Evidence that TRPC1 (transient receptor potential canonical 1) forms a Ca^{2+} -permeable channel linked to the regulation of cell volume in liver cells obtained using small interfering RNA targeted against TRPC1

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The TRPC1 (transient receptor potential canonical 1) protein, which is thought to encode a non-selective cation channel activated by store depletion and/or an intracellular messenger, is expressed in a number of non-excitabile cells. However, the physiological functions of TRPC1 are not well understood. The aim of these studies was to investigate the function of TRPC1 in liver cells using small interfering RNA (siRNA) to ablate the TRPC1 protein. Treatment of H4-IIIE liver cells with siRNA targeted against TRPC1 caused an approx. 50 % decrease in expression of the human TRPC1 protein in cells transfected with cDNA encoding human TRPC1, and a 50 % decrease in expression of the endogenous TRPC1 protein (assessed by Western blot and immunofluorescence). The decrease in endogenous TRPC1 protein in cells transfected with TRPC1 siRNA

was associated with a greater increase in cell volume (compared with the increase observed in control cells) immediately after cells were placed in a hypotonic medium, and an enhanced regulatory cell volume decrease after exposure to hypotonic medium. Treatment with siRNA targeted against TRPC1 also led to a 25 % inhibition of thapsigargin-stimulated Ca^{2+} inflow, a 40 % inhibition of ATP and maitotoxin-stimulated Ca^{2+} inflow, and a 50 % inhibition of maitotoxin-stimulated Mn^{2+} inflow. The idea that, in liver cells, TRPC1 encodes a non-selective cation channel involved directly or indirectly in the regulation of cell volume is consistent with the results obtained.

Key words: ATP, hypotonicity, maitotoxin, thapsigargin.

INTRODUCTION

Ca^{2+} plays a central role in the regulation of liver cell function, including the control of metabolic pathways, cell proliferation, bile flow and the maintenance of cell volume and shape (reviewed in [1]). Changes in liver cell volume are induced by alterations in osmolarity, ATP and other extracellular signals and metabolites (reviewed in [2]). The regulation of cell volume principally involves the movement of Cl^- , Na^+ and water through channels in the plasma membrane [3]. Changes in the concentration of free Ca^{2+} in the cytoplasmic space ($[\text{Ca}^{2+}]_{\text{cyt}}$) regulate K^+ and Cl^- channels and hence cell volume [4]. $[\text{Ca}^{2+}]_{\text{cyt}}$ is regulated principally by the inflow of Ca^{2+} through the plasma membrane Ca^{2+} channels and the uptake and release of Ca^{2+} by the endoplasmic reticulum and mitochondria (reviewed in [1]). Store-operated Ca^{2+} channels (SOCs) and intracellular messenger-activated non-selective cation channels (NSCCs) are chiefly responsible for facilitating Ca^{2+} inflow across the liver cell plasma membrane [1]. These cells may possess at least two types of SOC [5,6] and several types of NSCC and stretch-activated Ca^{2+} channel [1,7–9]. The predominant SOC has a high selectivity for Ca^{2+} over Na^+ and properties that are indistinguishable from those of Ca^{2+} release-activated Ca^{2+} channels in mast cells and lymphocytes [10]. Both SOCs [11] and stretch-activated NSCCs [7] are involved in the control of liver cell volume.

The polypeptides that compose SOCs and most NSCCs are presently not well described. However, there is evidence that members of the transient receptor potential (TRP) family of non-selective cation channels and Ca^{2+} channels comprise some SOCs

and NSCCs (reviewed in [12–14]). There is evidence that TRP canonical (TRPC) 1–3, TRP vanilloid ('TRPV') 1, 4 and 6, and TRP melastatin ('TRPM') 4, 5 and 7 are expressed in liver cells and/or liver tissue [9,15–20]. Considerable attention has been paid to TRPC1, which is expressed widely in liver cells and in many types of non-excitabile animal cell [12–14], and may contribute to the formation of NSCCs [9,21–23] and SOCs [24–26]. Since NSCCs and SOCs are involved in the initial response of hepatocytes to a change in extracellular osmolarity, and since the TRPC1 polypeptide may form NSCCs and/or SOCs, it was considered possible that TRPC1 is involved in hepatocyte volume regulation.

The aim of the present experiments was to elucidate the role of TRPC1 in plasma-membrane Ca^{2+} inflow and in the regulation of liver cell volume using small interfering RNA (siRNA) targeted against TRPC1 to ablate the TRPC1 protein. siRNA is a powerful sequence-specific reagent designed to suppress the expression of genes in cultured mammalian cells through a process known as RNA interference [27,28]. Recent studies in several laboratories have shown that siRNA designed to bind to a specific species of mRNA effectively enhances the degradation of that targeted mRNA [29,30]. The present results show that liver cells transfected with siRNA specific for TRPC1 exhibit an approx. 50 % decrease in expression of the TRPC1 protein and partial inhibition of plasma membrane Ca^{2+} inflow initiated by thapsigargin, ATP and maitotoxin, and partial inhibition of plasma membrane Mn^{2+} inflow initiated by maitotoxin. These changes are associated with an enhanced swelling in hypotonic medium and a faster regulatory-cell volume decrease. It is concluded that one of

Abbreviations used: TRP, transient receptor potential; TRPC, TRP canonical; hTRPC1, human TRPC1; NSCC, intracellular messenger-activated non-selective cation channel; SOC, store-operated Ca^{2+} channel; siRNA, small interfering RNA; DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum; Ca_o^{2+} , extracellular Ca^{2+} ; $[\text{Ca}^{2+}]_{\text{cyt}}$, free Ca^{2+} concentration in the cytoplasmic space.

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the functions of TRPC1 in liver cells is to mediate the inflow of Na^+ and Ca^{2+} to the cytoplasmic space and hence modulate the intracellular concentrations of Ca^{2+} and Na^+ . This may regulate cell volume directly or indirectly.

MATERIALS AND METHODS

Materials

Except where indicated below, reagents were obtained from the sources described previously [9,31,32].

Design and synthesis of siRNA

A target sequence for siRNA in mRNA encoding human TRPC1 (hTRPC1) was identified using the principles described by Elbashir et al. [27], including location at more than 100 nt downstream of the TRPC1 start codon and a G/C content of approx. 50%. The selected sequence, 5'-AAG CUU UUC UUG CUG GCG UGC-3', is identical in rat, human, mouse, rabbit and bovine TRPC1 mRNAs, and is located in the first ankyrin repeat in the N-terminal region of TRPC1 mRNA. A search of the National Centre for Biotechnology Information database (BLAST program) found no other mRNA species containing a similar sequence. The RNA sense sequence 5'-GCU UUU CUU GCU GGC GUG CdTdT-3' (where dT is 2'-deoxythymidine) and the antisense RNA sequence 5'-GCA CGC CAG CAA GAA AAG CdTdT-3' were synthesized, and the sense and antisense RNA sequences were annealed to give TRPC1-specific siRNA with a 2-nt overhang at the 3'-end (5'-GCU UUU CUU GCU GGC GUG CdTdT-3'; 3'-dTdT CGA AAA GAA CGA CCG CAC G-5'; Dharmacon Research, Lafayette, CO, U.S.A.). The TRPC1 siRNA utilized in this study was synthesized with symmetrical 2'-deoxythymidine residues in the 2-nt overhangs because it has been shown that uridine residues in the 2-nt 3' overhang can be replaced by 2'-deoxythymidine without affecting its activity [33].

Transfection of H4-IIE cells with TRPC1 siRNA and cDNA encoding hTRPC1

H4-IIE cells (derived from a rat liver hepatoma) were cultured as described previously [9,31,32]. Cells (0.5×10^6 /well) were seeded in a six-well-plate on the day before transfection, and maintained in Dulbecco's modified Eagle's medium (DMEM) containing foetal bovine serum (FBS) 10% (v/v) but free of antibiotics. Cells transfected with TRPC1 siRNA were prepared as follows. Oligofectamine™ (Invitrogen Life Technologies; 6 μl) was mixed dropwise with 54 μl of Opti-MEM medium (Invitrogen Life Technologies) in a 1.5-ml plastic centrifuge tube. TRPC1 siRNA (15 μl of a 20 μM solution, final concentration 150 nM, except where indicated otherwise in the text) was mixed dropwise with 125 μl of Opti-MEM medium in a second 1.5-ml plastic centrifuge tube. Each tube was incubated at 20 °C for 5 min. The diluted Oligofectamine™ and TRPC1 siRNA were then combined to form the transfection mixture (200 μl), which was incubated for a further 20 min at 20 °C. Cells growing at the bottom of one well of a six-well plate were washed twice with antibiotic- and serum-free DMEM, then 0.8 ml of this medium was added, followed by the transfection mixture, and the cells were incubated at 37 °C in 95% O_2 /5% CO_2 . After 5–6 h, a further 1 ml of antibiotic-free DMEM containing 20% (v/v) FBS was added to each well. The cells were then incubated at 37 °C for 48–72 h. For transfection with the single-stranded antisense

Figure 1 Treatment with siRNA targeted against TRPC1 reduces expression of hTRPC1 assessed by Western blot in cells transfected with cDNA encoding hTRPC1

(A) Western blots of proteins (40 μg of total protein/lane) extracted from cells transfected with cDNA encoding hTRPC1 plus TRPC1 siRNA (lane 1) or TRPC1 antisense RNA (lane 2), or from mock-transfected cells (lane 3). Cell transfection and Western-blot analysis were performed as described in the Materials and methods section. Membranes were blotted with the 1F1 mouse anti-TRPC1 monoclonal antibody and a mouse anti- β -actin monoclonal antibody. The β -actin band served as an internal control for protein loading and quantification. (B) The intensities of the hTRPC1 bands and of a 30 kDa unknown protein (selected as a control protein 'band selected for siRNA specificity test') in (A) expressed as a function of the intensity of the β -actin band. The results are the means $\pm$ S.E.M. from three separate experiments. Degrees of significance, determined using Student's *t* test for paired samples, were **P* < 0.05.

sequence of the TRPC1 siRNA (TRPC1 antisense RNA), cells were treated as described above for transfection with TRPC1 siRNA except that the TRPC1 siRNA was replaced by TRPC1 antisense RNA. Mock transfection was conducted as described above for siRNA transfection except that the TRPC1 siRNA solution was replaced by an equal volume of Opti-MEM.

Transfection with both cDNA encoding hTRPC1 and TRPC1 siRNA was achieved as follows. The following were mixed in three separate tubes. Lipofectamine 2000™ (Invitrogen Life Technologies; 12 μl) plus Opti-MEM medium (250 μl); pcDNA3.1(–)hTRPC1 cDNA (3 μg), pEGFP-C1 cDNA (0.3 μg) plus Opti-MEM medium (125 μl); and TRPC1 siRNA (15 μl of a 20 μM solution) plus Opti-MEM medium (125 μl). Each tube was incubated at 20 °C for 5 min. Half of the Lipofectamine 2000™ mixture was then added to each of the tubes containing hTRPC1 cDNA and TRPC1 siRNA, mixed, and the tubes incubated for a further 20 min at 20 °C. The cells from a single well of a six-well plate were washed twice with serum- and antibiotic-free DMEM, then serum- and antibiotic-free DMEM (0.5 ml) was added followed by the dropwise addition of the hTRPC1 cDNA/Lipofectamine 2000™ mixture followed by the TRPC1 siRNA/Lipofectamine 2000™ mixture. After a 5–6 h incubation at 37 °C, a further 1.0 ml of antibiotic-free DMEM containing 20% (v/v) FBS was added and the cells incubated

Figure 2 Treatment with siRNA targeted against TRPC1 reduces expression of hTRPC1 assessed by immunofluorescence in cells transfected with cDNA encoding hTRPC1

Cells were transfected with cDNA encoding hTRPC1 alone (**A, D, E**), hTRPC1 cDNA plus TRPC1 siRNA (**C, F**) or hTRPC1 cDNA plus TRPC1 antisense RNA (**B**), then incubated for 18–22 h and the hTRPC1 protein detected by immunofluorescence (IF), as described in the Materials and methods section. Magnification was 100 $\times$ in (**A**)–(**D**) and 200 $\times$ in (**E**) and (**F**). The results shown are those obtained in one of two separate experiments which each gave similar results.

for 18–22 h at 37 °C in 95% O₂/5% CO₂. Cells transfected with cDNA encoding hTRPC1 plus cDNA encoding TRPC1 antisense RNA were prepared as described above except that the TRPC1 siRNA was replaced by single-stranded TRPC1 antisense RNA. Cells transfected with only cDNA encoding hTRPC1 were prepared as described above except that the TRPC1 siRNA was replaced by the same volume of Opti-MEM. The final concentration of TRPC1 siRNA (150 nM) employed in most experiments was chosen on the basis of previous results reported by others [27–30].

Immunoprecipitation, Western-blot analysis and immunofluorescence

Western-blot analysis, and a combined immunoprecipitation Western-blot technique for the detection of heterologously expressed and endogenous TRPC1 in H4-IIIE cells, were performed as described previously [32]. The antibodies employed were: 1F1 monoclonal anti-TRPC1 [34] (primary antibody) and horseradish-conjugated mouse IgG (Sigma; secondary antibody) for the detection of TRPC1 in Western blots; mouse monoclonal

anti- β -actin (Sigma; primary) and horseradish-conjugated mouse IgG (secondary) for the detection of β -actin in Western blots; and 1.12 AP rabbit polyclonal anti-TRPC1 [9,32] (immunoprecipitation) and 1F1 mouse monoclonal anti-TRPC1 (Western blot) for the detection of TRPC1 using the combined immunoprecipitation and Western blot technique. Protein concentrations were measured using the Bradford method [35]. Membranes were imaged using a Bio-Rad imaging densitometer (model GS-700) and the intensities of the bands measured using Molecular Analyst® software version 2.1 (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

For immunofluorescence, cells grown on collagen-coated coverslips were rinsed three times with cold PBS. Following this, 2 ml of fixative [4% (w/v) paraformaldehyde in PBS] was added and the coverslips incubated for 30 min at 20 °C. The coverslips were then washed three times (5 min each) with cold PBS, the cells permeabilized by the addition of 1 ml of 1% (v/v) Triton X-100 in PBS, incubated for 4 min at 0 °C, the coverslips washed three times with cold PBS, and blocking solution (20% FBS in PBS) added. The coverslips were incubated with anti-TRPC1 antibody [2 μ g/ml in PBS containing 0.1% (v/v) Tween-20] at 4 °C for 2 h followed by three washes with PBS/Tween-20 before incubation with anti-rabbit or anti-mouse IgG secondary antibody conjugated with Cy3 (1:250 dilution). The coverslips were washed three times in cold PBS and mounted on slides in 50% glycerol in PBS (pH 8.6) or Immu-Mount medium (Jomar Diagnostics, Stepney, SA, Australia).

Fluorescence microscopy was performed using an Olympus AX-70 fluorescence microscope equipped with a Chroma 31002 filter (excitation wavelength at 515–550 nm, emission wavelength at 575–615 nm), a 10 $\times$ or a 200 $\times$ objective lens and a Hamamatsu ORCA cooled CCD camera. The mean immunofluorescence intensity per cell was determined by measuring the fluorescence intensity of each of 20 cells under each experimental condition using Scion Image software version 1.62 (Scion Corporation, Ferderick, MA, U.S.A.).

Measurement of cytoplasmic free Ca^{2+} concentration, Ca^{2+} inflow, Mn^{2+} inflow and cell size

[Ca^{2+}]_{cyt}, the rate and extent of Ca^{2+} inflow, and the rate of Mn^{2+} inflow were measured using fura-2 as the Ca^{2+} sensor as described previously [9,31]. For the measurement of cell size, cells were harvested, counted and the concentration adjusted to approx. 1×10^6 cells/ml. The cell suspension was then centrifuged at 700 *g* for 3 min at 20 °C, the cells washed twice in Hank's solution (137 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO_4 , 0.8 mM Na_2HPO_4 , 0.4 mM KH_2PO_4 , 4.2 mM NaHCO_3 , 5.5 mM glucose, 20 mM Hepes and 1.5 mM CaCl_2 , pH 7.4) at 37 °C, suspended in Hank's solution then aliquoted into two vials and maintained at 37 °C. To expose cells to a hypotonic medium, samples of cells in Hank's solution were diluted with an equal volume of 1.5 mM CaCl_2 (pH 7.4 at 37 °C) [36]. Cell size (the number of cells within a given size range) was estimated using a Coulter Counter (model TAIL, Coulter Electronics, Harpenden, Herts., U.K.) fitted with a 70 μ m orifice and population-count accessory [37,38]. Except for the time-course studies, three estimates of cell size were made within a period of 3 min for each incubation (Hank's solution or hypotonic medium) and the mean value determined (representing one experiment).

Statistical analysis

Unless indicated otherwise, the data have been expressed as means $\pm$ S.E.M. of the number of experiments indicated. Degrees

of significance between either paired or unpaired groups were determined using Student's *t* test.

RESULTS

To assess the ability of TRPC1-specific siRNA (TRPC1 siRNA) to inhibit expression of the TRPC1 protein, initial experiments were conducted with H4-IIE cells heterologously expressing the hTRPC1 protein. As shown previously [32], in Western blots of heterologously expressed TRPC1, this protein is detected as an 80 kDa band (Figure 1A, lane 3). Cells transfected with TRPC1 siRNA exhibited a 50% decrease in the amount of heterologously expressed hTRPC1 (Figure 1A, compare lanes 1 and 3; and Figure 1B). A similar reduction was observed in cells transfected with single-stranded TRPC1 antisense RNA (Figure 1A, compare lanes 2 and 3; and Figure 1B). Neither TRPC1 siRNA nor single-stranded TRPC1 antisense RNA caused any decrease in the amounts of (i) an unknown protein with a molecular mass of approx. 30 kDa observed in Western blots treated with the 1F1 anti-TRPC1 antibody or (ii) β -actin (Figure 1). A decrease in heterologously expressed hTRPC1 protein in cells transfected with TRPC1 siRNA or with single-stranded TRPC1 antisense RNA was also observed when immunofluorescence was used to detect heterologously expressed hTRPC1 [compare Figure 2C with Figures 2A and 2D (effects of TRPC1 siRNA) and compare Figure 2B with Figures 2A and 2D (effects of single-stranded TRPC1 antisense RNA)]. Examination of the distribution

Figure 3 Treatment with siRNA targeted against TRPC1 reduces expression of endogenous TRPC1 assessed by Western blot

(A) Proteins were extracted from cells transfected with siRNA targeted to TRPC1 (lane 1), TRPC1 antisense RNA (lane 2) or mock-transfected (lane 3), or were transfected with cDNA encoding hTRPC1 (lane 4), then subjected to immunoprecipitation using a polyclonal anti-TRPC1 antibody (1.12 AP) and Western-blot analysis using a monoclonal anti-TRPC1 antibody (1F1), as described in the Materials and methods section. The endogenous TRPC1 protein was detected as a band of 92 kDa and heterologously expressed hTRPC1 as a band of 80 kDa (arrows). The IgG_H band (detected by the secondary antibody) indicates that about the same amounts of protein were loaded on to each lane. The results shown are those obtained in one of two separate experiments which each gave similar results. (B) The mean intensity of the endogenous TRPC1 band in (A) expressed as a ratio of the intensity of the IgG_H band. The results shown are representative of (A) or the means from (B) two experiments.

Figure 4 Treatment with siRNA targeted against TRPC1 reduces expression of endogenous TRPC1 assessed by immunofluorescence

Cells were mock-transfected (**A, D, E**) or transfected with TRPC1 siRNA (**C, F**) or TRPC1 antisense RNA (**B**), incubated for 72 h, then endogenous TRPC1 was detected by immunofluorescence using a polyclonal 1.12 AP anti-TRPC1 antibody (**A–D**) and monoclonal 1F1 anti-TRPC1 antibody (**E, F**), as described in the Materials and methods section. In (**D**), the 1.12 AP anti-TRPC1 antibody was replaced by pre-immune serum. The results shown are those obtained in one of five separate experiments which each gave similar results.

of immunofluorescence in mock-transfected cells at higher magnification indicated that heterologously expressed hTRPC1 is located in the cytoplasmic space and the plasma membrane, but not in the nucleus (which in H4-IIIE cells occupies a large region of the cytoplasmic space; Figure 2E). Treatment with siRNA reduced expression of the protein in both of these locations (compare Figures 2F and 2E).

Transfection of cells with TRPC1 siRNA caused an approx. 50% reduction in endogenous TRPC1 when this was measured using a combined immunoprecipitation/Western blot procedure (Figure 3A, compare lanes 1 and 3; and Figure 3B). As shown previously [32], the apparent molecular mass of endogenous TRPC1 in H4-IIIE cells is 92 kDa compared with 80 kDa for heterologously expressed hTRPC1 (Figure 3A, compare lanes 3 and 4). A small reduction in the amount of endogenous TRPC1 was also

observed in cells treated with single-stranded TRPC1 antisense RNA (Figure 3A, compare lanes 2 and 3; and Figure 3B). In cells transfected with TRPC1 siRNA or with single-stranded TRPC1 antisense RNA, a reduction in the endogenous TRPC1 protein was also observed when TRPC1 was measured by immunofluorescence [compare Figure 4C with Figures 4A and 4D (effects of TRPC1 siRNA) and compare Figure 4B with Figures 4A and 4D (effects of TRPC1 antisense RNA)]. The mean immunofluorescence intensity per cell was 1320 ± 110 , 860 ± 30 , 1080 ± 40 and 460 ± 20 pixels/cell (means $\pm$ S.E.M.) for mock-transfected cells, cells transfected with TRPC1 siRNA, cells transfected with single-stranded TRPC1 antisense RNA and mock-transfected cells where the anti-TRPC1 antibody was replaced by pre-immune serum, respectively (the degrees of significance, using Student's *t* test for paired samples, were $P < 0.01$,

Figure 5 Treatment with siRNA targeted against TRPC1 enhances the increase in cell volume when the incubation medium is changed from a normal to a hypotonic medium

(A, B) Distribution of cells within different size ranges [the number of cells (as a percentage of the total number of cells) in a given size range plotted as a function of the size range] for cells incubated in normal (Hank's) medium (A) and in hypotonic medium (B). (C) The ratio (relative cell size) of the number of cells (percentage of total number of cells) with a diameter in the range 10.1–12.7 μm to the number of cells with a diameter in the range 8–10.1 μm , calculated from the results shown in (A) and (B). (D) The data shown in (C) expressed in terms of the relative cell size in hypotonic solution expressed as a ratio of the relative cell size in normal medium (the normalized relative cell size). Cells were transfected with TRPC1 siRNA, TRPC1 antisense RNA, or mock-transfected, incubated for 48–72 h, then transferred to normal or hypotonic medium for an average time of 1.5 min and cell size measured, as described in the Materials and methods section. The results are the means $\pm$ S.E.M. from three separate experiments. The degrees of significance, calculated using Student's *t* test for paired samples, for comparison of siRNA- or antisense RNA-transfected cells with mock-transfected cells were $*P < 0.05$. Degrees of significance for comparison of siRNA-transfected cells with antisense RNA-transfected cells were $P < 0.05$ in (C) and (D).

$P < 0.05$ and $P < 0.01$ for mock-transfected cells compared with cells transfected with TRPC1 siRNA, cells transfected with single-stranded TRPC1 antisense RNA and mock-transfected cells incubated with pre-immune serum, respectively; and $P < 0.01$ for comparison of cells transfected with TRPC1 siRNA with cells transfected with single-stranded TRPC1 antisense RNA).

Examination of mock-transfected cells at higher magnification showed that, as in the case of heterologously expressed hTRPC1, endogenous TRPC1 is located in the cytoplasmic space and at the plasma membrane (Figure 4E). A number of bright (punctate) spots of immunofluorescence suggest a concentration of TRPC1

in certain regions of the cytoplasmic space (Figure 4E). Treatment with TRPC1 siRNA caused a reduction in TRPC1 expression in most regions of the cell (compare Figures 4F and 4E).

Immunofluorescence detection of TRPC1 was used to perform a dose–response curve for suppression of the endogenous TRPC1 protein by TRPC1 siRNA. The concentrations of TRPC1 siRNA employed were 0 (mock-transfected), 30, 150 and 300 nM. The observed mean immunofluorescence intensity per cell was 1150 ± 150 , 920 ± 90 , 740 ± 90 and 720 ± 80 pixels/cell ($n = 5$), respectively (the value for mock-transfected cells in which pre-immune serum was used in place of anti-TRPC1 antibody was 370 ± 30 pixels/cell). The degree of significance for comparison of the values of each of 150 and 300 nM TRPC1 siRNA with mock-transfected cells was $P < 0.05$. These results indicate that maximum suppression of expression of the endogenous TRPC1 protein is observed using 150 nM TRPC1 siRNA (i.e. 15 μl of 20 μM stock siRNA). This concentration of TRPC1 siRNA was employed in subsequent experiments on Ca^{2+} and Mn^{2+} inflow.

Effects of ablation of the TRPC1 protein on cell volume regulation

Exposure of mock-transfected (control) cells to hypotonic medium for 1.5 min (average time) decreased the number of cells with a diameter in the range 8–10.1 μm , and increased the number of cells with a diameter in the range 10.1–12.7 μm (Figure 5). In cells transfected with TRPC1 siRNA, or with single-stranded antisense TRPC1 RNA, the effect of hypotonic medium in increasing cell size was enhanced (Figure 5).

The ability of cells exposed to hypotonic medium to undergo regulatory cell-volume decrease was assessed by measuring cell diameter as a function of the time elapsed after exposure of the cells to a hypotonic medium. Figure 6 shows that in mock-transfected (control) cells, as shown previously by others [38,39], after the initial increase in volume following hypotonic treatment, the cell volume returns towards the value for normal tonicity over a

Figure 6 Treatment with siRNA targeted against TRPC1 enhances the increase in cell volume and the regulatory cell volume decrease when the incubation medium is changed from a normal to a hypotonic medium

A plot of normalized relative cell size as a function of time elapsed after transferring the cells into hypotonic medium for mock-transfected cells and cells transfected with TRPC1 siRNA. Cells were transfected with TRPC1 siRNA or were mock-transfected, incubated for 48–72 h, then transferred to normal or hypotonic medium and cell size measured as described in the Materials and methods section. The definition of normalized relative cell size and measurement of this parameter are described in the legend of Figure 5. The results are the means $\pm$ S.E.M. from three experiments or the means from two experiments. Degrees of significance for comparison of siRNA- and mock-transfected cells, determined using Student's *t* test for paired samples were $*P < 0.05$.

Figure 7 Treatment with siRNA targeted against TRPC1 inhibits thapsigargin-, ATP- and maitotoxin-initiated Ca^{2+} inflow

Plots of $[\text{Ca}^{2+}]_{\text{cyt}}$ as a function of time for cells treated with thapsigargin (Tg; **A**), ATP (**B**) and maitotoxin (MTX; **C**). The cells were transfected with TRPC1 siRNA or were mock-transfected. Cells were incubated initially in the absence of added Ca_0^{2+} . Thapsigargin ($10 \mu\text{M}$), ATP (1 mM) or maitotoxin (450 pM) and Ca^{2+} (5 mM) were added at the times indicated. The results shown are representative of those obtained from nine (thapsigargin), three (ATP) and four (maitotoxin) separate experiments which each gave similar results.

period of 15 min. Cells transfected with TRPC1 siRNA exhibited a more rapid and complete return to normal volume (Figure 6).

Effects of ablation of the TRPC1 protein on plasma membrane Ca^{2+} and Mn^{2+} inflow

To evaluate the effects of ablation of the TRPC1 protein on Ca^{2+} inflow through SOCs and NSCCs, Ca^{2+} inflow was initiated by thapsigargin, ATP and maitotoxin. In rat liver cells, thapsigargin and ATP predominantly activate SOCs [10,11] while maitotoxin activates NSCCs [8,9]. Ca^{2+} inflow was measured in cells loaded

with fura-2, incubated in the absence of added extracellular Ca^{2+} (Ca_0^{2+}), then exposed to the sequential addition of agonist and Ca_0^{2+} . When compared with mock-transfected cells, cells transfected with TRPC1 siRNA exhibited: (i) a 25 % reduction in the initial rate of thapsigargin-induced Ca^{2+} inflow with no significant reduction in the peak of $[\text{Ca}^{2+}]_{\text{cyt}}$ observed after Ca_0^{2+} addition or in the amount of Ca^{2+} released from intracellular stores by thapsigargin (Figures 7A and Table 1); (ii) a 40 % reduction in the initial rate of ATP-initiated Ca^{2+} inflow with no significant reduction in the peak of $[\text{Ca}^{2+}]_{\text{cyt}}$ observed after Ca_0^{2+} addition or in the amount of Ca^{2+} released from intracellular stores by ATP (Figures 7B and Table 1); (iii) and a 40 % decrease in the rate of maitotoxin-initiated Ca^{2+} inflow and a 25 % decrease in the peak of $[\text{Ca}^{2+}]_{\text{cyt}}$ induced by maitotoxin (Figures 7C, Table 1).

Mn^{2+} inflow was measured in cells loaded with fura-2, incubated in the presence of 1 mM Ca_0^{2+} [9]. The basal rate of Mn^{2+} influx in cells transfected with TRPC1 siRNA was similar to that observed in mock-transfected cells (Figure 8A and Table 2). However, the rate of maitotoxin-initiated Mn^{2+} inflow, and the degree of stimulation of Mn^{2+} inflow by maitotoxin, were each decreased by about 50 % in cells transfected with TRPC1 siRNA (Figure 8B and Table 2).

DISCUSSION

The results reported here indicate that a reduction in the amount of TRPC1 protein in liver cells is associated with an exaggerated response of the cells to changes in cell volume induced by hypotonicity, and a reduction in thapsigargin-, ATP- and maitotoxin-initiated Ca^{2+} inflow, and in maitotoxin-initiated Mn^{2+} inflow. The magnitude of the decrease in the TRPC1 protein (approx. 50 %) is about the same as the magnitude of the decrease in ATP- and maitotoxin-initiated Ca^{2+} inflow (about 40 %), and in maitotoxin-initiated Mn^{2+} inflow (about 50 %).

The observations that TRPC1 siRNA inhibits the expression of hTRPC1 in cells transfected with cDNA encoding this protein, and does not inhibit the expression of an unrelated 30-kDa protein or β -actin, provide evidence that the actions of TRPC1 siRNA are specific for TRPC1. Moreover, results obtained by others indicate that siRNA targeted to a given mRNA species is quite specific in ablating the expression of the protein encoded by that mRNA species [27,33]. The observation that transfection with single-stranded TRPC1 antisense RNA also reduced the amount of TRPC1 is not surprising since the single-stranded antisense sequence of siRNA has been shown, in a number of instances, to effectively induce mRNA degradation [40–42]. The results of dose–response experiments indicate that maximum suppression of TRPC1, which can be obtained using this siRNA species, was 50 %. siRNAs targeted to alternative regions of TRPC1 would be required to obtain greater suppression of the TRPC1 protein [43].

The observation that TRPC1 siRNA decreases thapsigargin- and ATP-initiated Ca^{2+} inflow, and maitotoxin-initiated Ca^{2+} and Mn^{2+} inflow, suggests that TRPC1 is a component or regulator of more than one type of Ca^{2+} -inflow pathway. The present results obtained with siRNA are consistent with those obtained previously in this laboratory for heterologous overexpression of hTRPC1 in H4-IIE cells [9]. Thus overexpression of hTRPC1 was associated with an enhancement of both thapsigargin- and maitotoxin-initiated Ca^{2+} inflow and of maitotoxin-initiated Mn^{2+} inflow. In liver cells, thapsigargin and ATP initiate the activation of SOCs [10,11], while maitotoxin initiates the activation of at least one type of NSCC without activating SOCs [8,9]. The main SOC in H4-IIE cells is indistinguishable from Ca^{2+} -release-activated

Table 1 Effects of treatment with siRNA targeted against TRPC1 on thapsigargin-, ATP- and maitotoxin-initiated Ca^{2+} inflow

Initial (thapsigargin and ATP) and maximum (maitotoxin) rates of Ca^{2+} inflow and the peaks of Ca_0^{2+} -induced and agonist-induced increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ were measured as described in the Materials and methods section and in the legend of Figure 7. The results are means $\pm$ S.E.M. from the number of experiments indicated in parentheses. The degrees of significance, determined using Student's *t* test for paired samples, for a comparison with mock-transfected cells were **P* < 0.05 and †*P* < 0.01.

Agonist	Treatment	Rate of Ca^{2+} inflow (nM/s)	Peak of Ca_0^{2+} -induced increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ (nM)	Peak of increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ induced by agonist addition (nM)
Thapsigargin	Mock transfection	39 ± 4 (9)	1330 ± 60 (9)	140 ± 10 (9)
	siRNA transfection	30 ± 2 (9)†	1150 ± 140 (9)	160 ± 20 (9)
ATP	Mock transfection	9.9 ± 1.8 (3)	320 ± 50 (3)	380 ± 40 (3)
	siRNA transfection	6.1 ± 2.1 (3)*	210 ± 50 (3)	380 ± 80 (3)
Maitotoxin	Mock transfection	10.2 ± 1 (4)	1270 ± 80 (4)	—
	siRNA transfection	6.5 ± 0.3 (4)*	960 ± 70 (4)*	—

Figure 8 Treatment with siRNA targeted against TRPC1 inhibits maitotoxin-initiated Mn^{2+} inflow

Plots of quenching of fura-2 fluorescence as a function of time for basal Mn^{2+} inflow (A) and maitotoxin (MTX)-initiated Mn^{2+} inflow (B). The cells were transfected with TRPC1 siRNA or were mock-transfected cells. Maitotoxin (600 pM) and Mn^{2+} (100 μM) were added at the times indicated. The results shown are representative of those obtained for five separate experiments which each gave similar results.

Ca^{2+} channels in mast cells and lymphocytes and has a high selectivity for Ca^{2+} compared with Na^{+} [10]. However, when heterologously expressed in liver cells [9] and in other animal cells, TRPC1 has a low selectivity for Ca^{2+} compared with Na^{+} (reviewed in [12–14]). Even taking account of the capacity of TRPC1 to form heterotetramers with other TRPC proteins [44,45], it is unlikely that TRPC1 is a component of Ca^{2+} -release-activated Ca^{2+} channels. However, it is possible that TRPC1 is a component of an SOC with low selectivity for Ca^{2+} compared with Na^{+} . The most likely function for TRPC1 in H4-IIE cells is considered to be an NSCC, the activation of which can be initiated by

Table 2 Effects of treatment with siRNA targeted against TRPC1 on maitotoxin-initiated Mn^{2+} inflow

Rates of Mn^{2+} inflow were measured as described in the Materials and methods section and in the legend of Figure 8. Maitotoxin (600 pM), when present, was added 5 min before Mn^{2+} (100 μM). The maitotoxin-stimulated rate of Mn^{2+} inflow was calculated for each separate experiment (*n* = 5) by subtracting the basal rate of Mn^{2+} inflow from that observed in the presence of maitotoxin. The results are means $\pm$ S.E.M. from five experiments. The degree of significance, determined using Student's *t* test for paired samples, for a comparison of the values obtained for mock-transfected cells with the corresponding value for TRPC1 siRNA-transfected cells was **P* < 0.01.

Treatment	Rate of Mn^{2+} inflow (fluorescence units/min)		
	Basal	Maitotoxin present	Maitotoxin-stimulated rate
Mock-transfection	12.0 ± 1.6	31.0 ± 3.2	19.0 ± 2.1
TRPC1 siRNA-transfection	12.2 ± 0.9	$21.0 \pm 2.9^*$	$8.8 \pm 2.6^*$

thapsigargin and maitotoxin, and is most probably also initiated by other agonists. This NSCC may be formed from two types of TRPC1 polypeptide (e.g. TRPC1 and TRPC3) [44,45].

The results of immunofluorescence experiments indicate that endogenous TRPC1 is located in regions of the cytoplasmic space as well as at the plasma membrane of H4-IIE cells. Such a distribution has been observed for some other plasma membrane receptors and channels, and may reflect the processes which regulate the amount of protein at the plasma membrane [9,46,47]. However, it is possible, as suggested for some other TRP proteins, that some of the physiological actions of TRPC1 are exerted by the protein located at an intracellular site [26,48].

The effect of ablation of TRPC1 on the response of H4-IIE liver cells to a hypotonic medium was to enhance the increase in volume (swelling) when cells are initially placed in a hypotonic medium and to make recovery to normal cell volume faster and more extensive. The likely sequence of events following the introduction of hypotonic medium is, initially, the movement of water from the extracellular medium to the cytoplasmic space, accompanied by the movement of Cl^{-} , K^{+} and Na^{+} across the plasma membrane and the release of ATP. These changes then initiate processes involved in the reduction of cell volume, including K^{+} and Cl^{-} efflux through K^{+} and Cl^{-} channels, which may be Ca^{2+} -activated, the activation of protein kinase $\text{C}\alpha$ and phosphoinositide 3-kinase, and an increase in $[\text{Ca}^{2+}]_{\text{cyt}}$. The last-mentioned response, which may in part be due to the actions of extracellular ATP [2–4,11,39,49], is caused by the release of Ca^{2+} from intracellular stores and the activation of Ca^{2+} inflow through SOCs and stretch-activated Ca^{2+} channels. TRPC1 could

potentially participate in these processes as a component of SOC_s and/or stretch-activated NSCC_s [9,24–26], the Na⁺ channel [50], an intracellular Ca²⁺ channel [48] and/or as a component or regulator of InsP₃ receptors [26].

While the ablation of 50% of the endogenous TRPC1 protein did cause a clearly defined alteration in the response of H4-IIIE cells to hypotonic medium, the effect was relatively small. If the main role of TRPC1 were as a component of SOC_s and/or InsP₃-mediated Ca²⁺ release channels, the ablation of TRPC1 would have been expected to reduce the hypotonic medium-initiated increase in [Ca²⁺]_{cyt} and reduce the subsequent reduction in cell volume. However, the opposite effect, enhanced volume increase and recovery of cell volume, was observed. This suggests that, in connection with volume regulation, another function of TRPC1, for example as a component of a channel which principally admits Na⁺ [50], may be involved. The observed effects on cell-volume regulation may be a secondary consequence of the ablation of this function of TRPC1. Another possibility is that TRPC1 may be involved through its interaction with the actin cytoskeleton via the ankyrin-binding domains of TRPC1 [23,51], since it has been shown that the actin cytoskeleton is involved in cell-volume regulation [52].

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REFERENCES

- Barritt, G. J. (2000) Calcium signalling in liver cells. In *Calcium: the Molecular Basis of Calcium Action in Biology and Medicine* (Pochet, R., Donato, R., Haiech, J., Heizmann, C. and Gerlce, V., eds.), pp. 175–196. Kluwer Academic Publishers, The Netherlands.
- Haussinger, D. and Lang, F. (1991) Cell volume in the regulation of hepatic function: a mechanism for metabolic control. *Biochim. Biophys. Acta* **1071**, 331–350.
- Dunkelberg, J. C., Feranchak, A. P. and Fitz, J. G. (2001) Liver cell volume regulation: size matters. *Hepatology* **33**, 1349–1352.
- Graf, J. and Haussinger, D. (1996) Ion transport in hepatocytes: mechanisms and correlations to cell volume, hormone actions and metabolism. *J. Hepatol.* **24**, 53–77.
- Strigrow, F. and Bohnsack, R. (1994) Inositol 1,4,5-trisphosphate activates receptor-mediated calcium entry by two different pathways in hepatocytes. *Eur. J. Biochem.* **222**, 229–234.
- Fernando, K. C. and Barritt, G. J. (1995) Characterisation of the divalent cation channels of the hepatocyte plasma membrane receptor-activated Ca²⁺ inflow system using lanthanide ions. *Biochim. Biophys. Acta* **1268**, 97–106.
- Bear, C. E. and Li, C. H. (1991) Calcium-permeable channels in rat hepatoma cells are activated by extracellular nucleotides. *Am. J. Physiol.* **261**, C1018–C1024.
- Woods, N. M., Dixon, C. J., Yasumoto, T., Cuthbertson, K. S. R. and Cobbold, P. H. (1999) Maitotoxin-induced free Ca changes in single rat hepatocytes. *Cell. Signalling* **11**, 805–811.
- Brereton, H. M., Chen, J. L., Rychkov, G., Harland, M. L. and Barritt, G. J. (2001) Maitotoxin activates an endogenous non-selective cation channel and is an effective initiator of the activation of the heterologously expressed hTRPC1 (transient receptor potential) non-selective cation channel in H4-IIIE liver cells. *Biochim. Biophys. Acta* **1540**, 107–126.
- Rychkov, G., Brereton, H. M., Harland, M. L. and Barritt, G. J. (2001) Plasma membrane Ca²⁺ release-activated Ca²⁺ channels with a high selectivity for Ca²⁺ identified by patch-clamp recording in rat liver cells. *Hepatology* **33**, 938–947.
- Roe, M. W., Moore, A. L. and Lidofsky, S. D. (2001) Purinergic-independent calcium signaling mediates recovery from hepatocellular swelling: implications for volume regulation. *J. Biol. Chem.* **276**, 30871–30877.
- Zitt, C., Halaszovich, C. R. and Luckhoff, A. (2002) The TRP family of cation channels: probing and advancing the concepts on receptor-activated calcium entry. *Prog. Neurobiol.* **66**, 243–264.
- Vennekens, R., Voets, T., Bindels, R. J., Droogmans, G. and Nilius, B. (2002) Current understanding of mammalian TRP homologues. *Cell Calcium* **31**, 253–264.
- Minke, B. and Cook, B. (2002) TRP channel proteins and signal transduction. *Physiol. Rev.* **82**, 429–472.
- Hayes, P., Meadows, H. J., Gunthorpe, M. J., Harries, M. H., Duckworth, D. M., Cairns, W., Harrison, D. C., Clarke, C. E., Ellington, K., Prinjha, R. K. et al. (2000) Cloning and functional expression of a human orthologue of rat vanilloid receptor-1. *Pain* **88**, 205–215.
- Wissenbach, U., Bodding, M., Freichel, M. and Flockerzi, V. (2000) Trp12, a novel Trp related protein from kidney. *FEBS Lett.* **485**, 127–134.
- Peng, J. B., Chen, X. Z., Berger, U. V., Weremowicz, S., Morton, C. C., Vassilev, P. M., Brown, E. M. and Hediger, M. A. (2000) Human calcium transport protein CaT1. *Biochem. Biophys. Res. Commun.* **278**, 326–332.
- Launay, P., Fleig, A., Perraud, A. L., Scharenberg, A. M., Penner, R. and Kinet, J. P. (2002) TRPM4 is a Ca²⁺-activated nonselective cation channel mediating cell membrane depolarization. *Cell* **109**, 397–407.
- Montell, C., Birnbaumer, L. and Flockerzi, V. (2002) The TRP channels, a remarkably functional family. *Cell* **108**, 595–598.
- Runnels, L. W., Yue, L. and Clapham, D. E. (2001) TRP-PLIK, a bifunctional protein with kinase and ion channel activities. *Science* **291**, 1043–1047.
- Sinkins, W. G., Estacion, M. and Schilling, W. P. (1998) Functional expression of Trpc1 – a human homologue of the *Drosophila* Trp channel. *Biochem. J.* **331**, 331–339.
- Antoniotti, S., Lovisolo, D., Pla, A. F. and Munaron, L. (2002) Expression and functional role of bTRPC1 channels in native endothelial cells. *FEBS Lett.* **510**, 189–195.
- Engelke, M., Friedrich, O., Budde, P., Schafer, C., Niemann, U., Zitt, C., Jungling, E., Rocks, O., Luckhoff, A. and Frey, J. (2002) Structural domains required for channel function of the mouse transient receptor potential protein homologue TRP1beta. *FEBS Lett.* **523**, 193–199.
- Singh, B. B., Zheng, C., Liu, X., Lockwich, T., Liao, D., Zhu, M. X., Birnbaumer, L. and Ambudkar, I. S. (2001) Trp1-dependent enhancement of salivary gland fluid secretion: role of store-operated calcium entry. *FASEB J.* **15**, 1652–1664.
- Brough, G. H., Wu, S., Cioffi, D., Moore, T. M., Li, M., Dean, N. and Stevens, T. (2001) Contribution of endogenously expressed Trp1 to a Ca²⁺-selective, store-operated Ca²⁺ entry pathway. *FASEB J.* **15**, 1727–1738.
- Mori, Y., Wakamori, M., Miyakawa, T., Hermosura, M., Hara, Y., Nishida, M., Hirose, K., Mizushima, A., Kurosaki, M., Mori, E. et al. (2002) Transient receptor potential 1 regulates capacitative Ca²⁺ entry and Ca²⁺ release from endoplasmic reticulum in B lymphocytes. *J. Exp. Med.* **195**, 673–681.
- Elbashir, S. M., Harborth, J., Weber, K. and Tuschl, T. (2002) Analysis of gene function in somatic mammalian cells using small interfering RNAs. *Methods (Duluth)* **26**, 199–213.
- Hannon, G. J. (2002) RNA interference. *Nature (London)* **418**, 244–251.
- Holen, T., Amarzguioui, M., Wiiger, M. T., Babaie, E. and Prydz, H. (2002) Positional effects of short interfering RNAs targeting the human coagulation trigger tissue factor. *Nucleic Acids. Res.* **30**, 1757–1766.
- Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. and Tuschl, T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature (London)* **411**, 494–498.
- Auld, A., Chen, J., Brereton, H. M., Wang, Y. J., Gregory, R. B. and Barritt, G. J. (2000) Store-operated Ca²⁺ inflow in Reuber hepatoma cells is inhibited by voltage-operated Ca²⁺ channel antagonists and, in contrast to freshly isolated hepatocytes, does not require a pertussis toxin-sensitive trimeric GTP-binding protein. *Biochim. Biophys. Acta* **1497**, 11–26.
- Ong, H. L., Chen, J., Chataway, T., Brereton, H., Zhang, L., Downs, T., Tsiokas, L. and Barritt, G. (2002) Specific detection of the endogenous transient receptor potential (TRP)-1 protein in liver and airway smooth muscle cells using immunoprecipitation and Western-blot analysis. *Biochem. J.* **364**, 641–648.
- Tuschl, T. and Borkhardt, A. (2002) Small interfering RNAs. *Mol. Interventions* **2**, 158–167.
- Tsiokas, L., Arnould, T., Zhu, C. W., Kim, E., Walz, G. and Sukhatme, V. P. (1999) Specific association of the gene product of PKD2 with the TRPC1 channel. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 3934–3939.
- Bradford, H. F. and Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Adv. Exp. Med. Biol.* **69**, 395–404.
- Wang, Y. J., Gregory, R. B. and Barritt, G. J. (2002) Maintenance of the filamentous actin cytoskeleton is necessary for the activation of store-operated Ca²⁺ channels, but not other types of plasma-membrane Ca²⁺ channels, in rat hepatocytes. *Biochem. J.* **363**, 117–126.
- Roman, R. M., Wang, Y. and Fitz, J. G. (1996) Regulation of cell volume in a human biliary cell line: activation of K⁺ and Cl[−] currents. *Am. J. Physiol.* **271**, G239–G248.
- Roman, R. M., Wang, Y., Lidofsky, S. D., Feranchak, A. P., Lomri, N., Scharschmidt, B. F. and Fitz, J. G. (1997) Hepatocellular ATP-binding cassette protein expression enhances ATP release and autocrine regulation of cell volume. *J. Biol. Chem.* **272**, 21970–21976.

- 39 Feranchak, A. P., Fitz, J. G. and Roman, R. M. (2000) Volume-sensitive purinergic signaling in human hepatocytes. *J. Hepatol.* **33**, 174–182
- 40 Hohjoh, H. (2002) RNA interference (RNAi) induction with various types of synthetic oligonucleotide duplexes in cultured human cells. *FEBS Lett.* **521**, 195–199
- 41 Martinez, J., Patkaniowska, A., Urlaub, H., Lührmann, R. and Tuschl, T. (2002) Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. *Cell* **110**, 563–574
- 42 Schwarz, D. S., Hutvagner, G., Haley, B. and Zamore, P. D. (2002) Evidence that siRNAs function as guides, not primers, in the *Drosophila* and human RNAi pathways. *Mol. Cell* **10**, 537–548
- 43 Hamada, M., Ohtsuka, T., Kawaida, R., Koizumi, M., Morita, K., Furukawa, H., Imanishi, T., Miyagishi, M. and Taira, K. (2002) Effects on RNA interference in gene expression (RNAi) in cultured mammalian cells of mismatches and the introduction of chemical modifications at the 3'-ends of siRNAs. *Antisense Nucleic Acid Drug Dev.* **12**, 301–309
- 44 Lintschinger, B., Balzer-Geldsetzer, M., Baskaran, T., Graier, W. F., Rومانin, C., Zhu, M. X. and Groschner, K. (2000) Coassembly of Trp1 and Trp3 proteins generates diacylglycerol- and Ca^{2+} -sensitive cation channels. *J. Biol. Chem.* **275**, 27 799–27 805
- 45 Strubing, C., Krapivinsky, G., Krapivinsky, L. and Clapham, D. E. (2001) TRPC1 and TRPC5 form a novel cation channel in mammalian brain. *Neuron* **29**, 645–655
- 46 Kanzaki, M., Zhang, Y. Q., Mashima, H., Li, L., Shibata, H. and Kojima, I. (1999) Translocation of a calcium-permeable cation channel induced by insulin-like growth factor-I. *Nat. Cell Biol.* **1**, 165–170
- 47 Ma, D., Zerangue, N., Lin, Y. F., Collins, A., Yu, M., Jan, Y. N. and Jan, L. Y. (2001) Role of ER export signals in controlling surface potassium channel numbers. *Science* **291**, 316–319
- 48 Hassock, S. R., Zhu, M. X., Trost, C., Flockerzi, V. and Authi, K. S. (2002) Expression and role of TRPC proteins in human platelets: evidence that TRPC6 forms the store-independent calcium entry channel. *Blood* **100**, 2801–2811
- 49 Junankar, P. R., Karjalainen, A. and Kirk, K. (2002) The role of P2Y1 purinergic receptors and cytosolic Ca^{2+} in hypotonically activated osmolyte efflux from a rat hepatoma cell line. *J. Biol. Chem.* **277**, 40 324–40 334
- 50 Nilius, B. (2003) Calcium-impermeable monovalent cation channels: a TRP connection? *Br. J. Pharmacol.* **138**, 5–7
- 51 Lockwich, T. P., Liu, X., Singh, B. B., Jadowiec, J., Weiland, S. and Ambudkar, I. S. (2000) Assembly of Trp1 in a signaling complex associated with caveolin-scaffolding lipid raft domains. *J. Biol. Chem.* **275**, 11 934–11 942
- 52 Papakonstanti, E. A., Vardaki, E. A. and Stournaras, C. (2000) Actin cytoskeleton: a signalling sensor in cell volume regulation. *Cell. Physiol. Biochem.* **10**, 257–264

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