Trp proteins form store-operated cation channels in human vascular endothelial cells

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Received 23 August 1998

Abstract Members of the Trp protein family have been suggested as the structural basis of store-operated cation conductances. With this study, we provide evidence for the expression of three isoforms of Trp (hTrp1, 3 and 4) in human umbilical vein endothelial cells (HUVEC). The role of Trp proteins in store regulation of endothelial membrane conductances was tested by expression of an N-terminal fragment of hTrp3 (N-TRP) which exerts a dominant negative effect on Trp channel function presumably due to suppression of channel assembly. Depletion of intracellular Ca^{2+} stores with IP_3 (100 μM) or thapsigargin (100 nM) induced a substantial cation conductance in sham-transfected HUVEC as well as in HUVEC transfected with hTrp3. In contrast, HUVEC transfected with N-TRP failed to exhibit store-operated currents. Our results suggest the involvement of Trp related proteins in the store-operated cation conductance of human vascular endothelial cells.

Key words: Vascular endothelium; Store-operated cation channel; Trp channel; Protein; IP_3

1. Introduction

Hormone-induced release of Ca^{2+} from intracellular stores is known to determine the activity of ion channels in the plasma membrane. One particular group of channels is activated by the reduced filling state of the Ca^{2+} store (for review see [1]). These so-called store-operated channels serve refilling of the Ca^{2+} store by providing a Ca^{2+} entry pathway and may in addition control membrane potential and homeostasis of monovalent cations. Indeed, depletion of Ca^{2+} stores was found to induce highly selective Ca^{2+} conductances in mast cells [2,3] and Jurkat T cells [4] as well as non-selective cation conductances in other cell types such as pancreatic acinar [5] and vascular endothelial cells [6–8]. Thus, store-operated channels appear as a heterogeneous family of ion channels that share the principal property of being activated by depletion of intracellular Ca^{2+} stores.

As yet the molecular nature of these store-operated ion channels has not been unraveled. Nonetheless, the *Drosophila* transient receptor potential (trp) gene has been suggested to encode a store-operated ion channel [9]. Recently a number of vertebrate homologues of Trp have been cloned [10–14], and expression of these proteins has been demonstrated in a variety of tissues [10,15,16]. Heterologous expression of Trp proteins resulted in enhanced capacitative Ca^{2+} entry [11,12,17], and in detectable store-operated cation conductances [11,13,18–20]. The observed functional similarities between expressed Trp channels and native store-operated channels strongly suggest the involvement of Trp proteins in store-operated cation conductances and capacitative Ca^{2+} entry in mammalian tissues.

The vascular endothelium represents a tissue whose function is for a large part controlled by IP_3-mediated release of Ca^{2+} from intracellular stores and subsequent activation of store-operated channels. Hormones and neurotransmitters that stimulate intracellular IP_3 production, but also agents that deplete Ca^{2+} stores more directly, such as thapsigargin or ionomycin, were found to induce a Ca^{2+} conductance of low selectivity in endothelial cells [6–8,21].

The present study was designed to test whether Trp proteins contribute to the store-operated membrane conductances observed in vascular endothelial cells. We present evidence for the expression of three different homologues of Trp in endothelial cells derived from human umbilical vein (HUVEC), and demonstrate that transfection of HUVEC with the N-terminal domain of Trp which has been shown to dominantly suppress Trp-mediated membrane currents [20], specifically eliminates a store depletion-induced membrane conductance in these vascular endothelial cells.

2. Materials and methods

2.1. Cell culture

Endothelial cells from human umbilical vein (HUVEC) were isolated as described previously [21]. HUVEC as well as human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS. For RNA preparation, subconfluent cells were used. For transient transfection and electrophysiological experiments HUVEC (passage 1) were plated on coverslips and used just before confluence was obtained.

2.2. RT-PCR

RNA was prepared from HUVEC and HEK 293 cells using the RNeasy Mini-Kit from Qiagen, and 3 μg total RNA was reverse-transcribed into first-strand cDNA with the T-Primed First Strand Kit from Pharmacia. Aliquots of the cDNA products were used as templates for PCR amplification using primers specific for human trp homologues (Table 1).

Cycling conditions were: 3 min at 94°C followed by 30 cycles of 30 s

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Abbreviations: BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; ECV 304, cell line from human umbilical vein endothelium; GFP, green fluorescent protein; HUVEC, human umbilical vein endothelial cells; HEK 293, cell line from human embryonic kidney cells; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonic acid; N-TRP, N-terminal domain (residues 1–302) of hTrp3

PII: S0014-5793(98)01212-5

at 94°C/30 s at 58–64°C/30 s at 72°C, and a final extension at 72°C for 6 min. 'No-template' controls were run along all experiments. In some experiments full length trp cDNA constructs (U31110, U47050 and U50922) were used as templates to serve in terms of positive controls. PCR products were separated on 1% agarose gel by electrophoresis. Southern blots were performed and hybridized overnight at 42°C with digoxigenin-labeled oligonucleotides designed to bind to the expected PCR products internal to the PCR primers. Sequences of the oligonucleotides used as probes were: hTrp1: 5′-gacaatgttctaagttatcttcg-3′; hTrp3: 5′-gacagtgatgtagaatggaag-3′; hTrp4: 5′-gagacactgcagtccctgtt-3′, and mTrp4: 5′-tatatccgcatggtcagcaataag-3′. Results shown are representative of 4–8 RT-PCR experiments.

2.3. DNA constructs and cell transfection

Constructs used for expression in HUVEC were in the pcDNA3 vector (Invitrogen) or in the bicistronic expression vector pIRES-EGFP (Clontech). N-TRP consisted of amino acids 1–302 of hTrp3 (U47050). Subconfluent HUVEC were transiently transfected using Superfect reagent (Qiagen). Experiments were performed with cells which expressed green fluorescent protein (GFP) as a marker of successful transfection [21]. Expression of GFP was either directed by the pIRES-EGFP or by co-transfection with GFP in pcDNA3 at a molar DNA ratio of 4:1 (N-TRP or hTrp3/GFP). Sham transfected cells (control cells) expressed GFP only. Electrophysiological experiments were performed 2–3 days after transfection.

2.4. Current measurements

Membrane currents were recorded in conventional whole cell patch-clamp mode using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA, USA). Patch pipettes were fabricated from borosilicate glass (Clark Electromedical Instruments, Pangbourne, UK), and had resistances of 2–4 MOhm. The pipette solution contained (mM): 125 K^+/aspartate$^-$, 5 $MgCl_2$, 10 HEPES, 10 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA). The standard bath solution contained (mM): 137 NaCl, 2 $CaCl_2$, 5.4 KCl and 10 HEPES. The pH of all solutions was adjusted to 7.4 with *N*-methyl-D-glucamine. In some experiments NaCl or $CaCl_2$ was omitted in the bath solution and substituted by choline chloride, or $CaCl_2$ was increased to 10 mM. In order to induce rapid depletion of intracellular Ca^{2+} stores upon obtaining conventional whole cell configuration, the pipette solution was supplemented with 100 μM IP_3. Alternatively, the cells were exposed to extracellular thapsigargin (100 nM) to deplete Ca^{2+} stores. The effects of store depletion on membrane conductances were studied by applying slow voltage ramps (0.06 V/s; 0.2 Hz). All experiments were performed at room temperature. Averaged data are given as mean ± S.E.M. from the indicated number of experiments. Statistical analysis was performed using Student's *t*-test for paired values and differences were considered statistically significant at $P < 0.05$.

2.5. Materials

Tissue culture media were from Gibco-BRL (Vienna, Austria), all other chemicals from Sigma Chemical (Vienna, Austria). A green fluorescent protein construct was kindly provided by Dr. Ed C. Conley (Leicester, UK).

3. Results and discussion

3.1. Expression of Trp genes in human vascular endothelial cells

The presence of transcripts of trp genes in human vascular endothelial cells was tested with a RT-PCR strategy. Total RNA was prepared both from native human umbilical vein endothelial cells (HUVEC) which were previously characterized in terms of agonist-induced and store-operated membrane conductances [8,21], and from HEK 293, a cell line which was recently shown to express various Trp isoforms [16].

Specific primers were designed according to the cDNA sequences of Trp isoforms (Trp1, 3 and 4). Expression of Trp1 and Trp3 was tested with primers specific for the human isoforms hTrp1 [10] and hTrp3 [12]. For analysis of Trp4 expression, primers specific for a recently reported partial human sequence [12] as well as primers specific for mTrp4 [12] were used. PCR products were identified as Trp fragments by Southern blot hybridization with oligonucleotide probes designed to bind within the cDNA region flanked by the primers. Fig. 1 shows a Southern blot hybridization of PCR products obtained with RNA from HUVEC and HEK 293, with HUVEC RNA which was not subjected to reverse transcription (negative control) as well as with available target cDNA clones (hTrp1, hTrp3 and mTrp4) as template (positive control). PCR products of all three isoforms were obtained with reverse transcribed RNA preparations from HUVEC and HEK 293 cells but not in negative controls. Thus, our results indicate the expression of three human Trp species in human umbilical vein endothelium. This is consistent with a recent study demonstrating expression of multiple Trp related genes in bovine aortic endothelial cells [24]. Considering the ability of Trp proteins to provide store-operated cation conductances when expressed in mammalian host cells [11,13,20], it is tempting to speculate that the expression of Trp homologues is related to endothelial store-regulated cation conductances. In a recent study, however, Chang et al. [24] observed that hormone-induced changes in the Trp expression pattern of bovine endothelial cells are not associated with significant changes in the extent of store-operated Ca^{2+} entry. One explanation for these results may be that Trp isoforms substitute for each other in the formation of functional heteromultimeric channel complexes. Thus, reduced or eliminated expression of one specific Trp isoform may result in rather modest changes in channel properties rather than in suppression or complete 'knock-out' of the store-operated membrane conductance. Support for this hypothesis comes from a recent study which demonstrates that different Trp related proteins co-assemble to form store-operated channels with distinct properties [22].

To test for a functional role of Trp expression in vascular endothelium, we aimed at effective disruption of the function of Trp channels in this tissue. Our strategy for 'knock out' of Trp channel function was based on recent findings which demonstrate that assembly of functional Trp channels involves interaction within the N-terminal domain (N-TRP) of

Table 1

Gene	Acc. no.	Orientation	Primer sequence	Location
hTrp1	U31110	Forward	5′-ttctgtggattattgggatga-3′	2018–2038
		Reverse	5′-cagaacaaagcaaagcaggtg-3′	2502–2522
hTrp3	U47050	Forward	5′-atgctgcttttaccactgtag-3′	1985–2005
		Reverse	5′-tgagttagactgagtgaagag-3′	2413–2433
hTrp4	U40983	Forward	5′-cctggacattttgaagtttc-3′	26–48
		Reverse	5′-ctgcatggtcagcaatcag-3′	343–364
mTrp4	U50922	Forward	5′-tctgcagatatctctgggaaggatgc-3′	1698–1723
		Reverse	5′-aagctttgttcgagcaaatttccattc-3′	2086–2112

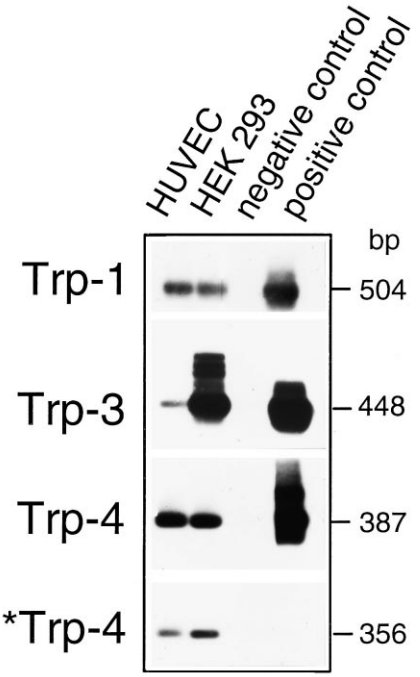

Fig. 1. Native HUVEC and the HUVEC derived cell line ECV 304 express multiple Trp isoforms. A: Separation of PCR products obtained with specific primers for hTrp1, hTrp3 and hTrp4. RNA preparations of HUVEC and ECV 304 with and without reverse transcription (Control). B: Southern blot hybridization of PCR products.

individual Trp proteins, and that consequently expression of N-terminal fragments of Trps suppresses Trp channel currents, presumably due to disruption of channel assembly [22]. Therefore, we tested whether expression of N-TRP affects endothelial store-operated currents.

3.2. Store-operated membrane currents in HUVEC

HUVEC respond to depletion of intracellular Ca^{2+} stores with a significant increase in the cation conductance of the plasma membrane [6,8]. In the present study we included 100 μM IP_3 and 10 mM of the Ca^{2+} chelator BAPTA in the pipette solution to rigorously deplete intracellular Ca^{2+} stores in conventional whole-cell patch clamp experiments. Mg^{2+}, which is known to inhibit Trp channel currents [22,25], was omitted from the bath solution.

A substantial inward current developed within 2–3 min after dialysis of cells with IP_3 in a bath solution containing 137 mM Na^+ and 2 mM Ca^{2+}. No significant changes in membrane conductances were observed when an IP_3-free pipette solution containing 30 nM free Ca^{2+} was used. The increase in inward current at −80 mV was larger and more stable with Na^+ (137 mM) than with Ca^{2+} (10 mM) as charge carrier (not shown). Typical membrane currents recorded in a Na^+-containing, Mg^{2+}-free solution are shown in Fig. 2. The upper panel shows the time course of the membrane current recorded at −80 mV. During dialysis of the cell with IP_3 plus BAPTA, the current at −80 mV increased gradually by about 100 pA. This current was dependent on extracellular Na^+ and blocked by 50 μM La^{3+} ($N = 7$), and resembled the previously reported thapsigargin-induced current of HUVEC [8]. The lower panel of Fig. 2 illustrates the current to voltage relationship of the IP_3-induced current that reversed at about neutral potential. Removal of extracellular Na^+ resulted in a reduction of current and shifted the reversal potential in hyperpolarizing direction indicating that the current is carried for a large part by Na^+. These results are consistent with previous reports suggesting that discharge of Ca^{2+} stores in vascular endothelial cells activates cation channels that are permeable for both Ca^{2+} and Na^+ ions [6–8]. Similarly, expressed hTrp3 was recently found to provide a non-selective cation conductance which is activated along with agonist-induced store-depletion [20]. The IP_3-induced membrane conductance was observed with high reproducibility (14 out of 15 cells) in HUVEC.

3.3. Inhibition of store-operated membrane currents by expression of the N-terminal domain of hTrp3

Since the transfection efficiency of native HUVEC is low (< 5%), expression of an easily detectable marker protein (GFP) was required to enable selection of successfully transfected cells. The N-terminal domain (residues 1–302) of hTrp3 was cloned into pcDNA3 and alternatively into the vector pIRES-EGFP which allows for simultaneous expression of

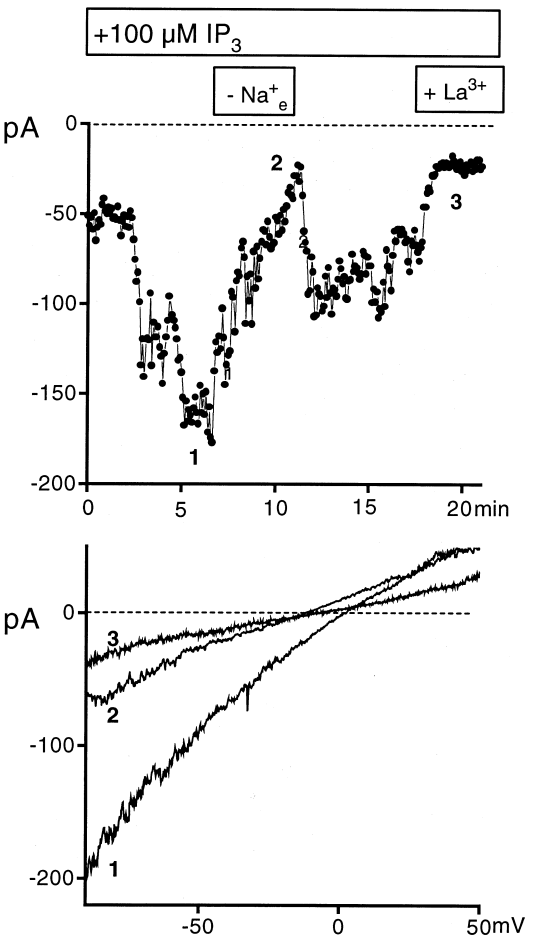

Fig. 2. Discharge by IP_3 of Ca^{2+} stores induces a cation current in HUVEC. HUVEC were dialyzed with IP_3 (100 μM) via the pipette solution, starting with rupture of the patch at time 0. Upper panel: Time-course of membrane current at −80 mV is shown for a cell which was transiently transfected with GFP. Removal of extracellular Na^+ and addition of La^{3+} (50 μM) is indicated. Lower panel: Current to voltage relationship derived by voltage-ramp protocols at the time points indicated.

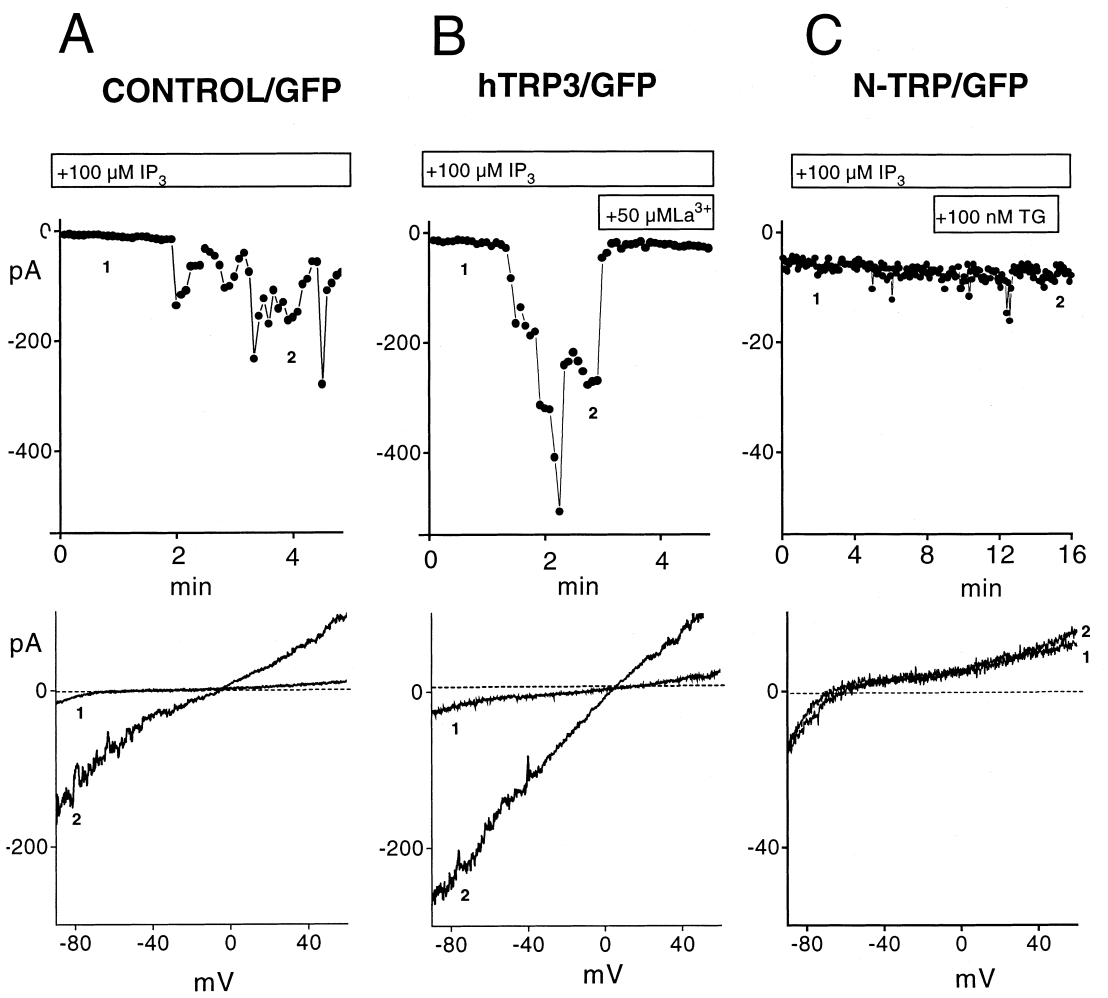

Fig. 3. IP_3-induced cation currents are blocked by expression of the N-terminal domain of hTrp3 but not by expression of full length hTrp3. Current responses during dialysis with IP_3 (100 μM) recorded from HUVEC transfected with GFP only (A), from HUVEC transfected with hTRP3 plus GFP (B) and from HUVEC transfected with N-TRP plus GFP (C). IP_3 was introduced via the pipette solution, starting with rupture of the patch at time 0. Upper panel: Time-course of membrane current at −80 mV. Addition of La^{3+} (50 μM) and thapsigargin (100 nM) is indicated. Lower panel: Current to voltage relationship derived by voltage-ramp protocols at the time points indicated.

proteins and the marker protein GFP. HUVEC were transiently transfected either with N-TRP in pcDNA3 plus pcDNA3-EGFP (4-fold molar excess of N-TRP), or with N-TRP in pIRES-EGFP. Control experiments were performed in cells transfected with pcDNA3-EGFP or pIRES-EGFP. In addition, a set of experiments was performed in HUVEC transfected with full length hTrp3 which is expected to interact with endogenous Trp proteins without preventing the formation of functional Trp oligomers. GFP-positive cells were challenged by intracellular administration of IP_3 plus BAPTA. As shown in Fig. 3A, cells transfected with GFP only responded to intracellular administration of IP_3 with a clear increase in the membrane current recorded at −80 mV (112 ± 22 pA; $N = 14$). A similar response resulting in a mean inward current of 119 ± 33 pA at −80 mV was elicited by extracellular administration of 100 nM thapsigargin in 4 out of 5 GFP-positive cells (not shown). Cells transfected with hTrp3 responded to dialysis with IP_3 in a manner similar to GFP-transfected controls exhibiting even slightly larger increases in membrane conductance (Fig. 3B). The observed increases in membrane conductance were completely blocked by 50 μM La^{3+} as shown in Fig. 3B. In clear contrast, membrane conductance of N-TRP-transfected HUVEC remained fairly stable during dialysis with IP_3 (100 μM; 10–20 min recording time; $N = 15$) as illustrated in Fig. 3C. Removal of extracellular Na^+ was without effect on membrane currents (not shown), and further challenge of the cells with thapsigargin (100 nM, $N = 4$) did not affect membrane conductance of N-TRP transfected cells (Fig. 3C).

It is noteworthy that the small inwardly rectifying K^+ conductance which is typically observed in HUVEC [21] was clearly present in N-TRP transfected cells (Fig. 3C; lower panel; note that the scaling of the *y* axis in Fig. 3C is different from Fig. 3A and B). Thus, the transfection of HUVEC with N-TRP suppressed selectively the store depletion-induced cation conductance whereas other membrane properties of HUVEC appeared unaffected. Fig. 4 summarizes the mean values of inward currents recorded at −80 mV initially (open columns) and 4–6 min after obtaining whole-cell configuration with IP_3 containing pipette solutions (filled columns). Dialysis with IP_3 induced a significant increase in membrane conductance of HUVEC transfected with GFP or with hTrp3 plus GFP. The IP_3-induced inward current at −80 mV was slightly but not significantly higher in cells transfected with hTrp3

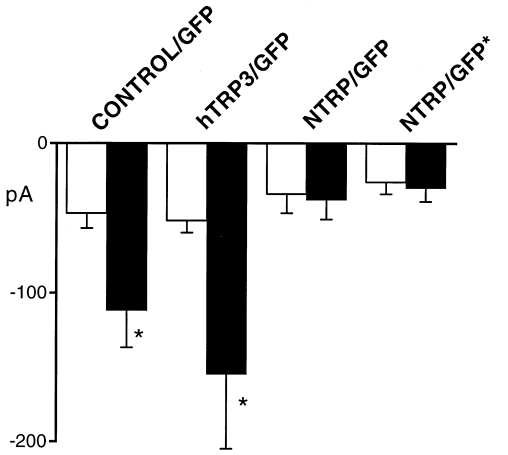

Fig. 4. Mean inward currents (at −80 mV), recorded initially (open columns) and 4–6 min after obtaining whole cell configuration with an IP_3-containing pipette solution (filled columns) in cells transfected with GFP only (control/GFP; $N=14$), hTrp3 plus GFP (hTrp3/GFP; $N=5$) and N-TRP plus GFP which was achieved either by co-transfection with N-TRP in pcDNA3 and pcDNA3-EGFP (NTRP/GFP; $N=11$) or by transfection with N-TRP in pIRES-EGFP (NTRP/GFP*; $N=5$). Mean values ± S.E.M.; * denotes statistically significant difference versus the initial current level.

compared to controls. In clear contrast, intracellular administration of IP_3 did not increase the membrane current in HUVEC transfected with N-TRP. Consistent results were obtained in experiments using pcDNA3-EGFP co-transfection [23] and expression via pIRES-EGFP (Fig. 4). Expression of N-TRP plus GFP using the pIRES vector involves translation of both genes from a single bicistronic mRNA. Therefore, it is concluded that expression of GFP was indeed associated with expression of N-TRP. The observation that expression of N-TRP prevents store-operated cation currents in HUVEC is in line with a recent study demonstrating N-TRP-mediated inhibition of currents through expressed Trp channels, which has been attributed to binding of the N-terminal fragment to complete Trp proteins and disturbance of the formation of functional oligomers [22]. However, other mechanisms related to the putative ability of N-TRP to interact with cellular proteins implicated in Trp channel activation, such as the IP_3 receptor or components of the cytoskeleton, may as well be considered. The interaction of N-TRP with the cytoskeleton has been hypothesized since conserved ankyrin-like repeats are present in N-TRP [26]. On the basis of the present results, we cannot exclude that inhibition of cation currents by N-TRP is due to interference with a mechanism of channel activation that involves the cytoskeleton. Nonetheless, two lines of evidence argue against an inhibitory mechanism at the level of IP_3-induced Ca^{2+} release. (i) N-TRP-transfected HUVEC were resistant not only to store depletion by IP_3 but also by thapsigargin demonstrating that the inhibitory effect of N-TRP is not restricted to store depletion by IP_3. (ii) In HEK 293 cells, which allow for efficient transient transfection ($>60\%$ positive cells), carbachol-induced, IP_3-mediated Ca^{2+} release was not affected by expression of N-TRP (unpublished observation).

Expression of full length hTrp3, i.e. N-TRP extended to a functional Trp protein, was inactive in terms of inhibition of endothelial cation conductances. This result indicates that the inhibitory effect of N-TRP is related to its deficiency in Trp channel function and supports the idea of a dominant negative effect of N-TRP on endogenous Trp channels. The well documented ability of N-TRP to bind to the N-terminal domain of other Trp proteins [22] appears as the most likely mechanism of the observed inhibitory effects. Interaction of N-TRP with endogenous Trp proteins is expected to hinder the assembly of functional Trp oligomers. By contrast, transiently expressed hTrp3 is expected to associate with endogenous Trps without essential impairment of channel function.

Over-expression of hTrp3 in HUVEC may change the isoform composition of Trp channels or even promote the formation of hTrp3 homomultimers. We observed that expression of hTrp3 in HUVEC failed to suppress or even slightly promoted IP_3-induced cation currents. This result is in line with the observation of a non-selective conductance in HEK 293 cells stably transfected with hTrp3 [20]. Moreover, hTrp3 over-expression clearly enhanced IP_3-mediated Ca^{2+} entry into HEK 293 cells [17]. It is therefore tempting to speculate about a prominent role of hTrp3 in terms of formation of non-selective store-operated channels. However, the RT-PCR experiments of this study as well as those of a previous study [16] indicate that hTrp3 is the prominent Trp isoform expressed in HEK 293 cells which exhibit only small endogenous store-operated currents [13]. In contrast, hTrp3 expression appears much less abundant in HUVEC, arguing against a role of hTrp3 homomultimers in the marked store-operated cation conductance of these cells. Interestingly, the interaction between different Trp and Trp-like proteins was found to be much stronger than the interaction between proteins of the same species [22]. Thus, the existence of heterooligomeric channels appears likely [22], and one may speculate that the N-terminal domain of hTrp3 may preferentially bind to other Trp isoforms, e.g. Trp1 and Trp4, thereby disrupting heterooligomeric channels comprised of these isoforms. It remains to be clarified to what extent individual isoforms of Trp contribute to the store-operated conductance of HUVEC.

In summary, the present study demonstrates that IP_3-mediated depletion of Ca^{2+} stores in HUVEC is associated with a non-selective cation conductance that is eliminated by expression of an N-terminal domain of Trp. The observed non-selective cation conductance resembled Trp currents not only in terms of its mechanism of activation but also in terms of its sensitivity to suppression by N-TRP [22]. Thus, the present study provides strong evidence for a Trp-related membrane conductance in native human endothelial cells and suggests a functional role of Trp proteins in vascular endothelium. This Trp-related ion conductance is likely to serve hormonal control of endothelial membrane potential and Ca^{2+} homeostasis, and may therefore play a significant role in endothelial physiology and pathophysiology.

Acknowledgements: This work was supported by the Fonds zur Förderung der Wissenschaftlichen Forschung: SFB Biomembranes (F708 and F715), P12667 and P12782 and the Austrian National Bank (project 6073). We wish to thank Dr. Ed C. Conley for providing a GFP clone as well as Mrs. R. Schmidt and Mrs. Ingrid Hauser for excellent technical assistance.

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