APB or the PLC inhibitor

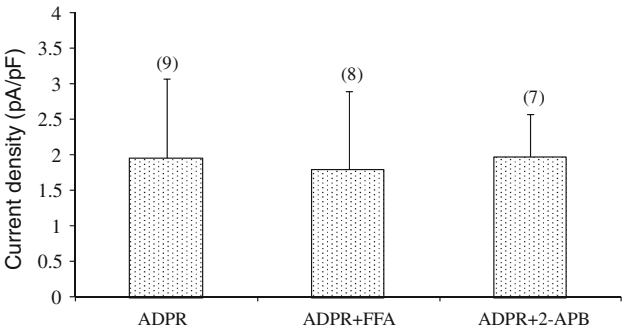

Fig. 5 Effects of extracellular FFA (0.2 mM in chamber) and 2-APB (0.05 mM in chamber) on ADPR (1 mM in pipette)-induced TRPM2 channel currents in native rat megakaryocytes. For each of the two applications studied in the cells, the initial current density divided by the cell capacitance, a measure of cell size, as well as maximal current density after dialysis with ADPR, ADP + FFA and ADPR + 2-APB was administered. Numbers in parentheses indicate the number of cells studied (mean ± SD)

FFA. Therefore, the PLC–IP_3 pathway may not be involved in Ca^{2+} gating by intracellular ADPR. In addition, although 2-APB is known to affect many proteins including TRP channels in addition to IP_3 receptors, it had no effect on TRPM2 opening by intracellular ADPR (Xu et al. 2005). I observed that ADPR-induced Ca^{2+} entry through TRPM2 is not inhibited by 2-APB using a cell system. Therefore, in this study, I believe that 2-APB act as an IP_3 inhibitor but not as a TRPM2 inhibitor.

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 pyrophosphates that degrade nucleoside diphosphates (Clapham 2007; Nazıroğlu 2011). Wehage et al. (2002) reported that 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 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 H_2O_2 is a strong oxidant, it is possible that intracellular H_2O_2 can oxidize NADH to NAD^+ in H_2O_2-treated cells.

In the present study I examined the effects of H_2O_2 and ADPR on activation of TRPM2 channels in megakaryocytes. The nonselective cation channel currents were induced by ADPR. The ADPR-induced activation in the TRPM2 channels is separately characterized by increased current levels. Activation of TRPM2 by H_2O_2 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 H_2O_2 is probably linked to the activity of the poly(ADP-ribose) 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, H_2O_2 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 me, why PARP-1 activation should be connected to activation of a plasma membrane channel (Perraud et al. 2005). Other groups have shown ADPR and H_2O_2-induced opening of TRPM2 channels directly (Wehage et al. 2002; Nazıroğlu and Lückhoff 2008a, 2008b). Indeed, currents through ADPR as well as an increase in free Ca^{2+} were consistently observed, although H_2O_2 did not induce a significant effect on the current. In rat megakaryocyte cells, my current responses in whole-cell configuration also raise the possibility of a direct and intracellular effect of ADPR on TRPM2 channels. The results of the current study support strongly the hypothesis of intracellular gating mechanisms in TRPM2 channels of megakaryocytes by ADPR.

In the present study, TRPM2 channels were not activated by H_2O_2. Similarly, Ishii et al. (2006) reported that H_2O_2-induced Ca^{2+} entry in native A172 cells was not observed. At least, H_2O_2 is not likely to be changed to NAD^+ metabolites such as NAD^+ and cyclic ADPR, which are activators of TRPM2, in the cell interior since extracellular H_2O_2 does not stimulate Ca^{2+} entry in TRPM channels of megakaryocytes. Therefore, Ca^{2+} influx in response to extracellular H_2O_2 is likely to be mediated via the IP_3 receptor but not via TRPM2. Future studies are needed to examine the mechanism of the H_2O_2-induced Ca^{2+} response in detail.

The differing results may arise from the use of different cell types to study TRPM channel activation by H_2O_2 and ADPR. On the other hand, the channel activation by ADPR and H_2O_2 appears to represent a cell-specific process in cells with endogenous expression of TRPM2. For example, the TRPM2 channel is activated by H_2O_2 in human embryonic kidney (HEK) 293 cells (Wehage et al. 2002), Chinese hamster ovary cells (Nazıroğlu and Lückhoff 2008a, b; Kühn et al. 2009) and rat primary striatal cultures (Fonfria et al. 2005) but not in the rat beta-cell line RIN-5F (Ishii et al. 2006), human neutrophil granulocytes (Heiner et al. 2006) or *Xenopus* oocytes (Toth and Csanady 2010). Kolisek et al. (2005) in HEK293 cells used cADPR, and the compound-stimulated TRPM2 channel was opened by cADPR. However, two recent studies did not support the results for neutrophil granulocytes (Heiner et al. 2006) and *Xenopus* oocytes (Toth and Csanady 2010), and TRPM2 channels did not open by stimulation with cADPR.

Activation of TRPM2 channels results in prolonged Ca^{2+} influx, which may lead to loss of cell viability. However, the relationship between channel activation, intracellular Ca^{2+} increase and apoptosis is still unclear in most cells (Wilkinson et al. 2008) via oxidative stress and ADPR, a problem compounded by uncertainty of the second messengers involved and the limited specificity of the pharmacological blockers (Clapham 2007). I investigated the effects of TRPM2 channel antagonists, 2-APB and FFA, on ADPR-induced currents in the cell systems. The 2-APB and FFA did not lead to an inhibition of ADPR-induced TRPM2 currents. The effectiveness of

these compounds at blocking the respective TRP channels has previously been confirmed in a variety of heterologous expression systems. Similarly, it has been reported that 2-APB (0.075 and 0.150 mM) exhibited no effects on ADPR-evoked TRPM2 responses at a whole-cell current level (Xu et al. 2005). Recently, it was reported that FFA did not reduce H_2O_2-induced damage of CA1-CA3 pyramidal neurons (Bai and Lipski 2010). Wilkinson et al. (2008) reported that clotrimazole failed to block H_2O_2-induced Ca^{2+} influx in transfected (HEK293/hTRPM2) cells and that FFA induced only a partial inhibitory effect. Hence, our 2-APB and FFA findings are supported by recent reports (Xu et al. 2005; Wilkinson et al. 2008; Bai and Lipski 2010).

In conclusion, these results demonstrated that intracellular ADPR is capable of activating TRPM2 in rat megakaryocytes. The agonist is likely to interact separately with TRPM2 channels, consistent with the idea that they bind to the Nudix domain, intracellularly. In addition, the TRPM2 channel antagonists 2-APB and FFA induced no effect on ADPR-induced currents, which most probably involve interaction of the inhibiting mechanism of the channels with IP_3 and PLC inhibitors and point to the existence of a conserved and functionally important drug-binding site on many members of this emerging family of channels (Xu et al. 2005).

Acknowledgments The study was performed in the Department of Cell Physiology and Pharmacology, University of Leicester (Leicester, UK). M. N. was supported by an academic visitor research fellowship of the Scientific and Technological Research Council of Turkey, Ankara (grant TUBITAK-2219). The author thanks Professor Martyn P. Mahout-Smith for comments on the manuscript.

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