Transient receptor potential vanilloid-2 mediates the effects of transient heat shock on human monocyte-derived dendritic cells

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Abstract

The goal of the current study was to investigate the effect of heat shock on human *in vitro* differentiated monocyte-derived dendritic cells (DCs) and to dissect the role of thermosensitive transient receptor potential (TRP) in the process. We have shown that, of the thermosensitive TRP channels, DCs express TRPV1, TRPV2 and TRPV4. We have furthermore presented evidence that a short heat shock challenge (43°C for 1 hr) decreased the endocytotic activity of the DCs and that this effect could be alleviated by the RNAi-mediated knockdown of TRPV2 but, importantly, not by the pharmacological (antagonists) or molecular (RNAi) suppression of TRPV1 and TRPV4 activities/levels. Likewise, the heat shock-induced robust membrane currents were selectively and markedly inhibited by TRPV2 "silencing" whereas modulation of TRPV1 and TRPV4 activities, again, had no effect. These intriguing data introduce TRPV2-coupled signaling a key player in mediating the cellular actions of heat shock on DCs.

Key words: thermosensitive transient receptor potential (TRP) ion channels; TRPV2; human dendritic cells; phagocytosis; RNA interference

1. Introduction

Thermosensitive members of the transient receptor potential (TRP) superfamily can be gated by changes in temperature, and include the "warm-sensitive" TRPV1, TRPV2, TRPV3 and TRPV4 channels (all of which belong to the vanilloid TRP family), as well as the cold-sensitive TRPA1 and TRPM8 (members of the ankyrin and melastatin families of TRP channels, respectively) [1]. The role of these channels in neuronal thermosensation is well described [2]; indeed, on sensory neurons, the molecules were shown to act as non-selective cation channels which can be activated by diverse temperature-ranges [3, 4].

However, a growing body of recent evidence points to the expression and the functional significance of many of these channels expressed by non-neuronal cell types [5]. Among these non-neuronal cells, we have been particularly interested in the biology of dendritic cells (DCs). With respect to temperature challenges, on various murine DC types, mild heat stress (temperatures below 42°C) has been shown to increase antigen uptake, enhance maturation, increase T-cell priming ability, and also increase pro-inflammatory cytokine production (reviewed in [6]). Likewise, on human monocyte-derived DCs, both long-term heat treatment (40°C applied for 24 hrs) [7] and repetitive, short heat-stress challenges (41-41.5°C, 15-30 min) [8] have been shown to stimulate the expression of differentiation and/or maturation DC markers (CD80, CD83, CD86) [7], increase the production of certain cytokines [7, 8] and promote the activity of DCs to activate allogenic naïve T cells [7, 8]. These results implicate that heat treatment may prime the pro-inflammatory response of DCs.

Interestingly, mostly contrasting the above data, we have previously shown that the activation of the heat-sensitive (>43°C) TRPV1 channel, expressed on human monocyte-derived DCs, by the natural agonist capsaicin resulted in a dose-dependent and TRPV1-specific inhibition

of cytokine-induced DC differentiation, phagocytosis of bacteria, activation of DCs, and pro-inflammatory cytokine secretion [9]. Yet, we lack information on the role of TRPV1 (which was shown to play a role in the transduction of the heat shock response of epidermal keratinocytes [10, 11]) and possibly other TRP channels reportedly expressed by DCs and other immune cells (see below) in mediating the effects of heat stress on human DC biology.

Therefore, in the current study, we aimed at first defining the thermo-TRP profile of human monocyte-derived DCs. Next, we assessed the role of the expressed ion channels in mediating the functional consequence of heat application. Quite unexpectedly, as presented below, we found that the cellular effects induced by heat challenges (43°C, 1 hr) are most probably mediated by TRPV2 (and not by the other thermo-TRPs expressed by the cells).

2. Materials and methods

2.1. DC cultures

Monocytes were isolated from buffy coats by immunomagnetic cell separation using anti-CD14-conjugated microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany), as described previously [9]. DC differentiation (which resulted in immature DCs, iDCs) was induced by supplementing AIMV medium (Invitrogen, Paisley, UK) with 80 ng/ml GM-CSF and 100 ng/ml IL-4 (both from Peprotech, London, UK). The same amount of cytokines was added at day 2, and the cells were cultured for another 3 days. Mature DCs (mDCs) were generated by supplementing the culturing medium of iDCs with a "pro-inflammatory cytokine cocktail" containing 80 ng/ml GM-CSF, 10 ng/ml TNF-α, 5 ng/ml IL-1β, 20 ng/ml IL-6 (all from Peprotech), and 1 μg/ml PGE2 (Sigma-Aldrich, St. Louis, MO). All experiments were performed from a minimum of three independent donors.

2.2. Heat shock treatment and determination of endocytotic activity

Heat shocked DCs were incubated at 43°C for 1h, while control cells were kept at 37°C. Endocytotic activity was then measured by the internalization of FITC-labeled dextran (Sigma-Aldrich). In brief, DCs were incubated with 1 mg/ml FITC-dextran at 37°C for 1 hr after heat shock treatment (negative control cells were treated with 1% sodium azide and kept at 0°C). Afterwards, the cells were washed three times with ice-cold PBS, and then stored on ice until the fluorescence intensity was measured by a FACScan flow cytometer (BD Biosciences Immunocytometry Systems, Franklin Lakes, NJ). When applicable, DCs were pre-incubated with capsazepine (CPZ, TRPV1 antagonist, Sigma-Aldrich) or HC 067047 (TRPV4 antagonist, Maybridge Ltd., Cambridge, UK) for 15 minutes before heat shock.

2.3. Determination of cytotoxicity (necrosis)

As described before [12], necrosis was determined by assessing the release of glucose-6-phophate-dehydrogenase (G6PDH) from the cells.

2.4. Immunocytochemistry

Acetone-fixed DCs were immunolabeled using rabbit anti-TRPV primary antibodies (1:200, Abcam PLC, Cambridge, UK) and a FITC-conjugated secondary antibody (1:200, Vector Laboratories, Burlingame, CA) as described before [9].

2.5. Quantitative real-time PCR (Q-PCR)

Q-PCR experiments were performed as described previously [13] by using Taqman primers and probes (Applied Biosystems, Carlsbad, CA).

2.6. Gene silencing using small interfering RNA (RNAi) probes

Cells were transfected on the second day of differentiation with specific Stealth RNAi oligonucleotides (40 nM, Invitrogen) against TRPV channels (trpv1: HSS111306, trpv2: HSS12144, trpv4: HSS126974), using Lipofectamine 2000 Transfection Reagent (Invitrogen). For controls, RNAi Negative Control Duplexes (scrambled RNAi; Invitrogen) were employed. In all cases, 3 sequences supplied by the manufacturer were applied separately. The efficacy of RNAi-driven ‘‘knockdown’’ was evaluated on day 3 after transfection by Western blotting as described previously [13] (see also Figure 2C), using the same antibodies applied for immunochemistry, although at greater dilutions (1:500). Optical density was normalized to β-actin (antibody from Sigma-Aldrich, applied in 1:1000 dilution) and expressed relative to cells transfected with scrambled RNAi. In each case, the most effective RNAi oligonucleotide was selected and then used in subsequent functional experiments.

2.7. Patch-clamp measurements

The standard whole-cell patch-clamp techniques were employed in voltage-clamp configuration. Whole-cell measurements were carried out using Axopatch-200A amplifiers connected to personal computers using Axon Instruments Digidata 1440 data acquisition boards (Axon Instruments, Foster City, CA). For data acquisition and analysis, the pClamp10 software package (Molecular Devices, Sunnyvale, CA) was used. Pipettes were pulled from GC 150 F-15 borosilicate glass capillaries (Clark Biomedical Instruments, Pangbourne, UK) in five stages and fire polished to gain electrodes of 2-5 MΩ resistance in the bath. iDCs were identified and selected for patch-clamp recording in a Nikon TE2000 microscope. The normal bath or extracellular (EC) solution given in mM was as follows: 145 NaCl, 5 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 5.5 glucose, and 10 HEPES (pH 7.35, 305 mOsm). The pipette solution was as follows: 140 CsCl, 5 EGTA and 10 HEPES (pH 7.20, ~295 mOsm). The temperature of the EC solution perfusing the DCs was maintained (TC2bip Temperature Controller, Cell MicroControls) at a constant level of either ~37°C or ~45°C. The bath solution was warmed up to ~37°C using a custom-made Petri dish. Thermal stimuli were applied using a preheated bath solution and temperature was monitored using a thermistor placed close to the cell. Bath perfusion around the measured cell was achieved using a gravity-flow perfusion system. Excess fluid was removed continuously. Ruthenium Red (RR, Sigma-Aldrich), CPZ and HC 067047 were applied by switching the perfusion system to a preheated bath solution containing the relevant antagonists.

2.8. Statistical analysis

When applicable, data were analyzed using a two-tailed un-paired *t*-test and $P < 0.05$ values were regarded as significant differences.

3. Results

3.1. Human DCs express TRPV1, TRPV2, and TRPV4

First, we determined the expression of thermosensitive TRP channels on DCs. Using Q-PCR, we found that specific TRPV1, TRPV2, and TRPV4 mRNA transcripts were expressed both in iDCs and mDCs (as well as in monocytes) (Figure 1A). The protein expression of these channels was also verified by immunocytochemistry (Figure 1B). Interestingly, the expression of TRPV3, TRPM8, and TRPA1 was not identified on any of the tested cells by Q-PCR (Figure 1A) or immunolabeling (data not shown).

3.2. Heat shock inhibits endocytosis by activating TRPV2

We then assessed the effect of heat shock on iDCs. Importantly, a short heat shock (43ºC for 1 hr) decreased their endocytotic capacity (Figure 2A) whereas, at the same time, it did not induce significant cell death (Figure 2B). Although TRPV1 was recently implicated in the transduction of heat shock effects on keratinocytes [10, 11]; and, moreover, we have previously reported that TRPV1 activation inhibits the phagocytotic/endocytotic activity of iDCs [9]; neither the TRPV1 antagonist CPZ, nor the RNAi mediated knockdown of this protein were able to prevent the endocytosis-inhibitory action of the heat shock (Figure 2A). Likewise, the application of HC 067047, an antagonist of TRPV4, and the RNAi mediated silencing of TRPV4 also failed to influence the effect of heat shock (Figure 2A). Of great importance, however, RNAi mediated knockdown of TRPV2 (as demonstrated in Figure 2C) markedly prevented the action of heat shock (Figure 2A) (due to the lack of commercially available, highly selective TRPV2 antagonists, we were unable to perform pharmacological experiments).

3.3. Heat shock-induced membrane currents are mediated by TRPV2

To determine whether heat shock directly activates TRPV2 channels found on the iDCs and to assess the functionality of the molecules, we performed patch-clamp experiments. Using a self-made thermostatable patch-clamp setup, we found that heating the cells above 43°C induced a robust membrane current. Importantly, this current could be significantly antagonized by the general TRPV antagonist Ruthenium Red as well as the RNAi mediated knock-down of TRPV2, but not by the applications of TRPV1 and TRPV4 antagonists (Figure 3).

4. Discussion

In this study, we have shown that human monocyte-derived DCs express TRPV1, TRPV2 and TRPV4 of the thermosensitive TRP channels. We have furthermore presented evidence that a short heat shock challenge (43°C for 1 hr) decreased the endocytotic activity of the DCs and that this effect could be alleviated by the RNAi-mediated knockdown of TRPV2 but, importantly, not by the pharmacological (antagonists) or molecular (RNAi) suppression of TRPV1 and TRPV4 activities/levels. Likewise, the heat shock-induced robust membrane currents were selectively and markedly inhibited by TRPV2 "silencing" (whereas modulation of TRPV1 and TRPV4 activities, again, had no effect).

Collectively, these findings strongly implicate that the cellular effects of heat shock on human DCs are mediated by TRPV2 ion channels. Intriguingly, on human mast cells, similar findings were recently published by Zhang et al [14]; namely, by using the human mast cell line HMC-1, TRPV2 has been shown to mediate the effects of mechanical, heat and red laser-light stimulation to induce degranulation. Of further importance, in murine macrophages, TRPV2 has been shown to be instrumental for proper phagocytosis, particle binding, and bacterial lipopolysaccharide induced cytokine production, as well as in podosome assembly [15, 16, 17]. In rats, TRPV2 has also been described on macrophages, Langerhans cells and DCs, although we lack functional data in this species [18]. It appears, therefore, that TRPV2 ion channels are key players in processing the cellular actions of harmful signals on immune cells.

Nevertheless, our presented results, especially in light of previous findings of the field, raise an intriguing question. Namely, how is it possible that TRPV2, which is reportedly activated by >52°C, mediates the cellular actions of a 43°C heat challenge? Originally, the temperature-gating properties of TRPV2 were described on sensory neurons and in heterologous systems

(where host cells ectopically expressed the channels) [4, 19]. Since we lack information on the related biophysical properties of the naïve TRPV2 channels found on non-neuronal cells, we cannot exclude the possibility that it is different from those found on sensory neurons; hence, the DC-expressed TRPV2 can be activated at somewhat lower temperatures than its sensory neuron-localized counterpart. Actually, differential sensitivities/affinities for various pharmacological agonists of TRPV1 expressed by neurons and non-neuronal cells have already been reported [20, 21]. Therefore, if this is proven to be true also for DCs, it may also explain the "unexpected" lack of involvement of TRPV1 in the current study which channel, otherwise (as we have shown before, [9]), is functional on human DCs and its activation by capsaicin results in similar cellular consequences (i.e. suppression of phagocytosis) to those of heat shock.

In addition, another explanation may also elucidate the unusual temperature-sensitivity of TRPV2 on DCs. Namely, in heterologous co-expression systems, TRPV channels have been shown to form heteromers, which exhibit intermediate temperature-gating properties [22]. Along these lines, we have initiated a preliminary biochemical study. In these experiments, we first performed immunoprecipitation of TRPV2 from human DC lysates by a TRPV2-specific antibody; then, the precipitate was subjected to Western blot analyses using TRPV1 or TRPV4-specific antibodies. As seen in Supplementary Figure 1, both TRPV1 and TRPV4 antibodies detected immunosignals in the TRPV2-precipitate which findings suggest that TRPV2-TRPV1 and TRPV2-TRPV4 heteromerization may take place in human DCs. Yet, it is noteworthy that, within these heteromers, TRPV2 apparently is the most "active" component since i) it is the most abundant TRPV channel expressed by human DCs (Figure 1A); ii) its molecular suppression fully prevented the actions of heat shock (Figure 2 and 3);

and iii) pharmacological or molecular inhibition of activities/levels of TRPV1 and TRPV4 did not really modify the heat shock effects (Figure 2 and 3).

Taken together, although further biophysical and biochemical studies are invited to uncover the molecular assembly and gating properties of thermo-TRPs expressed by human DCs, our presented results strongly argue for the central role of TRPV2 in mediating the cellular action of heat shock on these cells.

5. Acknowledgement

This project was supported by Hungarian ("Lendület" LP2011-003, TÁMOP-4.2.2-08/1/2008-0019, TÁMOP 4.2.1./B-09/1/KONV-2010-0007, TÁMOP-4.2.2./A-11/1/KONV-2012-0025, OTKA 78398, OTKA 101761) and EU (FP7-REGPOT-2008-1/229920) research grants.

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7. Figure legends

Figure 1. *Human monocyte-derived DCs express TRPV1, TRPV2 and TRPV4 of the thermosensitive TRP channels*

(**A**) Q-PCR analysis of thermosensitive TRP channels on monocytes, iDCs and mDCs. Data of channel expression was normalized to the level of GAPDH of the same sample and are expressed as mean±SEM (N=4). (**B**) TRPV1, TRPV2 and TRPV4 immunoreactivity on iDCs as determined by immunofluorescence (FITC, green). Nuclei were counterstained by DAPI (blue). Negative control cells were incubated with only the secondary antibody. Magnification: 400x.

Figure 2. *Heat shock decreases endocytotic activity of DCs*

(**A**) Endocytosis of iDCs as measured by FITC-dextrane internalization after heat shock (43°C for 1 hr). For the pharmacological studies, DCs were pre-incubated with 5 μM capsazepine (CPZ) or 1 μM HC 067047 for 15 minutes before heat shock. Control cells were kept at 37°C for 1 hr with or without the relevant antagonist(s). For RNAi experiments, cells were investigated three days after the transfection with the relevant nucleotides. Data are expressed as mean±SEM of four independent donors as the percentage of the control (100%, solid line). *$p<0.05$ compared to the control cells. #$p<0.05$ compared to heat shocked control. (**B**) G6PDH release from heat shocked iDCs. Data are expressed as mean±SEM of four independent donors as the percentage of the control (100%). (**C**) Evaluation of efficacy of RNAi by Western blotting. Optical density (OD) was normalized to β-actin and expressed as relative OD values compared to cells transfected with scrambled RNAi (SCR, control cells). Images present results of the three TRPV2-specific RNAi constructs (“3-5” RNAi) employed.

Figure 3. *Heat shock induced membrane currents on DCs are mediated by TRPV2*

(**A**) A representative trace of time-dependent changes of heat activated currents on iDCs at +100 and -100 mV holding potentials. Changes in current are normalized to average basal current measured at 37°C. The line marks application of 10 μM Ruthenium Red (RR). (**B**) Statistical analysis of heat-dependent currents on iDCs at +100 mV. Data are presented as mean ± SEM; N=9 for control measurements, N=4 for RR and RNAi groups, and N=8 for the HC067047+CPZ group (1 and 5 μM were applied, respectively). *$p<0.05$ compared to the heat shocked cells without inhibitors or RNAi silencing (Control).

8. Supplementary data

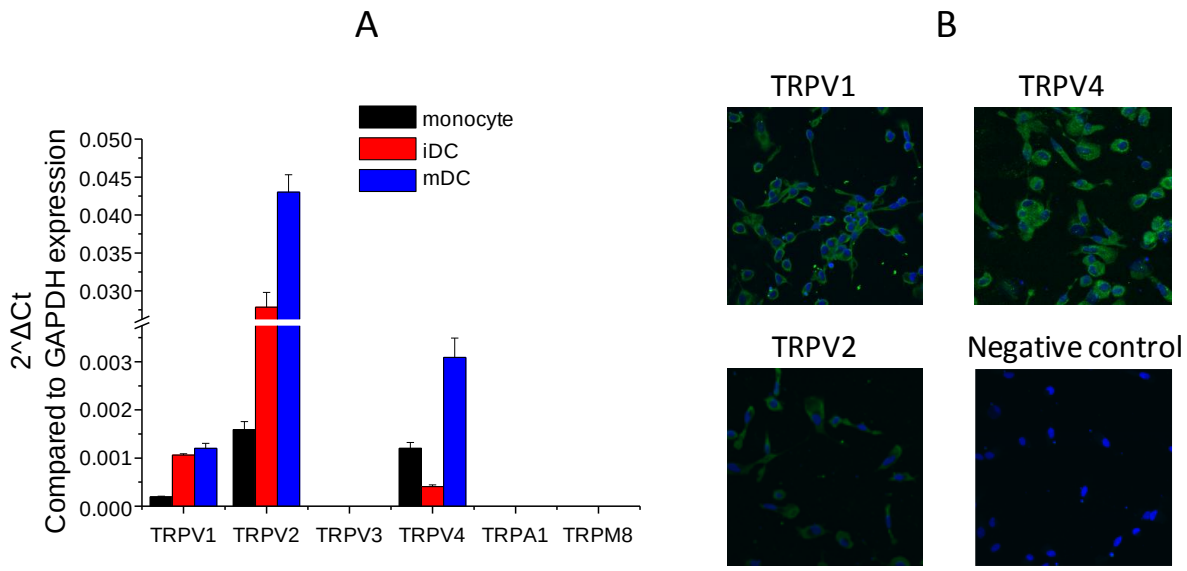
8.1. Supplementary materials and methods

Immunoprecipitation was carried out as described previously [23], with a TRPV2 antibody (Abcam). The precipitate was subsequently assayed by Western blotting with TRPV1, TRPV2 and TRPV4 antibodies (all from Abcam, TRPV2 was assayed with a different TRPV2 antibody).

8.2. Supplementary figure legends

Supplementary Figure 1. *Immunoprecipitation of DC proteins*

Protein samples prepared from iDCs were precipitated with a TRPV2 antibody. The precipitate was then assayed by Western blotting. Samples were stained using TRPV1 and TRPV4 antibodies, while a different TRPV2 antibody was used to validate the precipitation. Precipitation was performed on DCs obtained from three independent donors, marked D1-D3.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Negative control

Figure 1.

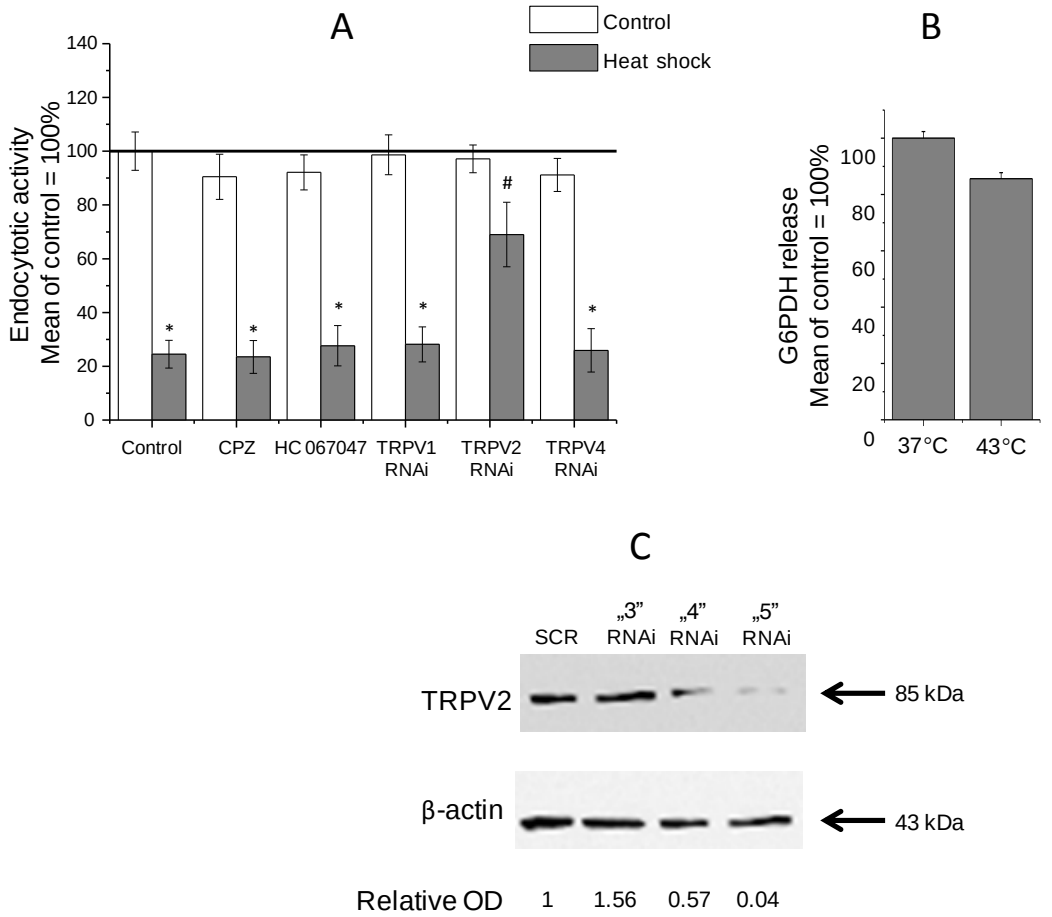
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Figure 2.

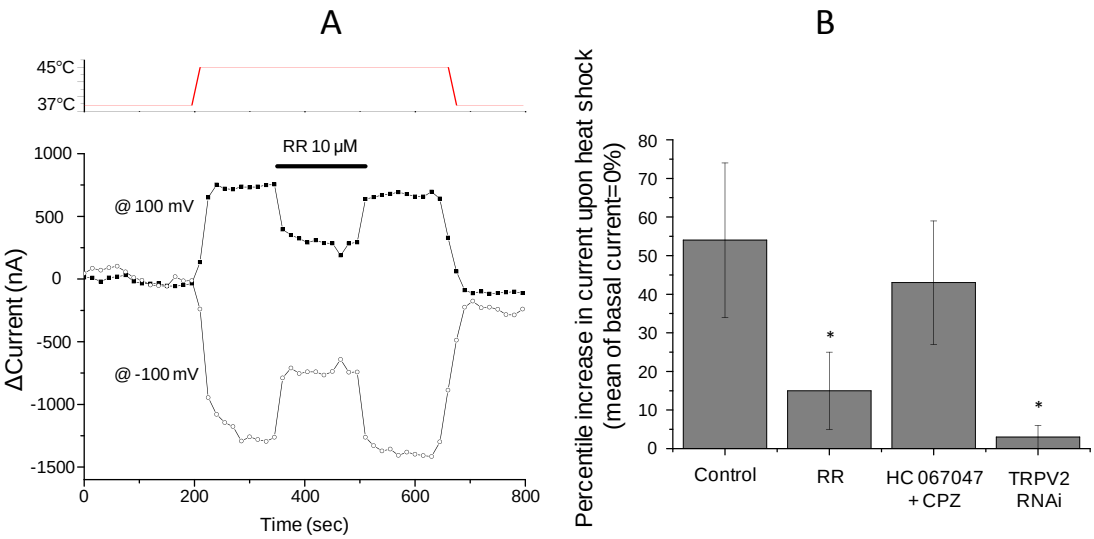
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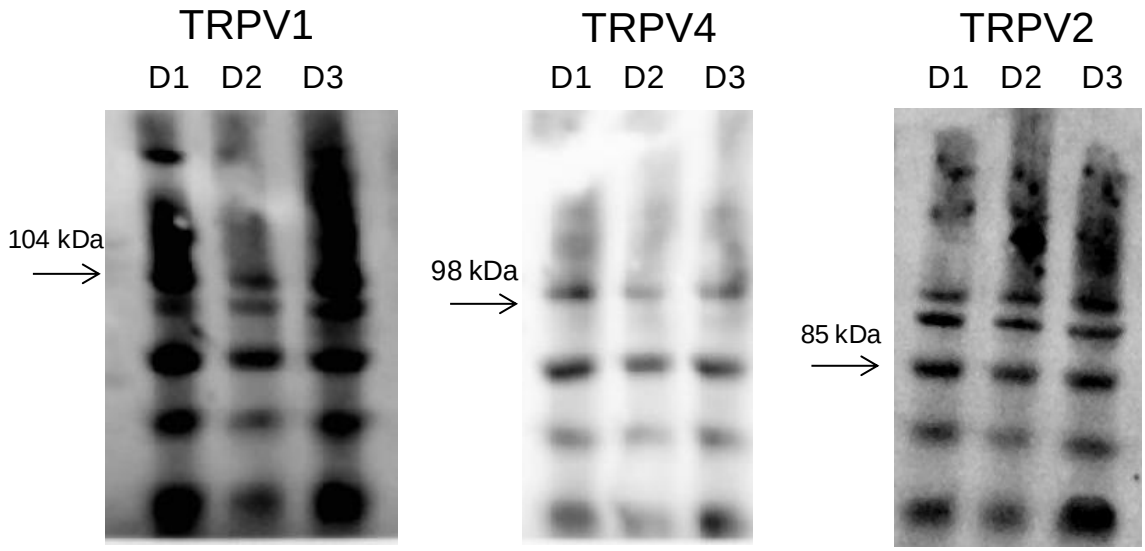
Figure 3.



Supplementary Figure 1.