H₂O₂-stimulated Ca²⁺ influx via TRPM2 is not the sole determinant of subsequent cell death

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Received: 19 July 2007 / Revised: 17 October 2007 / Accepted: 30 October 2007 / Published online: 28 November 2007
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Abstract Activation of transient receptor potential melastatin 2 (TRPM2), a non-selective, Ca²⁺-permeable cation channel, is implicated in cell death. Channel opening is stimulated by oxidative stress, a feature of numerous disease states. The wide expression profile of TRPM2 renders it a potentially significant therapeutic target in a variety of pathological settings including cardiovascular and neurodegenerative diseases. HEK293 cells transfected with human TRPM2 (HEK293/hTRPM2) were more vulnerable to H₂O₂-mediated cell death than untransfected controls in which H₂O₂-stimulated Ca²⁺ influx was absent. Flufenamic acid partially reduced Ca²⁺ influx in response to H₂O₂ but had no effect on viability. *N*-(*p*-Amylcinnamoyl) anthranilic acid substantially attenuated Ca²⁺ influx but did not alter viability. Poly(adenosine diphosphate ribose) polymerase inhibitors (*N*-(6-oxo-5,6-dihydro-phenanthridin-2-yl)-*N*, *N*-dimethylacetamide, 3,4-dihydro-5-[4-(1-piperidinyl) butoxy]-1(2H)-isoquinolinone and nicotinamide) reduced Ca²⁺ influx and provided a degree of protection but also had some protective effects in untransfected controls. These data suggest H₂O₂ triggers cell death in HEK293/hTRPM2 cells by a mechanism that is in part Ca²⁺ independent, as blockade of channel opening (evidenced by suppression of Ca²⁺ influx) did not correlate well with protection from cell

death. Determining the underlying mechanisms of TRPM2 activation is pertinent in elucidating the relevance of this channel as a therapeutic target in neurodegenerative diseases and other pathologies associated with Ca²⁺ dysregulation and oxidative stress.

Keywords TRPM2 · Hydrogen peroxide · Calcium · Cell death

Introduction

The transient receptor potential (TRP) ion channel superfamily is comprised of a diverse range of voltage-independent, Ca²⁺-permeable cation channels. TRP-melastatin 2 (TRPM2; formerly known as TRPC7 and LTRPC2) was first identified in 1998 [27] and subsequently recognised as a member of the TRPM (melastatin) family [25]. TRPM2 is highly expressed in the brain [6, 27] and also detected in vascular smooth muscle [41] and endothelial cells [42]. The channel is also expressed in a variety of immune ([22]; reviewed by [31]) and endocrine cells [14, 36].

Two extracellular signals are known to activate TRPM2: oxidative stress and tumour necrosis factor α [9]. The channel may also be temperature sensitive with body temperature acting as ‘an endogenous co-activator’ for TRPM2 [36]. Although there are suggestions to the contrary [38], Ca²⁺ influx via TRPM2 is thought to occur via production of adenosine diphosphate (ADP) ribose (ADPR) (reviewed by [20]). ADPR may arise from a mitochondrial source [32] or alternatively via activation of poly(ADPR) polymerase (PARP) with subsequent hydrolysis of poly(ADPR) by poly(ADPR) glycohydrolases liberating ADPR [5]. In addition to ADPR, other proposed intracellular activators or modulators of TRPM2 include the closely related cyclic

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ADPR (cADPR) and nicotinamide dinucleotide (NAD). Several authors have reported NAD-induced activation of TRPM2 [9, 18, 33]. However, the onset of NAD-induced activation is delayed (compared to ADPR), and much higher concentrations are required for channel opening. Because ADPR is a breakdown product of NAD, NAD-mediated gating may occur via ADPR (discussed by [20]). cADPR is generated from NAD by ADP-ribosyl cyclase and is broken down into ADPR by cADPR hydrolase. cADPR may synergise with ADPR to facilitate TRPM2 channel opening [18], although later work disputes this [11]. Data from Heiner et al. [11] also corroborate the previously reported importance of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) in mediating channel opening by ADPR [23], although elevated $[\text{Ca}^{2+}]_i$ alone is insufficient for channel opening. Calmodulin may be involved in this synergistic action of $[\text{Ca}^{2+}]_i$ [37].

The focus of this study was the role of TRPM2 in cell death induced by oxidative stress. Evidence in support of such a role already exists (reviewed by [24]), yet the underlying mechanisms are poorly understood, a problem compounded by uncertainty regarding the second messengers involved in TRPM2 activation and a lack of specific pharmacological blockers of the channel. Activation of TRPM2 results in Ca^{2+} influx and a loss of cell viability (for example, [9]). While transient elevations in $[\text{Ca}^{2+}]_i$ are vital in cell signalling pathways, excessive and/or prolonged rises in $[\text{Ca}^{2+}]_i$ are triggers for cell death [1]. Likewise, while oxidative stress has traditionally been regarded as deleterious to cell function, in recent years, reactive oxygen species have begun to emerge as important physiological signalling molecules [2] and can modulate or disrupt Ca^{2+} signalling pathways [4]. Thus, it is rational to hypothesise that oxidative stress-induced Ca^{2+} influx via TRPM2 is involved in subsequent cell death. In support of this, the inhibitory TRPM2-S variant described by Zhang et al. [43] inhibited both the rise in $[\text{Ca}^{2+}]_i$ and cell death induced by H_2O_2 when co-expressed with TRPM2. PARP inhibitors have also been observed to inhibit both H_2O_2 -induced rises in $[\text{Ca}^{2+}]_i$ and cell death mediated by TRPM2 [5]. Furthermore, use of small interfering ribonucleic acid targeted against TRPM2 prevents H_2O_2 -induced Ca^{2+} influx and restores cell viability in rat cortical neurones [16].

In light of the role of oxidative stress in the initiation and/or progression of many major diseases, the widespread expression of TRPM2, its activation by oxidative stress and its proposed role in mediating cell death, this channel represents a relevant therapeutic target in a diverse range of pathologies. However, at the present time, there is a paucity of specific blockers, and understanding of the precise details of TRPM2 activation mechanisms and how they lead to cell death is incomplete. This work aims to investigate the link between TRPM2 activation (by H_2O_2), Ca^{2+} influx and cell death using a number of compounds

each of which, although not fully selective, is reported to be a blocker of TRPM2.

Materials and methods

Generation of a stable cell line and cell culture

HEK293 cells were transfected with a pCINeo/IRES-GFP bicistronic expression vector containing the human TRPM2 (hTRPM2) gene (accession number NM 003307) using the PolyFect® Transfection reagent (Qiagen, Hybaid, Teddington, UK) according to the manufacturer's instructions. Stable hTRPM2-expressing HEK293 cell lines (HEK293/hTRPM2) were achieved by antibiotic selection over 4 weeks (media changed every 4–5 days) following addition of G-418 (1 mg ml^{-1}) to the media 3 days after transfection. Individual colonies were picked and grown to confluence in 25-cm^2 flasks, propagated into 75-cm^2 flasks and then examined for green fluorescent protein fluorescence and TRPM2 expression. The latter was confirmed by H_2O_2 -induced fluorimetric responses (see below), and once clones had been positively identified, G-418 selection pressure was reduced to $200 \text{ } \mu\text{g ml}^{-1}$. All data presented here were taken from one clone. HEK293/hTRPM2 and wild-type (WT; control) HEK293 cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Basic growth media (in which both cell types were plated for all experiments) were composed of minimum essential media with Earle's salts and L-glutamine supplemented with 9% (v/v) fetal calf serum (Gibco, Esher, Surrey UK), 3.6 mM NaOH and 1% (v/v) non-essential amino acids. The media for maintaining WT HEK293 cells for future passage also contained $50 \text{ } \mu\text{g ml}^{-1}$ gentamicin, 100 U ml^{-1} penicillin G, $100 \text{ } \mu\text{g ml}^{-1}$ streptomycin and $0.25 \text{ } \mu\text{g ml}^{-1}$ amphotericin, and the media used for maintaining hTRPM2/HEK293 cells additionally contained $200 \text{ } \mu\text{g ml}^{-1}$ G-418. Cells were harvested from culture flasks by trypsinisation and plated onto coverslips or into 96-well plates 24–96 h before experiments. All cell culture reagents were obtained from Gibco-BRL (Paisley, UK) unless otherwise stated.

Ca^{2+} measurements

Cells were loaded with fura-2 by incubation with $4 \text{ } \mu\text{M}$ fura-2/AM at room temperature in the dark in 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES)-buffered saline (HBS = 135 mM NaCl, 5 mM KCl, 1.2 mM MgCl_2 , 5 mM HEPES, 10 mM glucose, 30 mM sucrose, 2.5 mM CaCl_2 , pH 7.4, $21\text{--}24^\circ\text{C}$). After 40 min, this solution was replaced with fresh HBS, and cells were used in experiments after 20–60 min. Data are presented as the 340/380-nm fluorescence ratio ($R_{340/380}$) or as percent changes in $R_{340/380}$ (responses

were measured as the difference between basal and stimulated $R_{340/380}$. $[Ca^{2+}]_i$ was measured from single fura-2-loaded cells cultured on poly-lysine-coated glass coverslips as described previously [35]. Cells were continuously perfused, and measurements were made at room temperature. Alternatively, $[Ca^{2+}]_i$ was measured at room temperature or 37°C from fura-2-loaded cell populations cultured to 100% confluency in poly-lysine-coated, clear-bottomed, black-walled 96-well plates (Greiner) using a FlexStation II® (Molecular Devices, Berks, UK). Fura-2 was excited at 340/380 nm every 5 or 10 s, and data were acquired using SoftMax Pro software (Molecular Devices). Where Ca^{2+} was excluded from HBS, ethylene glycol-*bis*(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid was added at 1 mM.

Cell viability

Cell viability was assessed using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay [26]. Cells were cultured to between 50 and 75% confluency in clear 96-well plates. After treatment(s), MTT (5 mg ml⁻¹ in PBS) was added to each well with a final concentration of 0.5 mg ml⁻¹ and incubated at 37°C. After 3 h, an equal volume of acidic isopropyl alcohol (0.04 M HCl) was added to each well and mixed to dissolve blue formazan crystals. Absorbance was measured in a spectrophotometer at a test wavelength of 570 nm and a reference wavelength of 630 nm. Absorbance recorded at 570 nm was used to indicate cell viability, and values were converted to percent changes from the relevant controls. Apoptosis and necrosis were estimated by fluorescence microscopy using kits from Biotium and Biovision (Cambridge Biosciences, UK) as per the manufacturer's instructions. Apoptosis was estimated by binding of annexin V conjugated to either fluorescein isothiocyanate or Cy5. Necrosis was estimated by the binding of ethidium homodimer. The total cell number was estimated from Hoechst 33342 binding.

Statistics

Data were analysed by one-way analysis of variance (ANOVA) followed by Dunnett's test or by two-way ANOVA followed by Bonferroni, as appropriate. Each experimental manoeuvre was performed at least three times. Data are presented as means±SEM.

Materials and reagents

Unless stated otherwise, all drugs and reagents were obtained from Sigma (UK), and all cell culture components were obtained from Gibco-BRL: PolyFect® Transfection

Fig. 1 Activation of TRPM2 by H_2O_2 induces a rise in $[Ca^{2+}]_i$ (a) not seen in untransfected wild-type cells (b) or in the absence of extracellular Ca^{2+} (c). Each panel shows average traces ($n \geq 7$ cells; $\pm$ SEM) recorded from fura-2-loaded cells at room temperature. **a** Application of 1 mM H_2O_2 in Ca^{2+} -containing (2.5 mM) perfusate triggered a rise in $[Ca^{2+}]_i$ in HEK293 cells transfected with hTRPM2 (HEK293/hTRPM2), which was not seen in untransfected wild-type controls (b) or in HEK293/hTRPM2 cells in the absence of extracellular Ca^{2+} (c) perfusate contained 0 mM Ca^{2+} and 1 mM EGTA

Fig. 2 H_2O_2 caused a concentration-dependent increase in $[Ca^{2+}]_i$ in HEK293/hTRPM2 cells. Each *panel* shows traces recorded from single fura-2 loaded HEK293/hTRPM2 cells at room temperature.

Each trace represents a recording from a single cell. Each *panel* is a single experiment. H_2O_2 (0–1 mM) was applied in Ca^{2+} -containing (2.5 mM) perfusate

reagent (Qiagen, Hybaid), fetal calf serum (GlobePharm), fura-2/AM (Molecular Probes, UK), trolox (Calbiochem, UK), *N*-(*p*-amylcinnomoyl)anthranilic acid (ACA; Calbiochem), 3,4-dihydro-5-[4-(1-piperidinyloxy)]-1(2H)-

isoquinolinone (DPQ; Calbiochem) and *N*-(6-oxo-5,6-dihydro-phenanthridin-2-yl)-*N,N*-dimethylacetamide (PJ34; Calbiochem). Apoptosis and necrosis kits were from Biotium and Biovision (Cambridge Biosciences, UK).

Results

H₂O₂-mediated Ca²⁺ influx via TRPM2

Application of exogenous H₂O₂ (1 mM) to HEK293/hTRPM2 cells evoked a rise of [Ca²⁺]_i, which was absent in untransfected HEK293 cells (Fig. 1a and b). As previously reported for TRPM2 [33], this increase was the result of Ca²⁺ influx because it was not seen in the absence of extracellular Ca²⁺ (Fig. 1c). The observed effect of H₂O₂ on [Ca²⁺]_i was concentration dependent: Single-cell recordings at room temperature (Fig. 2) showed that cells responded at and above 30 μM H₂O₂. Increasing the concentration of H₂O₂ increased both the magnitude and speed of responses up to 300 μM H₂O₂, although a further increase to 1 mM was without additional effect. At 37°C, the H₂O₂-stimulated rise in [Ca²⁺]_i seen in HEK293/hTRPM2 cells was both larger and more rapid in onset than at room temperature (Fig. 3a and b) but was still absent from untransfected control cells (data not shown). Given that TRPM2 activation is temperature sensitive [36] and that MTT assays (below) were performed at 37°C, all subsequent Ca²⁺ recordings were made at 37°C.

H₂O₂-mediated cell death

H₂O₂ also mediated a concentration-dependent effect on cell death (Fig. 4). After 24 h exposure to H₂O₂ (30 or 100 μM), the reductions in cell viability observed in HEK293/hTRPM2 cells were significantly greater than those observed in untransfected cells, although at 100 μM H₂O₂ and above, significant loss of viability was also seen in untransfected cells (Fig. 4a). The time course of cell death revealed that HEK293/hTRPM2 cells showed significantly increased susceptibility to H₂O₂-induced cell death (compared to untransfected controls) as early as 2 h after H₂O₂ application (Fig. 4b). Cell death was mediated at least in part by oxidative damage, as the anti-oxidant trolox provided partial protection against H₂O₂-mediated death. Thus, in HEK293/hTRPM2 cells, co-application of trolox (200 μM) with 100 μM or 1 mM H₂O₂ increased viability to 150±17 and 287±45% of that seen in its absence at these respective concentrations of H₂O₂. Trolox also enhanced the viability of untransfected control cells when co-applied with 1 mM H₂O₂, to 236±52% of control value observed with 1 mM H₂O₂ alone. In agreement with previous studies [9, 45], exposure of HEK293/hTRPM2 cells to H₂O₂ induced evidence of both apoptotic and necrotic cell death. Thus, as determined in three separate trials, after 2 h exposure to 100 μM H₂O₂, 12.7±2.0% of cells showed positive staining for labelled annexin V binding (indicative of apoptosis), and 10.7±3.7% of cells were stained with ethidium homodimer (indicative of necrosis). These values

Fig. 3 The effect of temperature on H₂O₂-mediated Ca²⁺ influx via TRPM2. Recordings made from populations of fura-2-loaded cells at room temperature (RT) and 37°C. **a** The increase in Ca²⁺ induced by 1 mM H₂O₂ in HEK293/hTRPM2 cells ($n=4$ experiments; $\pm$ SEM) was larger and more rapid in onset at 37°C compared to RT. **b** At 37°C, in the presence of extracellular Ca²⁺ (2.5 mM), H₂O₂ (0–1 mM) induced a concentration-dependent increase in [Ca²⁺]_i in HEK293/hTRPM2 cells. Data expressed as percent changes from response to 1 mM H₂O₂ ($n=4$ experiments; $\pm$ SEM). Analysis by one-way ANOVA followed by Dunnett's test (asterisk, $P<0.05$; double asterisk, $P<0.01$)

are likely to be underestimated, as this degree of H₂O₂ exposure caused approximately 35% of cells to become detached.

Inhibition of TRPM2

Previous studies have correlated inhibition of oxidative stress-induced Ca²⁺ influx via TRPM2 with enhanced viability [7, 16, 43, 44]. We therefore investigated whether H₂O₂-induced Ca²⁺ influx in HEK293/hTRPM2 cells was

Fig. 4 HEK293/hTRPM2 cells are more vulnerable to H₂O₂-induced cell death than untransfected controls. Cell viability was assessed by the MTT assay ($n=3$ experiments; $\pm$ SEM). Data are expressed as percent changes from control (0 μ M H₂O₂). **a** 24 h exposure to H₂O₂ (0–1 mM) caused loss of viability in untransfected control and HEK293/hTRPM2 cells in a concentration-dependent fashion. HEK293 cells were more vulnerable to cell death at 30 and 100 μ M

H₂O₂. **b** HEK293/hTRPM2 cells were also more vulnerable to cell death induced by H₂O₂ than untransfected controls at a range of time points before 24 h. Analysis by one-way ANOVA followed by Dunnett's test (asterisk, $P<0.05$; double asterisk, $P<0.01$) or by two-way ANOVA followed by Bonferroni (double cross, $P<0.01$; triple cross, $P<0.001$) as appropriate. NS Non-significant

involved in enhanced oxidative cell death. To do this, we tested reported inhibitors of TRPM2: clotrimazole [13, 40], flufenamic acid (FFA) [12], ACA [19] and PARP inhibitors [5]. We have used these compounds and investigated their effects on both H₂O₂-induced Ca²⁺ influx via TRPM2 and also cell viability after 24 h treatment with H₂O₂. Compounds were applied both simultaneously with H₂O₂ and for 3 min before as well as during H₂O₂ exposure (the latter protocol is referred to as 'pre-exposure'). Pre-exposure to clotrimazole (20 μ M) had no significant effects on H₂O₂-induced Ca²⁺ influx into HEK293/hTRPM2 cells (data not shown), so its effects on cell viability after H₂O₂ exposure were not pursued.

When applied simultaneously to HEK293/hTRPM2 cells with 1 mM H₂O₂, FFA (200 μ M) significantly reduced subsequent Ca²⁺ influx to $67\pm8\%$ (of the control response with 1 mM H₂O₂; Fig. 5a). No inhibitory effects were seen at 100 or 300 μ M H₂O₂. However, when cells were pre-

exposed to FFA, significant inhibitory effects were seen at 100 and 300 μ M H₂O₂ with the rise in [Ca²⁺]_i being reduced to 41 ± 8 and $70\pm10\%$ (of the control responses with 100 and 300 μ M H₂O₂), respectively. Although a reduction in the response was also seen at 1 mM H₂O₂, this was not significant. However, whether applied before or at the same time as H₂O₂, no restorative effects on cell viability were seen after 24 h treatment with 100 μ M or 1 mM H₂O₂ (Fig. 5b). ACA (20 μ M) had no effect on H₂O₂-induced Ca²⁺ influx when applied simultaneously with H₂O₂, but when cells were pre-exposed to this compound, Ca²⁺ influx in response to 100 or 300 μ M or 1 mM H₂O₂ was significantly and substantially reduced (Fig. 6a). However, either simultaneous application of or pre-exposure to ACA had no protective effects on cell viability after 24 h exposure to 100 μ M or 1 mM H₂O₂.

The PARP inhibitors PJ34 (1 μ M), DPQ (10 μ M) and nicotinamide (1 mM) all effectively blocked or substantial-

Fig. 5 Flufenamic acid has some effect on H₂O₂-induced Ca²⁺ influx (a) via TRPM2 but offers no protection against H₂O₂-induced cell death (b). Flufenamic acid (FFA; 200 μM) was applied to HEK293/hTRPM2 cells simultaneously with or for 3 min before (as well as during exposure to) H₂O₂ (0.1–1 mM). a Recordings were made from fura-2 cell populations (37°C; *n*=4 experiments; ±SEM; data

expressed as percent changes from response to 1 mM H₂O₂). b Cell viability was assessed after 24 h exposure to H₂O₂ (37°C; *n*=3 experiments; ±SEM; data expressed as percent changes from control of 0 μM H₂O₂). Analysis by two-way ANOVA followed by Bonferroni (cross, *P*<0.05; triple cross, *P*<0.001)

ly reduced Ca²⁺ influx into HEK293/hTRPM2 cells when applied simultaneously with H₂O₂ (Fig. 7a). PJ34 totally abolished Ca²⁺ influx in response to all three concentrations of H₂O₂. DPQ ablated Ca²⁺ influx in response to 100 μM H₂O₂ and reduced the responses with 300 μM and 1 mM H₂O₂ to 31±3 and 34±7% (of the control responses with 300 μM and 1 mM H₂O₂), respectively, whereas nicotinamide eliminated the H₂O₂-induced rise in [Ca²⁺]_i at 100 and 300 μM H₂O₂ and reduced the response seen with 1 mM H₂O₂ to 34±7% (of the control response with 1 mM). These agents also offered some protection against H₂O₂-induced HEK293/hTRPM2 cell death. When co-applied with 100 μM H₂O₂ for 24 h, all three PARP inhibitors significantly increased viability (approximately two- to threefold compared to control cells treated with 100 μM H₂O₂ only; Fig. 7b) but not after exposure to 1 mM H₂O₂. It was also observed that PARP inhibition could partially but significantly restore viability in untransfected control cells after treatment with 1 mM H₂O₂.

Discussion

The present study was undertaken to investigate the link between oxidative stress-induced Ca²⁺ influx through TRPM2 and cell death. Because TRPM2 is widely expressed and also implicated in oxidative stress-induced cell death, there is broad therapeutic potential for blockers of this channel. However, those reported to date have numerous other biological activities, and studies have failed to address whether in addition to blockade of TRPM2-mediated currents or rises in [Ca²⁺]_i, there was any reduction in cell death [12, 13, 19, 28]. Thus, for example, FFA has been reported to block a variety of ion channels in addition to TRPM2 [28] such as Ca²⁺-activated Cl[−] channels [39] and voltage-activated K⁺ channels [21]. Conversely, it may also stimulate activity of some channels [15, 29]. In addition, ACA is a phospholipase A₂ inhibitor and also has inhibitory effects on a number of other TRP channels [10], and both DPQ and PJ34 are potent PARP

Fig. 6 ACA inhibits H₂O₂-induced Ca²⁺ influx (**a**) via TRPM2 but offers no protection against H₂O₂-induced cell death (**b**). *N*-(*p*-amylcinnomoyl)anthranilic acid (ACA; 20 μM) was applied to HEK293/hTRPM2 cells simultaneously with or for 3 min before (as well as during exposure to) H₂O₂ (0.1–1 mM). **a** Recordings were made from fura-2 cell populations (37°C; *n*=4 experiments; ±SEM;

data expressed as percent changes from response to 1 mM H₂O₂). **b** Cell viability was assessed after 24 h exposure to H₂O₂ (37°C; *n*=3 experiments; ±SEM; data expressed as percent changes from control of 0 μM H₂O₂). Analysis by two-way ANOVA followed by Bonferroni (cross, *P*<0.05; triple cross, *P*<0.001)

inhibitors. Nicotinamide also prevents cADPR signalling [34]. This notwithstanding, such tools are currently the only available and so were employed in the present work.

Our initial studies demonstrated that H₂O₂ activated Ca²⁺ influx via functional TRPM2 channels in a concentration- and temperature-sensitive manner after successful transfection of HEK293 cells. Thus, at room temperature, H₂O₂ concentrations of 30 μM elicited detectable rises of [Ca²⁺]_i in the majority of cells, and the onset and number of responsive cells increased with concentration, so that at 300 μM and 1 mM H₂O₂, almost all cells responded maximally. Ca²⁺ influx was enhanced at 37°C compared to room temperature. In particular, the onset of the response was much more rapid. In agreement with single-cell measurements of [Ca²⁺]_i made at room temperature, recordings made from cell populations at 37°C also point to a [H₂O₂] threshold for Ca²⁺ influx between 30 and 100 μM, with influx reaching maximum between 300 μM and 1 mM. This temperature-sensitive, H₂O₂-mediated Ca²⁺ influx via TRPM2 corroborates what has previously been reported for this channel [9, 36].

A significant loss of viability of HEK293/hTRPM2 cells was observed at 30 μM H₂O₂, and cell death was maximal between 300 μM and 1 mM H₂O₂ (Fig. 4). This is similar to the threshold and maximal concentrations of H₂O₂ observed for Ca²⁺ influx. However, while H₂O₂ failed to elicit a rise in [Ca²⁺]_i in untransfected control cells, it was effective in inducing cell death, even after short periods of exposure. These data indicate that loss of viability cannot be attributed solely to TRPM2 activity, although Ca²⁺ influx via this channel may potentiate oxidative cell death.

To investigate this further, we used a variety of compounds, which have been reported to block TRPM2 activation by H₂O₂. Clotrimazole failed to block Ca²⁺ influx in our hands, and FFA had only a partial inhibitory effect. In contrast, when applied to cells just before (as well as during) H₂O₂ application, ACA proved to be very effective in blocking the rise in [Ca²⁺]_i induced by 100 and 300 μM and 1 mM H₂O₂. However, when we looked at cell viability over 24 h, no protective effects were seen (Figs. 5 and 6). This suggests that the initial rise in [Ca²⁺]_i arising from Ca²⁺ influx through activated TRPM2 is *not* respon-

Fig. 7 PARP inhibition prevents H_2O_2 -induced Ca^{2+} influx (**a**) via TRPM2 and offers some protection against cell death (**b** and **c**). PJ34 (1 μM), DPQ (10 μM) or nicotinamide (1 mM) were applied to HEK293/hTRPM2 cells (and untransfected controls) simultaneously with H_2O_2 (0.1–1 mM). **a** Recordings were made from fura-2 HEK293/hTRPM2 cell populations (37°C; $n=4$ experiments; $\pm\text{SEM}$; data expressed as percent changes from response to 1 mM H_2O_2). **b**

Viability of HEK293/hTRPM2 and untransfected cells was assessed after 24 h exposure to H_2O_2 (37°C; $n=3$ experiments; $\pm\text{SEM}$; data expressed as percent changes from control of 0 μM H_2O_2). Analysis by one-way ANOVA followed by Dunnett's test (double asterisk, $P<0.01$) or by two-way ANOVA followed by Bonferroni (cross, $P<0.05$; double cross, $P<0.01$; triple cross, $P<0.001$) as appropriate

sible for subsequent cell death. We also examined the effects of PARP inhibition on H_2O_2 -induced Ca^{2+} influx and cell death. Previous work has shown that PJ34 and DPQ are effective at both inhibiting the rise in $[\text{Ca}^{2+}]_i$ and restoring cell viability in TRPM2-expressing HEK293 cells [5]. We also used nicotinamide, which has been reported to prevent cADPR-mediated signalling [34] but is also an effective PARP inhibitor. All three compounds significantly reduced and/or eliminated the rise in $[\text{Ca}^{2+}]_i$ induced by H_2O_2 over all concentrations tested. Additionally, they were able to partially restore HEK293/hTRPM2 cell viability

after exposure to 100 μM H_2O_2 . However, PARP inhibition was also partially protective in untransfected cells (Fig. 7).

Fonfria et al. [5] propose that oxidative stress-induced activation of PARP is important for TRPM2 channel opening. Our data are consistent with a PARP-dependent mechanism for H_2O_2 -induced Ca^{2+} influx through TRPM2. We observed that PARP inhibitors could prevent Ca^{2+} influx through TRPM2 triggered by 100 and 300 μM or 1 mM H_2O_2 . However, while these compounds reduced HEK293/hTRPM2 cell death after 24 h exposure to 100 μM H_2O_2 , no protection was seen at 1 mM H_2O_2

despite ablation of the initial increase in $[Ca^{2+}]_i$. Furthermore, PARP inhibition also offered some protection against H_2O_2 -induced cell death in untransfected control cells. These findings further indicate that H_2O_2 -induced Ca^{2+} influx via TRPM2 is not the sole determinant of cell viability and also that the mechanism by which PARP inhibition protects TRPM2-expressing cells from cell death is not fully TRPM2 dependent.

Evidence suggests that H_2O_2 may exert effects on $[Ca^{2+}]_i$ and/or cell survival, which are independent of TRPM2. For example, H_2O_2 can release Ca^{2+} from intracellular stores (for example, [8, 30, 46]), which would affect subsequent Ca^{2+} signalling events, including those involved in regulating cell fate, and could also be important for TRPM2 activation because channel opening is dependent on $[Ca^{2+}]_i$ [23]. However, we observed only an extremely small, slow rise of $[Ca^{2+}]_i$ evoked by H_2O_2 in the absence of extracellular Ca^{2+} (Fig. 1c). H_2O_2 -induced activation of PARP could also mediate effects on cell fate independently of its purported role in TRPM2 activation (via ADPR production). PARP is activated by deoxyribonucleic acid strand breaks, and poly(ADP-ribosylation) is essential for maintaining genomic integrity. However, with higher levels of genotoxic stress, over-activation of PARP occurs, and this leads to cell death via both necrotic and apoptotic mechanisms [3, 17]. Thus, the concentration of H_2O_2 used could be important in determining the role of PARP activation (and hence inhibition) in determining cell fate.

In conclusion, this study raises important issues regarding TRPM2 activation, Ca^{2+} influx and cell death. Firstly, the relationship between Ca^{2+} influx and cell death is clearly not simple—preventing the rise in $[Ca^{2+}]_i$ does not necessarily preserve cell viability. Secondly, we question the function of PARP in mediating TRPM2 activation by H_2O_2 . Blocking this enzyme reliably prevented Ca^{2+} influx but not cell death in HEK293/hTRPM2 cells and also afforded some protection to untransfected controls. This casts further uncertainty as to whether the rise in $[Ca^{2+}]_i$ triggers subsequent cell death and also implies a role for PARP in cell death, which is independent of TRPM2 activation.

Acknowledgements This work was supported by grants from the British Heart Foundation and the Alzheimer's Research Trust. We are grateful to Dr. Elizabeth Straszynski for expert help with cell viability assays.

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