Activation of membrane TRPM2 channel currents in primary Kv1.3-deficient mice megakaryocyte by agonist ADP-Ribose

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List of abbreviations
ADPR, adenosine diphosphatase ribose
CHO, chinese hamster ovary
CRG-G1, Cambridge rat insulinoma G1
HEK, human embryonic kidney
NMDG, N-methyl-D-glucamine
ROS, reactive oxygen species
TRP, transient receptor potential
TRPM2, melastatin-like transient receptor potential 2

Abstract

Since the mechanisms that lead to melastatin-like transient receptor potential 2 (TRPM2) channel activation in response to ADP-Ribose (ADPR) are not understood, I tested the effects of ADP-Ribose on TRPM2 cation channel currents in native bone marrow megakaryocyte of mice.

Kv1.3-deficient mice megakaryocyte cells were freshly isolated from bone marrow and the cells studied with the conventional whole-cell patch clamp technique. ADPR (1 mM) was applied intracellularly through the pipette.

Non-selective cation currents in the mice were consistently induced by ADPR. Current density of ADPR in the cells were significantly ($p<0.001$) higher than in control. The time courses of ADPR effects in the mice were characterized by a delay of 3.66 ± 0.69.

In conclusion, TRPM2 channels were constitutively activated by intracellular ADPR currents in mice native megakaryocyte.

Keywords

TRPM2 channels, ADP-Ribose, bone marrow, megakaryocyte.

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Introduction

The TRPM2 channel protein has two distinct domains functioning as ion channel and ADP-ribose (ADPR)-specific pyrophosphatese. Primary gating mechanism of TRPM2 is through the binding of intracellular ADPR (Perraud et al., 2001). The TRPM2 channel is also 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 cell such as neutrophil (Heiner et al., 2006) and megakaryocyte (Carter et al., 2006) can also be gated by ADPR.

The ion channel phenotype of the mammalian platelet has proven difficult to study using direct electrophysiology 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 the platelet, 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+} influx in the platelet (Tolhurst et al., 2008). However, there is one report on role of ADPR in molecular pathways of mice megakaryocytes/platelets TRPM2 channels (Carter et al., 2006).

The molecular mechanism by which ADPR leads to gate of TRPM2 channels in megakaryocyte need to be elucidated in detail. To study the role of ADPR in TRPM2 channels we used an experimental model in which ADPR was applied to mice native megakaryocyte cells.

Material and methods

Preparation of cell samples

We used male Wistar rat Kv1.3/L K^+ knockout male mice (10-12 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 the femoral and tibial bones using standard extracellular bath solution (see electrophysiology). The bone marrow samples were slowly cycled in ependorf tubes for 2-3 hours. Cells were seeded on patch-clamp chamber and electrophysiological studies were carried out 2-3 hrs after remove in cells

Electrophysiology

Patch-clamp techniques have been described in detail elsewhere (Carter et al., 2006). The 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, USA). Pipettes were made of borosilicate glass. Membrane currents during voltage ramps were filtered 1 kHz and acquired rate of 5 kHz, using Digidata 1200 A/D converter and pclamp 6 (Axon CNC Molecular Device Corporation). Membrane currents were also digitized at a slower rate (60 Hz, filtered at 30 Hz) using Cairn Research Ltd (Faversham, Kent) acquision system to provide a 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, 10 glucose, pH adjusted with NaOH to 7.35. Na^+ was replaced by 150 mM NMDG (N-methyl-D-glucamine) and the pH was adjusted with HCl and NMDG-Cl. Some electrophysiological recording, salines were designed 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, 10 glucose, pH adjusted with NaOH to 7.35. The pipette solution contained in mM: 150 caesium chlorur, 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 and stored at -20 °C. Before the experiment, ADPR (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. For rat studies 2-APB and FFA were diluted in K+ free extracellular solution. Dilutions of 2-APB and FFA stock solution in mice studies we used standard extracellular bath solution because they are Kv1.3/L K^+ knockout mice (K^+ free). All experiments were carried out in room temperature.

Data are expressed as mean ± SD. Statistical significance between groups was assessed with Student's t-test. A vale of $p < 0.05$ was considered significant.

Results

Effects of ADP-ribose

The TRPM2 channels were expressed in rat and mice native bone marrow megakaryocyte and studied with the patch-clamp technique in conventional whole-cell mode. The channels were stimulated by ADPR (0.3-1 mM) applied to the cytosolic side of the channels by diffusion through

the patch pipette. Low level (0.3 mM) ADPR doses have been using in transfected cell systems for gating the TRPM2 channel (Nazıroğlu and Lückhoff, 2008a and b). The amount of the ADPR was not able to gate TRPM2 channels (data not shown). However, after application of 1 mM ADPR, we were able to gating TRPM2 channels in mice and rat megakaryocyte cells. Representative experiments on the channels in the mice are shown in Figure 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 0.5 nA in the inward direction. These currents were reversibly blocked by the large impermeable cation NMDG which abolishes the inward but not the outward component of the currents (see Figure 1B). No currents were seen in the absence of ADPR (Figure 1A). The values for the current densities of control cells in the mice were 0.17 ± 0.1 (n=3) pA/pF (-70 mV holding potential) in the absence of ADPR. The values for the current densities of ADPR in mice megakaryocyte cells were 7.45 ± 2.26 (n=4) pA/pF (-70 mV holding potential) in the presence of ADPR. Currents densities were significantly ($p<0.001$) higher in ADPR group than in control. The observed current characteristics were in complete agreement to previous studies on TRPM2 in various laboratories including our own one (Carter et al., 2006; Tolhurst et al., 2008).

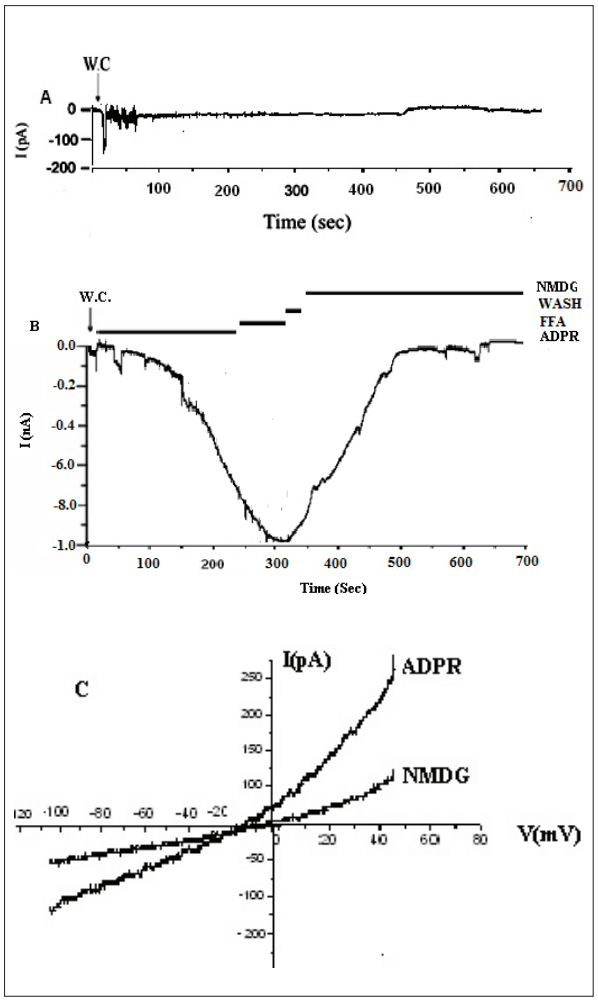

Figure 1. Effects of intracellular ADPR on TRPM2 channels in native Kv1.3/L K^+ knockout mice megakaryocyte. The time where the normal bath solution (145 mM Na^+) was changed to a solution with NMDG as main cation (150 mM, no Ca^{2+} present) is shown. The holding potential was –70 mV. **A.** Original recordings from control cell. **B**. Ca^{2+} selectivity of TRPM2. A cell expressing TRPM2 and stimulated with ADPR (1 mM in pipette) and the channel was not inhibited by FFA (0.1-0.2 mM in normal bath solution). **C.** Current voltage relationships of ADPR currents through TRPM2 in presence of various extracellular cations (same experiments as in panels B).

Effects of TRPM2 antagonist in megakaryocytes

We next tested whether the FFA and 2-APB would prevent or attenuate the induction of TRPM2 currents by ADPR. First we tested two (0.1 and 0.2 mM) concentrations of extracellular FFA in the cells. FFA did not exert inhibition even its highest available concentration (Figure 1). We attempted to examine the effects of 2-APB (0.05 mM) on the ADPR-induced TRPM2 currents. Unexpectedly, the 2-APB did not also lead to an inhibition of ADPR-induced TRPM2 currents, which suggests that the noninhibiton was mediated by Ins3 receptors The results of rat experiments are presented in Figures 1B and 1C in which only cells are

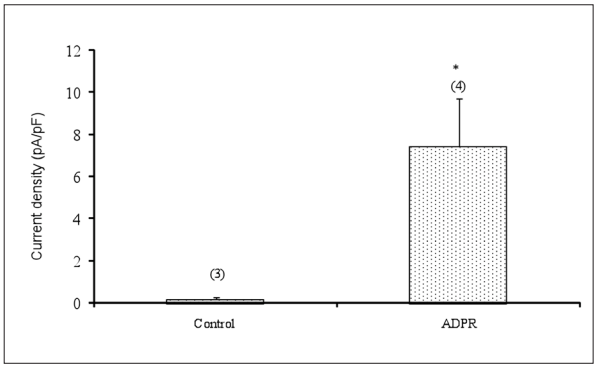

Figure 2. Effects of ADPR (1 mM in pipette) on currents of TRPM2 channel in native Kv1.3/L K^+ knockout mice megakaryocyte. For the applications studied in the cells, the initial current density, divided by the cell capacitance, a measure of cell size as well as maximal current density after dialysis with ADPR was administrated. The numbers in parentheses indicate the number of cells studied. Significant ($p<0.001$) stimulation of currents versus control (without ADPR) is indicated with *asterisk.* (mean ± SD).

included. In all cases, however, the currents were blocked by substation of NMDG for Na^+.

Discussion

We found that ADP-ribose stimulated Ca^{2+} gate in the megakaryocyte cells. In the current study, ADPR-induced Ca^{2+} gate was not blocked either the IP_3 receptor inhibitor 2-APB or PLC inhibitor FFA. Therefore, the PLC-IP_3 pathway may not be involve in Ca^{2+} gate by the intracellular added 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). We observed that ADPR-induced Ca^{2+} entry through TRPM2 is not inhibited by 2-APB using a native cell system. Therefore, in this study, we 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 pyrophosphatases that degrade nucleoside diphosphates (Nazıroğlu, 2010). 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-terminal of TRPM2. TRPM2 is also known to respond to intracellular ADPR, a metabolite of NAD^+, via direct binding to the Nudix domain (Hara et al., 2002; Wehage et al., 2002).

In the present study we examined the effects ADPR on activation of TRPM2 channel in magakaryocyte. The present study found that the non-selective 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 transferring multiple ADPR groups to proteins. Evidence for this intracellular pathway resulting in TRPM2 activation has been confirmed by the use of inhibitors of poly(ADP- ribose) polymerase (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 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 H_2O_2-induced opening of TRPM2 channel directly (Wehage et al., 2002; Naziroglu and Luckhoff, 2008a and b). Indeed, currents through ADPR as well as an increase in free Ca^{2+} were consistently observed although H_2O_2 did not induce significant effect on the current. In rat and mice megakaryocyte cells, our current responses in whole cell configuration, also raises the possibility of a direct and intracellular effect of ADPR on TRPM2 channels. The results of the current study support strongly the hypothesis of separately and direct (intracellular) gating mechanisms in TRPM2 channels of the megakaryocyte by ADPR.

Activation of TRPM2 channels results in prolonged Ca^{2+} influx that may lead to loss of cell viability. However, the relationship between channel activation intracellular Ca^{2+} increase and apoptosis is still unclear in most of cells (Nazıroğlu, 2010) via oxidative stress and ADPR, a problem compounded by uncertainty of the second messengers involved and the limited specificity of the pharmacological blockers (Nazıroğlu, 2007). We investigated effects of TRPM2 channel antagonist namely 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 in 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 currents level (Xu et al., 2005). Recently it was reported that FFA did not reduce H_2O_2-induced damage of CA1-CA3 pyramidial 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 FFA induced only a partial inhibitory effect. Hence, our 2-APB and FFA findings were supported by recent reports (Xu et al., 2005; Wilkinson et al., 2008; Bai and Lipski, 2010).

In conclusion, these results demonstrated that ADPR is capable of activating TRPM2. In addition, TRPM2

channels antagonists, 2-APB and FFA, induced no effect on ADPR-induced currents and most probably involving interaction the inhibiting mechanism of the channels with IP3 and PLC inhibitor and pointing to the existence of a conserved and functionally important drug-binding site on many remembers of this emerging family of channels (Xu et al., 2005).

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