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ORIGINAL ARTICLE

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Differential activation of the volume-sensitive cation channel TRP12 (OTRPC4) and volume-regulated anion currents in HEK-293 cells

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Abstract The detection of changes in volume and osmolality is an essential function in vertebrate cells. A novel member of the transient receptor potential (trp) family of ion channels, which is sensitive to changes in cell volume, has been described recently. Heterologous expression of TRP12 in HEK cells resulted in the appearance of a swelling-activated cation current. The permeability sequence of this cation current for various monovalent cations, as determined from shifts in reversal potential upon extracellular cation substitution, was $P_K > P_{Cs} > P_{Na} > P_{Li}$, corresponding to an Eisenman-IV sequence characteristic for a weak-field-strength site. Surprisingly, over-expression of this channel in HEK cells was accompanied by a dramatic down-regulation of the volume-regulated anion channel (VRAC), which is activated by cell swelling in non-transfected cells. In contrast to VRAC, TRP12 could not be activated at constant volume by a reduction of intracellular ionic strength or by intracellular perfusion with guanosine 5'-*O*-(3-thiotriphosphate (GTPγS). The kinetic and pharmacological profile of VRAC and TRP12 currents were also different.

Keywords TRP channels · Volume regulated anion channels · Channel interaction · Volume sensor · Volume regulation

Introduction

Ion channels that sense changes in cell volume are well characterized functionally. In particular the biophysical properties and activation mechanisms of volume-regulated anion channels (VRAC) have been studied extensively [10, 11, 16, 19]. VRAC can also be activated at constant volume by reducing intracellular ionic strength [1, 3, 12, 17, 26] or by guanosine 5'-*O*-(3-thiotriphosphate) (GTPγS) [9, 25], but probably not by membrane stretch. The molecular nature of this channel and its activation mechanism during cell swelling have not yet been resolved [13, 18].

Volume-regulated potassium channels have also been described recently [5, 8], a molecular candidate may be found in the two-pore K^+ channel family TASK [7]. Three probably identical members of the family of transient receptor potential (trp) cation channels, i.e. osmosensitive, transient receptor potential channel-4 (OTRPC4) [20], TRP12 [27] and the vanilloid receptor-related, osmotically activated channel (VR-OAC) [6], are volume sensitive. This channel is expressed abundantly in kidney, but also in lung, liver, spleen, fat tissue, heart, brain and in endothelial cells [6, 20, 27]. It is activated by cell swelling and is permeable for Ca^{2+} and monovalent cations with a P_{Ca}/P_{Na} permeability ratio of 0.8 and a single channel conductance of 30 pS (inwards) and 88 pS (outwards) [20]. Activation of this channel by cell swelling is not mediated by membrane stretch [20] and it is not clear how the TRP12 channel senses an increase in cell volume or a change in osmolarity. We therefore compared the activation pattern of TRP12 at constant volume with that of the much better studied VRAC in non-transfected and TRP12-transfected HEK cells under identical conditions. Besides obvious differences in the activation pattern of both channels, we also observed almost complete down-regulation of VRAC in TRP12-transfected cells.

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Materials and methods

Expression plasmids

We used the recombinant bicistronic expression plasmid pdiTRP12 that carried the entire protein-coding region for TRP12 and the green fluorescent protein (GFP) [27].

Cells and transfection

Human embryonic kidney cells, HEK293, were grown in DMEM containing 10% (v/v) human serum, 2 mM L-glutamine, 2 U/ml penicillin and 2 mg/ml streptomycin at 37 °C in a humidity-controlled incubator with 10% CO_2. HEK293 cells, transiently transfected with the above vector and expressing TRP12, were identified visually in the patch-clamp apparatus by their GFP fluorescence. GFP was excited at a wavelength of 425–475 nm. A 495-nm dichroic mirror was used for GFP. The emitted light was passed through a 500-nm long-pass filter. GFP-negative cells from the same batch were used as controls, because we have shown previously that currents in cells transfected with the same expression plasmid carrying the protein-coding region of GFP are not different from those in non-transfected cells, thus excluding non-specific effects of the plasmid and GFP expression [2].

Solutions

The standard extracellular solution contained (in mM): 150 NaCl, 6 KCl, 1 $MgCl_2$, 1.5 $CaCl_2$, 10 glucose, 10 HEPES, buffered at pH 7.4 with NaOH. The osmolality of this solution measured using a vapour-pressure osmometer (Wescor 5500, Schlag, Gladbach, Germany), was 320±5 mOsm. During the experiment, cells were perfused with a solution containing (in mM): 85 NaCl, 6 CsCl, 1 $MgCl_2$, 1.5 $CaCl_2$, 10 glucose, 10 HEPES, buffered pH 7.4 with NaOH. The appropriate amount of mannitol was added to this solution to adjust the osmolality as required to 200–400 mOsm. The normal hypotonic stimulation was 240 mOsm (25% reduction). The pipette solution contained (in mM): 40 CsCl, 100 Cs-aspartate, 1 $MgCl_2$, 1.93 $CaCl_2$, 5 EGTA, 4 Na_2ATP, 10 HEPES, pH 7.2 with CsOH (290 mOsm). The ionic strength of this pipette solution (Γ_p) was 155 mM. For experiments with a decreased ionic strength we used the following pipette solution (in mM): 40 CsCl, 30 Cs-aspartate, 2 $MgCl_2$, 1.93 $CaCl_2$, 5 EGTA, 4 Na_2ATP, 10 HEPES, 140 sucrose, pH 7.2 with CsOH (290 mOsm). This solution has an ionic strength of 125 mM. In a series of additional experiments, we substituted 8 mM NaCl for Na_2ATP. The free $[Ca^{2+}]$ in all solutions was buffered at 100 nM (5 mM EGTA and 1.93 mM $CaCl_2$). For pipette solutions with a different Γ_p, the concentration of Cs-aspartate was decreased and sucrose added to keep the osmolality constant. GTPγS (Sigma) was applied via the pipette solution at 100 µM. These protocols are used routinely in our laboratory [12, 25, 26]. Tamoxifen (Sigma) and 5-nitro-2-(3-phenylpropylamino)-benzoic acid, (NPPB, RBI) were applied from 50 mM stock solutions in DMSO. Extra- and intracellular solutions contained Cs^+-salts to inhibit the K^+ currents that are present in a few, but not all, HEK cells. All experiments were performed at room temperature (20–23 °C).

Electrophysiological recordings

Whole-cell membrane currents were monitored with an EPC-9 (HEKA Elektronik, Lambrecht, Germany, 8-pole Bessel filter 2.9 kHz) using ruptured patches. Patch electrodes had a DC resistance of 2–6 MΩ. An Ag-AgCl electrode was used as reference. We employed two different voltage protocols. The first consisted of 400-ms linear voltage ramps from –150 to +100 mV applied from a holding potential of –30 mV (0.8 ms sampling interval, 5 s between ramps, 512 points); the second of voltage steps from –100 mV to +100 mV (increment 20 mV, holding potential of –50 mV, 1 ms sampling interval, 6 s between steps, 2048 points). Between 50% and 70% of the series resistance was compensated electronically to minimise voltage errors. Time courses of the whole-cell current at –100 and +100 mV were derived from the averaged current amplitudes measured during voltage ramps in a narrow window around these potentials. Cell membrane capacitance (C_m) was read from the compensation circuitry of the EPC-9 amplifier.

Ca^{2+} measurements

For cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_i$) measurements, cells were loaded with Fura-II by incubating them for 20 min at 37 °C in a standard extracellular solution containing 2 µM Fura-2/AM. The dye was excited alternately at wavelengths of 360 and 390 nm through a filter wheel rotating at 2 Hz. The fluorescence was measured at 510 nm and corrected for autofluorescence, measured from the cell-free background. Apparent free $[Ca^{2+}]$ was calculated from the fluorescence ratio R according to: $[Ca^{2+}] = K_{eff} \cdot \frac{R-R_0}{R_1-R}$, where K_{eff} is the effective binding constant, R_0 the fluorescence ratio at zero $[Ca^{2+}]$ and R_1 that at high $[Ca^{2+}]$. The calibration constants were determined experimentally for the given set-up and the actual experimental conditions used.

Data analysis

Electrophysiological data were analysed using the WinASCD software (G. Droogmans, Leuven). Pooled data are given as means±SEM from *n* cells. The significance of differences between means was established using Student's *t*-test for paired samples.

Results

Background currents in non-transfected and TRP12-transfected HEK293 cells

Figure 1A shows background currents in a non-transfected and in a TRP12-transfected HEK293 cell in response to voltage steps. The currents in the non-transfected cells were outwardly rectifying with densities of –6±1.5 pA/pF at –100 mV and 22±7 pA/pF at +100 mV and reversed at –14±5 mV (n=6). Part of the current apparently passed through VRAC, since increasing the extracellular osmolarity to 380 mOsM by adding 80 mM mannitol to the extracellular solution reduced the current amplitude at +100 mV by approximately 60%. The difference current before and after cell shrinkage reversed at –23±5 mV (n=4) and showed outward rectification, a typical feature of VRAC described for many cell types (for reviews, see [11, 16, 19]). The remaining current had a nearly linear current/voltage (I/V) curve, an Eisenman IV permeability sequence ($K^+ > Cs^+ > Na^+ > Li^+$, [4]) and the inwards current component was almost completely abolished by replacing extracellular Na^+ by *N*-methyl-D-glucamine ($NMDG^+$, not shown). It therefore probably passed through non-selective cation channels with the same permeability sequence described previously in these cells [14].

The background current in TRP12-expressing cells (Fig. 1) was significantly larger than in control cells. These currents with densities of –30±5 pA/pF at –100 mV and 64±9 pA/pF at +100 mV were weakly outwardly rectifying and reversed at a more positive potential (+2±1 mV, n=17) than in non-transfected cells (Fig. 1B). Current noise was clearly enhanced at large negative and positive potentials. Shrinking of the cells by addition of 80 mM mannitol reduced the current by 86±7% and shifted the reversal potential towards more negative values. The inwards current also disappeared in

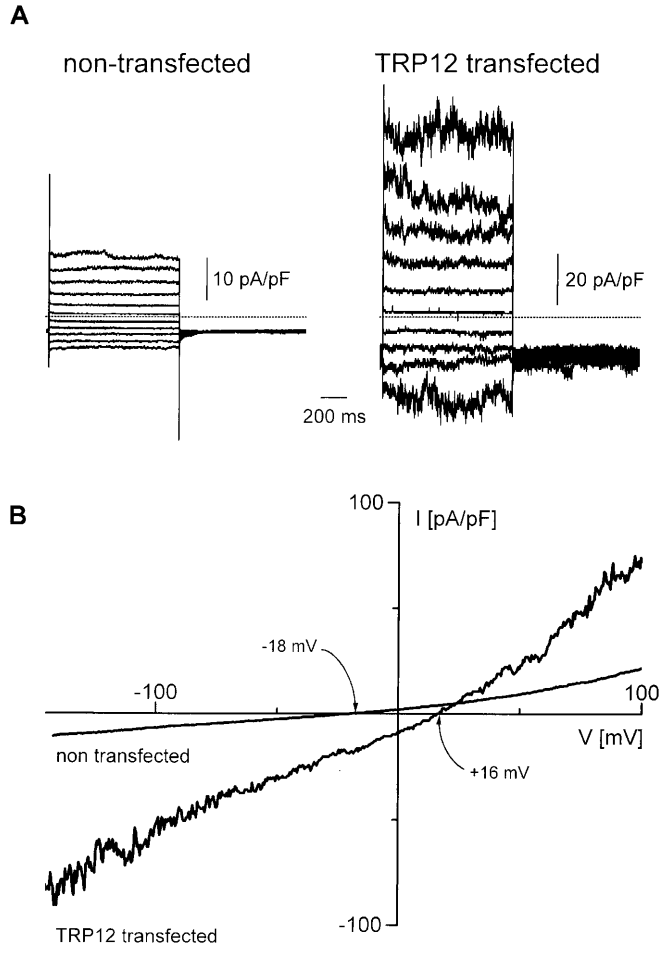

Fig. 1A, B Background current in a non-transfected and a TRP12-transfected HEK 293 cell. **A** Current traces in response to voltage steps from a holding potential of –50 mV to test potentials ranging from –100 to +100 mV in increments of 20 mV in a non-transfected (*left*) and a TRP12-transfected HEK 293 cell (*right*). **B** Instantaneous current/voltage (*I*/*V*) curves from a non-transfected and a TRP12-transfected HEK 293 cell during a voltage ramp from –150 to +100 mV

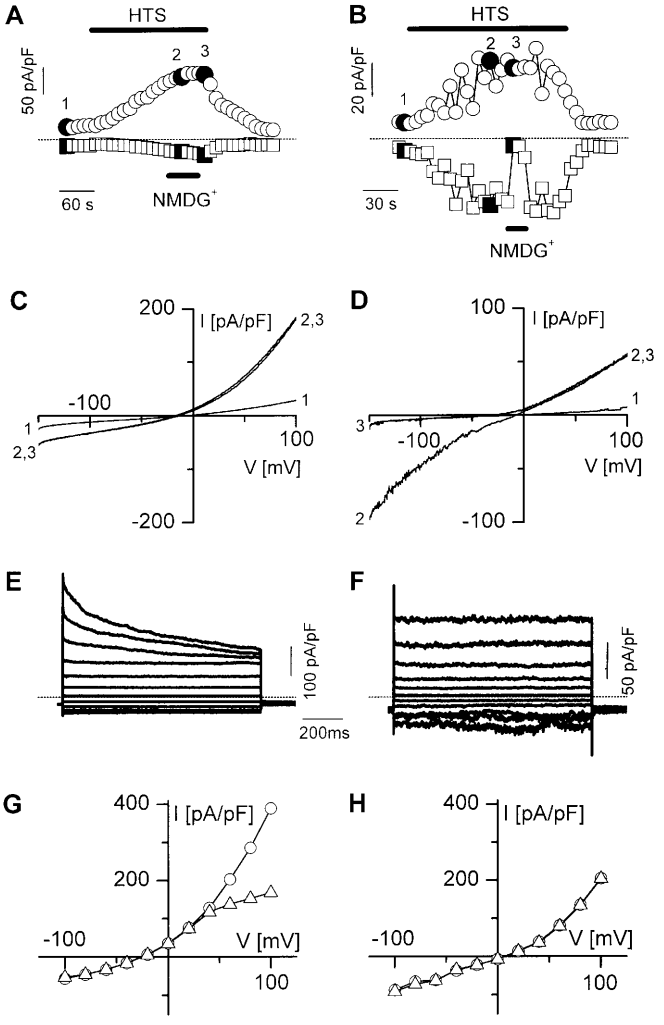

Fig. 2A–H Currents activated by a hypotonic stimulus (*HTS*) in a non-transfected (**A**, **C**, **E**, **G**) and a TRP12-transfected (**B**, **D**, **F**, **H**) HEK 293 cell. **A**, **B** Time course of current activation at –100 and +100 mV. Current values were obtained from voltage ramps applied every 5 s. For the period indicated by the *horizontal bars*, *N*-methyl-D-glucamine (*NMDG*$^+$) was substituted for extracellular Na^+. **C**, **D** Representative *I*/*V* curves from voltage ramps (–150 to +100 mV) applied at the times indicated (*1–3*) in **A** and **B**. **E**, **F** Current traces in response to voltage steps (see Materials and methods) in a non-transfected and in a TRP12-trasnfected HEK 293 cell. **G**, **H** *I*/*V* curves constructed from the currents at the beginning (*circles*) and end (*triangles*) of the voltage pulses in **E** and **F**

extracellular $NMDG^+$ solutions, suggesting that it is a cationic current, and similar to the swelling-activated TRP12 (OTRPC4) current in hypotonic solutions [6, 20, 27]. These findings are therefore consistent with a functional TRP12 current in isotonic solutions. The permeability sequence of this cation current for various monovalent cations, as determined from shifts in reversal potential upon extracellular cation substitution, was Eisenman IV with P_K:P_{Cs}:P_{Na}:P_{Li}=1.9±0.22:1.33±0.098:1:0.88±0.023 (*n*=6).

Resting $[Ca^{2+}]_i$ was 258±72 nM (*n*=10) in TRP12-transfected cells, which is significantly higher than the 62±9 nM (*n*=5) in non-transfected cells.

Currents activated by cell swelling in non-transfected and TRP12-transfected HEK 293 cells

Hyposmotic cell swelling induced a slowly activating and outwardly rectifying current in control HEK cells. This current reversed at –19±3 mV (*n*=12) and was not affected when $NMDG^+$ was substituted for Na^+ (Fig. 2A, C). This finding suggests that HEK cells lack a functional endogenous TRP12 current that is activated by cell swelling. These data are consistent with the reported lack of endogenous expression of TRP12 in these cells [20]. The current showed clear inactivation at positive potentials (Fig. 2E, G) and was blocked completely by 20 µM tamoxifen and 50 µM NPPB (data not shown, *n*=4). It obviously had the same phenotype as the previously described swelling-activated Cl^- current ($I_{Cl,swell}$) through VRAC [10, 13, 16, 19]. The swelling-activated current at

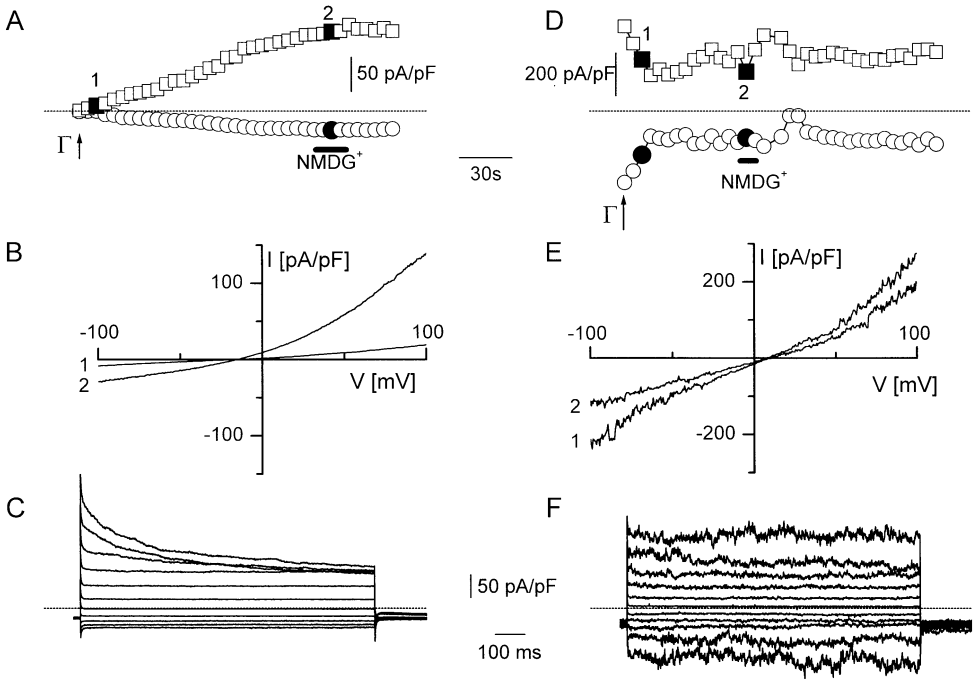

Fig. 3A–F Currents activated by a reduction of intracellular ionic strength in a non–transfected (**A**–**C**) but not in a TRP12-transfected HEK 293 cell (**D**–**F**). **A**, **D** Time course of the current at –100 and +100 mV after breaking into the cell with a pipette solution of reduced ionic strength (70 mM Cs^+-aspartate replaced by 140 mM sucrose, indicated by *arrow*). Data were obtained from currents recorded during repetitively applied voltage ramps. For the periods indicated by the *horizontal bars*, extracellular Na^+ was replaced by $NMDG^+$. **B**, **E** Representative *I/V* curves from the voltage ramps marked by the *filled symbols* in **A** and **D** (same cells as in **A** and **D**). **C**, **F** Currents elicited by voltage steps from –100 mV to +100 mV from a holding potential of –50 mV and applied after the ramp protocol, as described in Materials and methods (same cells as in **A** and **D**)

+100 mV had a density of 312±24 pA/pF (*n*=12), a not significantly different from the value in HEK293 cells transfected with GFP alone (248±43 pA/pF, *n*=6). The failure of GFP expression alone to affect VRAC has already been described in detail in endothelial and Caco-2 cells [2, 21, 22]. The current density at –100 mV was –5±2 pA/pF (*n*=12) and was not affected by substituting $NMDG^+$ for Na^+, indicating that cell swelling did not activate any cation current at this potential.

In TRP12-transfected cells, swelling activated a current that reversed close to 0 mV (2.2±1 mV, *n*=25). The current density at –100 mV was –55±5 pA/pF (*n*=12) and was largely abolished by substituting $NMDG^+$ for extracellular Na^+, suggesting that the current at his potential was carried mainly by cations (Fig. 2B and D). Figure 2 (F, H) shows the currents elicited by a step protocol. The kinetic profile of the current in the non-transfected cell corresponded to that of VRAC. The current in the TRP12-transfected cell was independent of time and showed a much higher noise level than in the non-transfected cell. These data therefore suggest that VRAC is either absent or at least dramatically down-regulated in TRP12-expressing cells. Currents activated by hypotonicity in TRP12-expressing cells were inhibited by a 2-min exposure to 400 mOsm hypertonic solutions. Inhibition at +100 mV was 56±8% and 72±7% at –100 mV (*n*=4). Interestingly, VRAC was completely inhibited by the same increase in osmolarity (see also [24]). The current in TRP12-expressing cells was insensitive to tamoxifen up to 20 µM, but 50 µM NPPB blocked it by 76±12% (*n*=4, at +100 mV). The swelling-activated current had a density of 135±24 pA/pF (*n*=20) at +100 mV. The permeability sequence at this potential was Eisenman IV, which is the same as for TRP12-transfected cells under isotonic conditions, i.e. P_K:P_{Cs}:P_{Na}:P_{Li}=2.1±0.3:1.25±0.11:1:0.92±0.05 (*n*=4).

We tested whether TRP12 currents could be activated in the absence of intracellular ATP which is required for activation of VRAC [10, 11, 15, 16, 19].The maximal currents after cell swelling (+79±34 pA/pF at +100 mV and –44±11 pA/pF at –100 mV, *n*=3) were not significantly different from those obtained in pipette solutions containing 4 mM Na_2ATP. These data are in agreement with previous findings that the TRP12 equivalents, OTRPC4 and VR-OAC, can be activated in the complete absence of ATP in the pipette solution [6, 20].

Cell swelling increased $[Ca^{2+}]_i$ in both non-transfected and TRP12-transfected cells, but the increase was much more pronounced in TRP12-transfected cells (492±14 nM, *n*=10) than in non-transfected cells (95±8 nM, n=5, *P*<0.05), confirming earlier reports [20, 27].

TRP12-mediated currents are not activated by reduced ionic strength or GTPγS

The absence of VRAC currents in TRP12-transfected HEK cells is intriguing and could point to competition

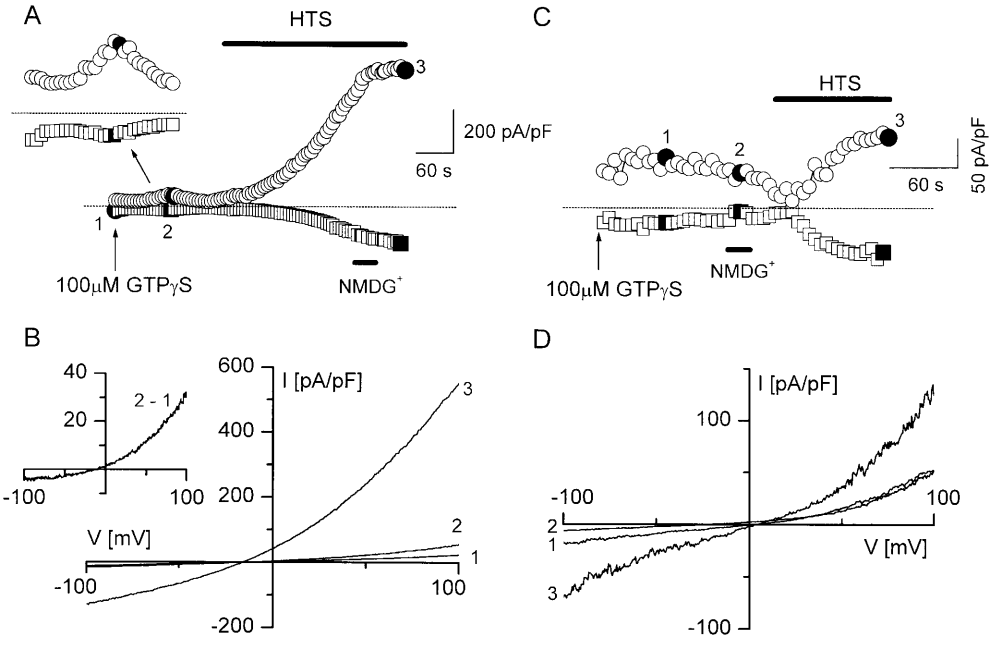

Fig. 4A–D Guanosine 5'-*O*-(3-thiotriphosphate) (*GTPγS*) activates a current in non-transfected but not in TRP12-transfected HEK 293 cells. **A**, **C** Time course of the currents in a non-transfected (**A**) and TRP12-transfected (**C**) cell after breaking into the cell with a pipette solution containing 100 μM GTPγS and subsequent exposure to a hypotonic stimulus (*HTS*). The *inset* in **A** shows the indicated section of the trace at a greater scale. **B**, **D** Representative *I*/*V* curves from voltage ramps applied at the times marked with *filled symbols* and the *numbers 1–3* in **A** and **C**. The *inset* in **B** shows the difference current (*2–1*), representing the *I*/*V* curve of the GTPγS-activated current

for a common volume sensor or to a common activation cascade shared by both channels. To discriminate between both possibilities, we tested whether the protocols that activate VRAC at constant volume in endothelial cells also activate TRP12.

In the first protocol, VRAC was activated by dialysing the cell with a pipette solution of reduced ionic strength, but the same osmolarity as the extracellular solution to prevent cell swelling. In endothelial cells, this protocol activates a current that is inhibited by cell shrinkage and shows typical $I_{Cl,swell}$ features, such as the Eisenman-I halide permeation sequence and a similar pharmacological profile, i.e. block by tamoxifen, NPPB or mibefradil [17, 26]. A current with a biophysical profile identical to that of $I_{Cl,swell}$ was also activated in a non-transfected HEK cell dialysed with an isosmotic pipette solution of reduced ionic strength (125 mM). This current was outwardly rectifying, reversed at –18±5 mV (*n*=9), inactivated at positive potentials and was insensitive to substitution of $NMDG^+$ for extracellular Na^+ (Fig. 3A–C). The same protocol did not, however, activate any current in TRP12-expressing cells (Fig. 3D–E). Subsequent exposure of non-transfected and transfected cells to a 25% reduction in extracellular tonicity (hypotonic stimulus, HTS) activated a typical VRAC current, with the characteristic outwards rectification (current trace 2 in Fig. 3B) and the response to the step protocol (Fig. 3C), in non-transfected cells (928±210 pA/pF at +100 mV, *n*=9), and a cation current, but not VRAC, in TRP12-transfected cells (165±85 pA/pF, *n*=9). The latter current was independent of time, and showed an increased noise level (Fig. 3F).

Dialysis of a non-transfected HEK cell with GTPγS activated a small, transient VRAC current (GTPγS-induced current 28±8 pA/pF, *n*=8, +100 mV; Fig. 4A, B, and insets) that was, however, smaller than in endothelial cells [25]. A subsequent HTS induced a large VRAC current (388±45 pA/pF, *n*=8). The transient activation was virtually absent in a TRP12-transfected cell dialysed with GTPγS (GTPγS-"induced" current 2±4 pA/pF, *n*=6; Fig. 4C, D), whereas the subsequent hypotonic challenge was still able to activate an additional (cationic) current (82±22 pA/pF at +100 mV; Fig. 4C, D).

Both protocols that activated VRAC at constant cell volume in non-transfected HEK cells failed to activate both VRAC and the cation current in TRP12-transfected cells. These data therefore suggest that the activation cascade of TRP12 is different from that of VRAC, and that the functional down-regulation of VRAC by TRP12 is independent of the mode of activation.

Discussion

TRP12 and the related channels OTRPC4 and VR-OAC, which are widely expressed in vertebrates, might play a role in the sensing of cell volume or changes in osmolarity and be involved in electrogenesis by depolarizing cells. The higher resting $[Ca^{2+}]_i$ in TRP12-expressing cells may point to another putative role of this channel as an influx route for Ca^{2+}.

Activation of this channel by cell swelling occurs along a pathway that is clearly different from that of VRAC, for which activation by cell swelling or reduced ionic strength proceed along the same activation cascade. In contrast, the TRP12 channel could not be activated at constant volume by a reduction of ionic strength or by GTPγS. Also the inhibition by cell shrinkage seems to be different for VRAC and TRP12 currents. Exposure of

cells that had been challenged by a hypotonic solution almost immediately reduced VRAC. The inhibition of TRP12 was less complete.

The mechanisms of activation also differ with respect to intracellular ATP. TRP12 currents were not changed significantly in the absence of ATP in the patch pipette, whereas VRAC activation requires ATP [11, 15, 19]. This feature of TRP12 is in agreement with data on OTRPC4 and VR-OAC [6, 20]. Obviously, TRP12 is, more robust with respect to changes intracellular ATP, possibly implying a minor role of phosphorylation events in the activation cascade of this channel.

The rather unexpected down-regulation of VRAC in TRP12-expressing cells is apparently independent of the activity of TRP12 channels, because VRAC currents activated by reduced ionic strength and GTPγS in isotonic solution, i.e. without activating TRP12, were completely abolished also. The mechanism of this down-regulation is not clear, and many possibilities are open for further investigation. Because intracellular Ca^{2+} plays only a permissive role in the activation of VRAC, it is unlikely that the different $[Ca^{2+}]_i$ in control and TRP12-transfected cells is responsible for the differences in VRAC currents. The most straightforward explanations are that expression of TRP12 suppresses that of VRAC, or theat there is a protein-protein interaction between VRAC and TRP12, e.g. competition for a putative volume sensor. Both hypotheses are hard to substantiate because the molecular nature of VRAC is not yet known. Interference between the activation cascades of the channels is, however, unlikely, because VRAC inhibition occurs independently of the TRP12 activation cascade. The effect of cell swelling on VRAC activation in TRP12-expressing cells is probably also not mediated via a modified volume response of these cells to hypotonicity, because activation of VRAC at constant volume was also suppressed.

Interestingly, swelling also activates a gadolinium- and lanthanum-sensitive Ca^{2+} influx independent of store depletion in sensory neurons [23]. It is intriguing to speculate that TRP12 channels could be involved, and that these channels also play a major role under pathophysiological conditions of cell swelling.

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