Expression and functional role of bTRPC1 channels in native endothelial cells

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Abstract We have analyzed the expression and localization of bovine transient receptor potential-C1 (bTRPC1) in bovine aortic endothelial cells, and its possible involvement in the store-independent calcium influx induced by basic fibroblast growth factor (bFGF). RT-PCR experiments confirmed the existence of two btrpc1 mRNA isoforms; conversely, the btrpc3 gene was not transcribed. Anti-TRPC1 antibody revealed the presence of the protein in the membrane-rich compartment only. Application of anti-TRPC1 during the response to bFGF caused a partial but significant reduction of calcium entry. This is the first evidence of TRP channel involvement in a non-capacitative calcium influx induced by a biologically relevant agonist such as the angiogenic factor bFGF in native endothelial cells.

Key words: Transient receptor potential-C1; Basic fibroblast growth factor; Calcium influx; Endothelial cells

1. Introduction

The recently identified transient receptor potential (TRP) family of channel proteins has been suggested to be involved in calcium influx triggered by extracellular agonists in several excitable and non-excitable cell types [1–4].

This family of proteins is widely associated to store-dependent, or capacitative, calcium entry, a widespread process whose mechanism(s) of activation and physiological role are still not completely understood. However, it is now accepted that TRP proteins represent a highly heterogeneous group, including both store-dependent and store-independent (or non-capacitative) channels [1,4]. The archetypal TRP from *Drosophila melanogaster* is activated by PLC [1]; the other *Drosophila* channel, TRPL, is insensitive to store depletion, and opened by fatty acids, such as arachidonic and linolenic acid [5]. The mammalian homologs TRPC3, TRPC6 and TRPC7 are directly opened by diacylglycerol, even if the interpretation of the experimental data is not unequivocal [6,7]. Other groups have proposed more complex patterns of activation, suggesting that other intracellular messengers and co-factors may be involved [8]. Notably, recent evidence supports the hypothesis of an enzymatic activity of some members of the family, that would be involved in the regulation of the properties of the pore [9]. The heterogeneity of this family extends to the biophysical properties: while some members (such as *Drosophila* TRP) are reported to be quite selective for calcium, others (including TRPC1, TRPC3, TRPC4) are non-selective cationic channels, permeable to Ca^{2+}, Na^{+} and K^{+}, carrying an outwardly rectifying current [10–12].

Most of the data have been obtained on proteins heterologously expressed in transfected cells [13–18]. This has two major drawbacks: on one hand, it limits the information about the physiological role of endogenously expressed TRP channels in the different cell types; on the other, transfected TRP subtypes may form heteromultimers with other members of the family or interact with accessory proteins expressed by the host cell, thus complicating the interpretation of the experimental results.

Recently, endogenous trpc genes (and in some cases the related protein) have been described in some cell types, such as smooth muscle cells [19], salivary glands cells, in which they may be involved in the control of secretion [20,21], B lymphocytes, with a suggested role in cell viability [22], and vascular endothelial cells [23–25].

In endothelial cells, calcium entry is a critical physiological event: it is triggered by vasoactive agents and by growth factors, binding respectively to G-protein coupled and tyrosine kinase receptors [26,27].

A well studied agonist for these cells is basic fibroblast growth factor (bFGF) or FGF-2, a peptidic growth factor belonging to the family of FGFs, that exerts a potent motogenic and proliferative effect on endothelial cells [28]. Binding to specific tyrosine kinase receptors, this factor is able to induce different transduction pathways, among them the recruitment and activation of cytosolic phospholipase A2 and release of arachidonic acid and its metabolites, the so called eicosanoids. These processes are the basis of the angiogenic role of the factor in proliferative diseases such as cancer [29].

In a previous work we showed the ability of bFGF to induce a non-capacitative calcium influx in a line of bovine aortic endothelial cells (BAE-1 cells) in culture [27]. This influx is dependent on the tyrosine kinase activity of bFGF receptors and it is due to the opening of calcium-permeable cationic channels. Similar events are triggered by arachidonic acid in the same cells, and this pathway is involved in the control of cell proliferation [30].

Since on the same cell type Chang et al. [23] reported the expression of bovine trpc1 (btrpc1) and btrpc3 genes, we sought to assess the role of the related proteins in the non-capacitative calcium influx induced by bFGF.

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2. Materials and methods

2.1. Cell culture

BAE-1 (European Cell Culture Collection, UK) were maintained in Dulbecco's modified Eagle's medium (DMEM, BioWhittaker, Verviers, Belgium) supplemented with 10% heat-inactivated fetal calf serum (FCS, BioWhittaker), 50 μg/ml gentamycin and 2 mM glutamine, at 37°C, in a humidified atmosphere of 5% CO_2 in air.

For electrophysiological and cytofluorimetric measurements, monolayers were growth arrested by shifting exponentially growing cells to DMEM containing 1% FCS 24–72 h before measurements. Only nonconfluent, single isolated cells were used, at passages 2–8. External solutions containing the agents were applied by a microperfusion system (delay 3–5 s).

Unless otherwise specified, products were from Sigma (St. Louis, MO, USA); bFGF was from Amersham Pharmacia Biotech (UK).

2.2. RT-PCR

Total RNA was prepared from BAE-1 cells, both starved (24 h in 1% FCS DMEM) and exponentially growing (10% FCS DMEM) with the SV Total RNA Isolation System from Promega (Madison, WI, USA). 1 μg of total RNA was reverse-transcribed into first-strand cDNA by using random hexameric primers (0.5 μg) and AMV Reverse Transcriptase (Reverse Transcription System, Promega). Aliquots of the cDNA products were used as templates for PCR amplification. Expression of btrpc1 gene was studied according to data and procedures published for BAE cells by Chang et al. [23], who cloned the btrpc1 gene (GenBank accession number AF012900). Two primers were used, specific for btrpc1, that bracket the splice region in the N-terminal domain and reveal the presence of two mRNA isoforms, btrpc1 a (longer) and btrpc1 b (shorter): 5′-GAACATAAATTGCGTAGATG-3′ (left) and 5′-CGATGAGCAGCTAAAATGACAG-3′ (right).

Cycling conditions were: 2 min at 94°C followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C, and a final extension of 7 min at 72°C. Control reactions were performed in the absence of cDNA. PCR products were separated on 2% agarose gels by electrophoresis and visualized by staining with ethidium bromide.

To study the expression of the btrpc3 gene in BAE-1 cells, we tried three different sets of specific primers reported in the literature. The first was the set described by Chang et al. [23], that cloned a partial cDNA sequence of btrpc3 (accession number AF012902): 5′-TGACTTCCGTTGTGCTCAAATATG-3′ (left) and 5′-CCTTCTGAAGCCTTCTCCTTCTGC-3′ (right). The second set of primers was reported by Kamouchi et al. [11], that cloned another fragment of btrpc3 cDNA sequence (accession number AJ006781): 5′-CAATCTGGTACGAGAACC-3′ (left) and 5′-CAGTCCAAGTGAACTGTG-3′ (right). The third set was designed on the human trpc3 sequence (accession number U47050) by Balzer et al. [31], working on porcine aortic endothelial cells: 5′-ATGCTGTTTTACCACTGTAG-3′ (left) and 5′-TGAGTTAGACTGAGTGAAGAG-3′ (right).

Control mRNA for the metabolic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified with the following primers: 5′-GGCATCGTGGAGGGACTTATGA-3′ (left) and 5′-ATGCCAGTGAGCTTCCCGTTGAGC-3′ (right) [32].

Oligonucleotides were synthesized by Sigma Genosys.

2.3. Immunoblotting

Subconfluent monolayers of BAE-1 cells, plated in 10 cm Petri dishes, were washed with cold PBS, added with 1 mM phenylmethylsulfonyl fluoride (PMSF), and then lysed in a buffer containing 20 mM Tris, pH 7.4, 0.1 M NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 0.5% sodium deoxycholate, 2 μg/ml aprotinin, 1 mM PMSF, and homogenized on ice by passing through a syringe needle. Lysates were centrifuged for 5 min at $12\,000\times g$ and supernatants boiled 5 min in a sample denaturing buffer. 100 μg of total proteins per lane was separated by electrophoresis on a 8% SDS–polyacrylamide gel, transferred to a polyvinylidene fluoride (PVDF) membrane and blocked overnight in Tris-buffered saline (pH 7.5) plus 5% bovine serum albumin and 1% Tween 20.

The PVDF membrane was incubated with the α-TRPC1 primary antibody (Alomone Labs, Israel, diluted 1:300), for 1 h at 25°C, washed three times with Tris-buffered saline, and then incubated with horseradish peroxidase-conjugated secondary antibody (Dako, Denmark, 1:3000), followed by a second set of three washes with Tris-buffered saline. Bands were visualized by chemiluminescence with ECL Western Blotting Detection System (Amersham).

As a control, α-TRPC1 was preincubated overnight at room temperature with the corresponding antigen peptide (Alomone, 5:1 peptide:antibody), and subsequently used for immunoblotting.

Membrane and cytosolic fractions were obtained lysing subconfluent BAE-1 cells in 25 mM Tris (pH 7.4), 0.3 M sucrose, 1 mM PMSF and 2 μg/ml aprotinin on ice and homogenized as above. Debris was removed by centrifugation at $8000\times g$ for 15 min at 4°C. The resulting supernatant was centrifuged at $100\,000\times g$ for 1 h at 4°C to obtain a pellet (membrane fraction), that was resuspend in the same buffer, and a supernatant (cytosolic fraction). 100 μg of both fractions were separated by electrophoresis and processed as described above.

2.4. Immunocytochemistry

BAE-1 cells were plated on plastic at a density of 5000 cells/cm^2 in 10% FCS DMEM; after 24 h, cells were washed twice with PBS, fixed for 45 min in 4% paraformaldehyde at room temperature, washed twice in PBS, then blocked with normal goat serum (10% in PBS) and incubated overnight with primary antibody (α-TRPC1, 1:200 final concentration). Antigen was visualized by incubation with appropriate biotinylated secondary antiserum, the Vector kit (ABC kit, Vector Laboratories, Burlingame, CA, USA), 3,3′-diaminobenzidine (0.015%) and hydrogen peroxide (0.008%) as chromogen. As a control, α-TRPC1 was preincubated with the corresponding antigen peptide as described above for immunoblotting. Stained cells were observed on a Olympus IX50 inverted microscope (Olympus Optical Co, Hamburg, Germany).

2.5. Calcium measurements

Cells on glass coverslips were loaded with the acetoxymethyl ester form of indo-1 (Molecular Probes, Eugene, OR, USA; 45 min incubation with 2.5 μM indo-1 AM at 37°C). The medium was then replaced with standard Tyrode solution, of the following composition, in mM: NaCl 154; KCl 4; $CaCl_2$ 2; $MgCl_2$ 1; Na–HEPES 5; glucose 5.5; NaOH to pH 7.4. The coverslips were placed on an inverted IM-35 Zeiss microscope with a fluorescence objective (Nikon 100×). Diaphragms were used to observe single cells. Fluorescence signals were taken at excitation wavelength of 380 nm and emissions of 400 and 480 nm using a spectrophotometer from Cairn Ltd., Newnham, UK. Due to the uncertainties of the cytosolic free calcium concentration ($[Ca^{2+}]_i$) calibration in our experiments, $[Ca^{2+}]_i$ is expressed as a ratio, R, of emitted fluorescence (400/480), corrected for background and autofluorescence online. The increment, ΔR, was calculated as a difference between the basal level and the peak of the response. All experiments were performed at 22–24°C.

Student's t-test was used to determine statistically significant differences among the groups. The level of significance was $P < 0.05$. All values are given as mean ± S.D.

2.6. Electrophysiology

Cells used in the experiments were flat and firmly attached to the dish (35 mm dishes; Corning, Denmark) and bathed in standard Tyrode solution. Patch-clamp pipette contained, in mM: KCl 15, K-aspartate 118, $MgCl_2$ 3, EGTA 5, HEPES 5, NaGTP 0.4, Na_2ATP 5, Na_2phosphocreatine 5, KOH to pH 7.3.

Whole-cell patch-clamp recordings were performed using electrodes of 3–8 MΩ resistance connected to an EPC-7 amplifier (List, Germany). Data were stored on a DAT recorder system (Sony, Japan) and sampled at 200 ms intervals (5 Hz). Voltage clamp protocols and off-line analysis were performed with pCLAMP software (Axon Instruments, Burlingame, CA, USA). All experiments were performed at 22–24°C.

3. Results

3.1. Expression of transcripts for btrpc1 by BAE-1 cells

RT-PCR experiments in BAE-1 cells using the primers specific for btrpc1 revealed the presence of two fragments of 282 and 180 bp, confirming the existence of two mRNA isoforms produced by alternative splicing, btrpc1 a and btrpc1 b (Fig. 1A). The lengths are in agreement with those described by

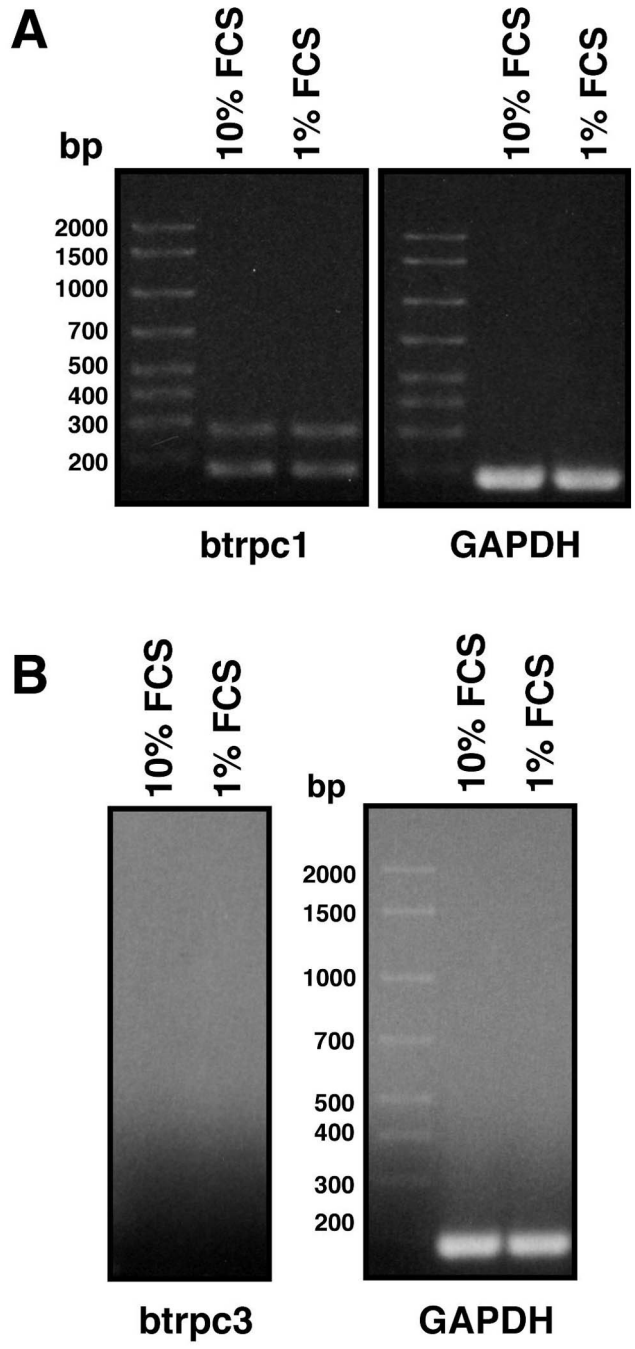

Fig. 1. btrpc expression in BAE-1 cells. A: mRNA detected by RT-PCR in exponentially growing (10% FCS) and serum-starved (1% FCS) BAE-1 cells. Both isoforms of btrpc1 were present, distinguishable upon their size differences (282 and 180 bp). Predicted size of GAPDH PCR product was 188 bp. B: btrpc3 mRNA was detected neither in exponentially growing (10% FCS) nor in serum-starved (1% FCS) BAE-1 cells. The positive control GAPDH mRNA could be amplified in the same samples.

Chang et al. in the same cell type, that they have shown to endogenously express btrpc1 [23]. Comparable levels of expression could be observed both in growth-arrested and in exponentially growing cells (Fig. 1A).

We next checked for the presence of btrpc3 transcripts, as reported in the same cell type [23]. In our conditions, the expression of btrpc3 mRNA was not detectable neither in starved cells (1% FCS DMEM for 24 h) nor in exponentially growing cells (10% FCS) (Fig. 1B). Different working conditions were tried, by changing $MgCl_2$ concentration, cycling parameters and sets of primers. Starting with the same cDNA, the control mRNA of the metabolic enzyme GAPDH was amplified, excluding problems within the sample (Fig. 1B).

3.2. Expression of bTRPC1 protein and its plasma-membrane localization

Expression of bTRPC1 protein was assessed by the use of a commercial rabbit polyclonal antiserum (α-TRPC1, Alomone Labs, Israel) against a peptide corresponding to the sequence QLYDKGYTSKEQKDC of the human protein (amino acids 557–571), as described [33]; the amino acid sequence is highly homologous in bovine (14/15 identical: proline in place of serine). According to the current models based on the hydrophobicity of the amino acid sequence of TRPC1, this antigen should be localized near the outer mouth of the pore, between the fifth and the sixth transmembrane segments [19,34]

Western blot experiments were performed in order to identify the expression and localization of the protein. A band of

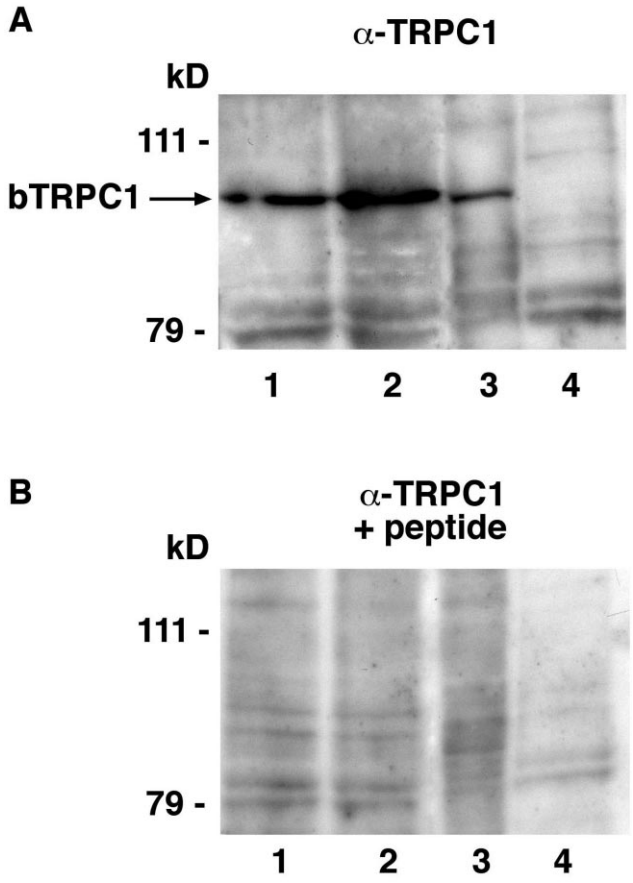

Fig. 2. bTRPC1 protein expression in BAE-1 cells. A: SDS–PAGE and immunoblotting with α-TRPC1 were performed on total lysate from serum-starved (lane 1) and exponentially growing (lane 2) BAE-1 cells. Lanes 3 and 4 represent respectively membrane preparation and cytosolic fraction of exponentially growing cells. The arrow indicates reactivity to α-TRPC1. B: Western blot analysis performed with the same protein samples as in (A). Immunoreactivity to α-TRPC1 was competed off by preincubation of the antibody with the antigen peptide, underlying the specificity of the primary antibody.

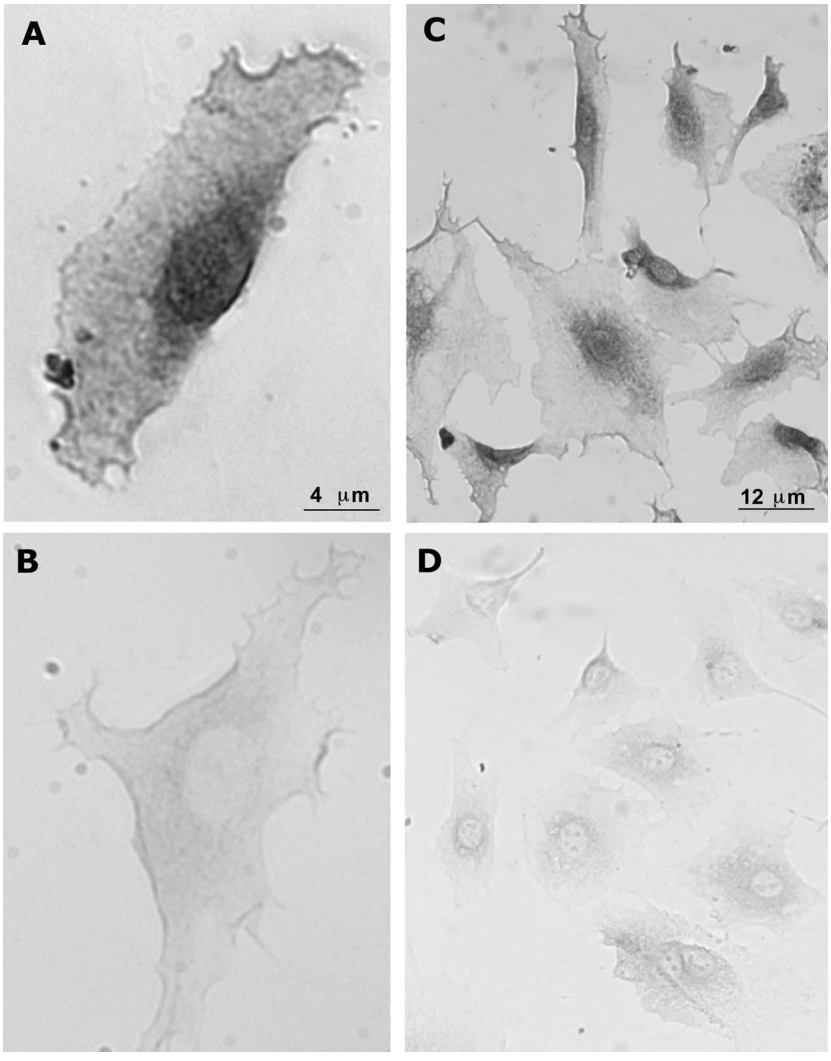

Fig. 3. Immunolocalization of bTRPC1 protein in BAE-1 cells. A,C: Immunoreactivity for bTRPC1 protein in non-permeabilized cells; B,D: staining with α-TRPC1 was competed off by preincubation of the antibody with the antigen peptide; A,B show a BAE-1 cell at higher magnification.

about 100 kDa molecular weight was detectable in the total lysate from both serum-starved and exponentially growing cells (Fig. 2A, lanes 1 and 2). The band was not detectable by Western blot staining with secondary antibody only (data not shown).

When the membrane and soluble fractions were separated, the protein was still present in the membrane-rich compartment, while not detectable in the soluble fraction (Fig. 2A, lanes 3 and 4 respectively).

To assess the specificity of the staining, preincubation with the control antigen peptide (Alomone, 5:1 peptide:antibody) was performed: the labelling was competed off by peptide, as expected for a competitive effect (Fig. 2B); the additional bands present both in Fig. 2A,B were also present in blots performed, as control, with secondary antibody only, underlying an aspecific staining by this secondary antibody (data not shown).

As a further test of the plasma-membrane localization of bTRPC1, we performed immunocytochemical experiments by incubating non-permeabilized cells with the α-TRPC1 antibody. A plasma-membrane-associated staining pattern was clearly evident in BAE-1 cells (Fig. 3A,C), that could be abolished by staining cells with α-TRPC1 preincubated with the antigen peptide (Fig. 3B,D).

3.3. bFGF-induced calcium entry is partially inhibited by α-TRPC1

In a previous work we have described the ability of bFGF to trigger a calcium entry from the extracellular medium in BAE-1 cells through the opening of store-independent calcium channels [27].

In order to check the potential involvement of bTRPC1 channels in the calcium influx observed in response to bFGF, we performed single-cell cytosolic calcium measurements using the indo-1 probe.

Application of 1:100 α-TRPC1 to the external bath in resting conditions failed to exert any detectable effect on basal Ca^{2+} levels ($n = 6$; Fig. 4A). Interestingly, the addition of the antibody during the response to 100 ng/ml bFGF (corresponding to the saturating dose [27]) caused a partial reduction of the response in 17 out of 26 of the cells tested: α-TRPC1, in the continuous presence of bFGF, significantly ($P < 0.05$) inhibited the factor-induced calcium increase. The mean increment of $R(400/480)$, ΔR, at the peak of the re-

sponse to bFGF was 0.22 ± 0.12; at the end of antibody addition, in the continuous presence of the agonist, it was 0.14 ± 0.10 ($n = 17$). In individual cells in which the reduction occurred, the percentage of reduction ranged from 15 to 71% (mean: $41 \pm 29\%$). In many cases the antibody was washed out in the continuous presence of bFGF and the effect could be reverted (Fig. 4B).

To exclude any aspecific effects of α-TRPC1 that may involve also other mechanisms of calcium permeation, we employed a polyclonal rabbit antiserum (produced against rat immunoglobulins; kind gift of Dr. C. Dati); this serum did not have any effects on basal calcium levels ($n = 14$, Fig. 5A) nor on the bFGF induced calcium influx ($n = 8$, Fig. 5B).

As a further test, whole-cell patch-clamp experiments were performed, in the voltage clamp mode at a V_{hold} of −50 mV, in order to exclude an indirect effect of the antibody on inwardly rectifying potassium currents that are highly expressed in these cells; indeed a reduction of these currents might lower the driving force for calcium, thus affecting calcium influx. As tested by comparison of voltage ramps (from −150 to +50 mV) applied before and after superfusion of the antibody into the bath, no effect could be detected in resting conditions, where inward rectifying potassium currents are the main component (data not shown).

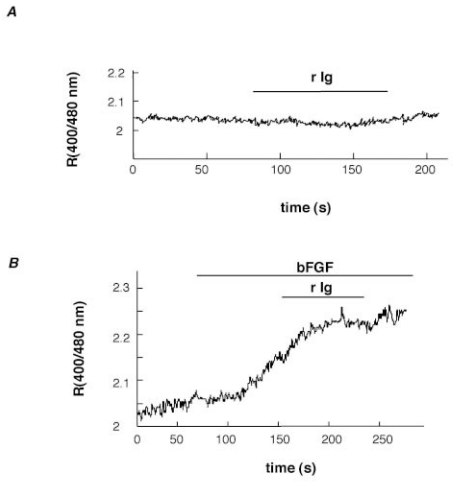

Fig. 5. Lack of effects of the non-specific rabbit polyclonal antiserum (r Ig). Single-cell $[Ca^{2+}]_i$ measurements with indo-1 probe. A: Superfusion with the rabbit polyclonal antiserum did not exert any effect on resting calcium levels. B: bFGF-induced $[Ca^{2+}]_i$ increase was not affected by the addition of the rabbit polyclonal antiserum.

4. Discussion

In spite of the great amount of data that has accumulated in the last years on the molecular nature of TRP proteins, much has still to be understood about the pathways that underlie their activation; while the store-operated paradigm has gained wide consensus, it is now apparent that other, more direct mechanisms linking receptor activation to channel opening can explain the experimental findings obtained for several TRP subtypes in several experimental models [1,5,10]. Another issue which still needs a more detailed study is the functional relevance of these channels, and specifically of the calcium influx that can be associated to the activation of many of them.

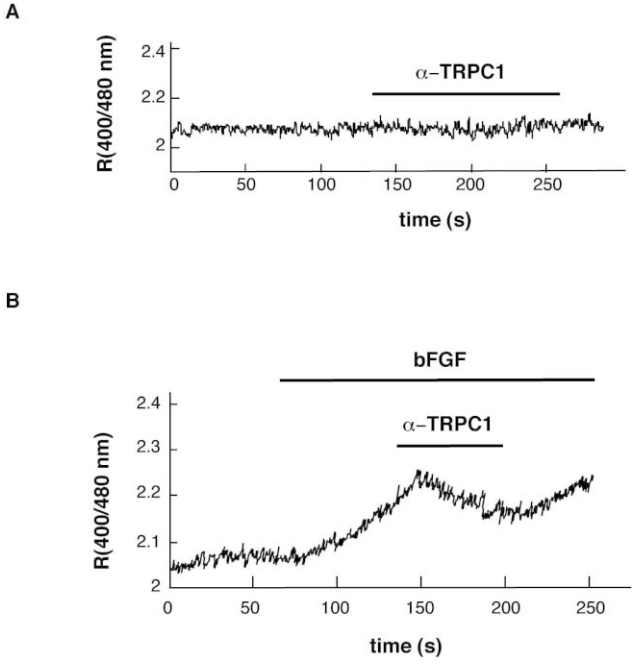

Fig. 4. Inhibition of bFGF-induced calcium influx by α-TRPC1 in BAE-1 cells. Single-cell $[Ca^{2+}]_i$ measurements with indo-1 probe. A: Application of α-TRPC1 (1:100) failed to exert any detectable effect on basal calcium levels. B: Stimulation with 100 ng/ml bFGF induced an increase in $[Ca^{2+}]_i$, that was partially and reversibly inhibited by α-TRPC1 (1:100) addition to the bath during the response.

These limitations in our knowledge are probably due at least to two reasons. The first is the great heterogeneity of these molecules: their properties and tissue distribution are highly variable [1,35]. The second is that functional properties have been studied mostly on channels heterologously expressed in transfected cells [13–18]. This approach may have relevant consequences, because it is widely accepted that several features of ionic channels are dependent on cell-specific factors, such as accessory proteins. Moreover, since the actual subunit composition of mammalian TRPC homologs is unknown, endogenous proteins in mammalian cells heterologously expressing trpc genes may contribute to channel structure, conferring to it different features, such as thapsigargin sensitivity [10].

Endogenous trpc mRNA has been detected in vascular endothelial cells [23–25]. In BAE cells, Chang et al. presented evidence for the endogenous expression of btrpc1, btrpc3, btrpc4 and btrpc5 mRNA [23].

In the same cell type, we have previously shown that growth factors, and in particular bFGF, binding to tyrosine kinase receptors, induce a non-capacitative calcium entry [27]. bFGF is a potent mitogenic factor for endothelial cells (including BAE-1 cells) and is strongly involved in angiogenic processes. In the present work, we provide evidence for the presence of bTRPC1 proteins in BAE-1 cells, for their membrane localization and for their involvement in the calcium influx activated by bFGF. We have centered our efforts on btrpc1 and btrpc3, the two subtypes better characterized in this cell type [23]. Moreover, both types have been associated

with non-capacitative calcium influx, at least in some experimental models [1,10].

Interestingly, we found expression of btrpc1 mRNA (and protein), but not of btrpc3. bTRPC1 is expressed in exponentially growing cells (medium containing 10% FCS), as well as in serum-starved ones; thus, apparently, its expression is not dependent on cell cycle.

Chang et al. [23] described also the presence of btrpc3 transcripts in BAE cells, and showed that its expression varied following hormonal stimulation. The discrepancies with our results may be ascribed to the different culture conditions (the data by Chang et al. were obtained on confluent cells and by exposition to different patterns of external agonists).

Western blot and immunocytochemical analyses give the first evidence for the native expression of bTRPC1 protein in endothelial cells; moreover, we show that it is localized at the plasma membrane, while no detectable protein levels are present in the cytosol. This is the first description of endogenous expression of bTRPC1 protein in native endothelial cells, and one of the first for native tissues [19,33]. In cytofluorimetric calcium measurements, the antibody against bTRPC1 (α-TRPC1) protein did not exert any detectable effect on basal cytosolic calcium level; on the other hand, when α-TRPC1 was applied in presence of bFGF, a significant reduction of the response to the factor was measured, suggesting an involvement of bTRPC1 channels in the calcium influx induced by the factor. The effect of the antibody could be reverted by washing it out; while this is not the general case for the action of antibodies on membrane proteins such as channels, it is in agreement with the findings reported by other authors for different antibodies (see e.g. [36]).

The observation that α-TRPC1 can block only a fraction of the response triggered by bFGF, and that in some cells the response is not apparently affected by the antibody, suggests that other channels (either belonging to the TRPC family or not) can be activated by the factor in these cells. Alternatively, bTRPC1 may form heteromultimers with other members of the family [37].

Electrophysiological measurements in the whole-cell configuration were performed as controls aimed to exclude any aspecific effects on membrane properties. In particular, an inhibition of an inwardly rectifying potassium current, highly expressed in these cells, may reduce the driving force for calcium; this could mimic an inhibitory effect on calcium entry. The lack of effect of the antibody on potassium currents allows us to exclude this possibility.

Thus, bTRPC1 appears to be involved in the generation of non-capacitative calcium influx, in accordance with the observations by other groups [10].

Taken together, the data presented here provide the first evidence for an involvement of a channel belonging to the TRPC family in a calcium influx activated by a biologically relevant agonist such as bFGF in native endothelial cells.

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