Concomitant and hormonally regulated expression of *trp* genes in bovine aortic endothelial cells

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Abstract Recent findings have suggested that the vertebrate *trp* family of channel proteins is the structural basis for Ca^{2+} influx through the capacitative calcium entry (CCE) pathway. We have discerned, in bovine aortic endothelial cells, the concomitant expression of four such vertebrate genes: *trp-1* (two splice variants), *trp-3*, *trp-4* and *trp-5*. Exogenous hormones rendered dynamic effects on the transcript levels of these genes. Most notably, β-estradiol significantly down-regulated *trp-4* while *trans*-retinoic acid dramatically up-regulated *trp-5*; yet these hormones rendered little change in CCE. These findings suggest that the extent of a given *trp* channel's participation in CCE is not reflected in alterations of its transcript level.

Key words: Capacitative calcium entry; Fura-2; Steroid hormone; Semi-quantitative PCR; Vascular endothelium; Thapsigargin

1. Introduction

Elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) following activation of plasmalemmal receptors is a common second messenger response to external stimuli for excitable and non-excitable cells. This phenomenon is attributed to activation of receptor-linked G proteins that mediate IP_3 production, IP_3-stimulated release of internal Ca^{2+} stores, and subsequent activation of plasmalemmal Ca^{2+} influx. This latter influx activity, referred to as 'capacitative calcium entry' (CCE) [1], is activated by the depleted state of internal stores to facilitate their repletion and is also believed to be important for other Ca^{2+}-dependent cellular processes [2]. Intracellular IP_3 may also activate plasmalemmal influx independent of internal store mobilization [3]. Several voltage-independent Ca^{2+} entry mechanisms have been characterized that may subserve CCE, and their biophysical and functional distinctions might be attributed to cell-specific distinctions [4].

Two related ion channels involved in IP_3-dependent visual transduction of *Drosophila*, *trp* and *trpl*, were thought to manifest functional characteristics reminiscent of vertebrate CCE [5,6]. Indeed, heterologous expression of the cloned *trp* cDNA in insect Sf9 cells resulted in membrane channel activities which were Ca^{2+}-permeable and sensitive to internal store depletion, albeit with ion selectivities which were distinct from vertebrate CCE pathways [7]. Channel activities rendered by *trpl*, on the other hand, were activated by a receptor-mediated mechanism involving intracellular IP_3 or a direct G protein-linked mechanism [8,9,23,24]. These findings strongly suggested that *trp/trpl* share structural resemblances with vertebrate channel proteins that subserve CCE. Subsequent searches in the human expressed sequence tag databases identified cDNA sequences that exhibited significant homologies with the *Drosophila* channel proteins [10–12]. These sequences are splice variants of the same gene transcript, which was named either *TRPC1* or *htrp-1* to signify its relationship with the *Drosophila* gene. The encoded protein is reminiscent of *trp/trpl* in that it is characterized by a N-terminal region that contains several ankyrin-like repeats, a central hydrophobic region with six to eight transmembrane helices, and a presumptive pore region which resembles that of voltage- and cyclic nucleotide-gated cation channels. This gene is a member of a family that contains at least six members, and available structural information for the other family members indicates that they share the same general transmembrane topology as *trp-1* [13,14,17]. Within this family, *trp-1*, *trp-2* and *trp-4* are known to exhibit structural variants through alternative mRNA splicing [11,12,15,16,22]; two known variants of *trp-1* encode prematurely truncated proteins which are probably non-functional.

Heterologous expression of full-length, vertebrate *trp* cDNAs has further implicated their relationships to cellular CCE. Exogenous *trp-1*, *trp-3*, *trp-4* and *trp-6* individually provided increased Ca^{2+} influx activity in eukaryotic cell hosts following internal store depletion or intracellular IP_3 application [12,14,17,18]. Moreover, combined expression of all six vertebrate *trp* genes in the antisense orientation led to decreased cellular CCE activity [14]. Similarly, antisense expression of mouse *trp-4* also led to inhibited CCE [18] Thus, these genes encode proteins which are either the pore-forming subunits of the CCE pathway or integral, accessory subunits thereof. In the present study, we sought to further assess the relationship between *trp* genes and CCE by comparing hormone-induced changes in gene expression and cellular CCE activity.

2. Materials and methods

2.1. Endothelial cell culture

Cells were maintained in culture with Dulbecco's modified Eagle's medium (low glucose; Life Technologies, Inc.) supplemented with 10% fetal bovine serum (HyClone) and 1% penicillin/streptomycin (Life Technologies, Inc.), and kept at 37°C in a humidified incubator with 5% CO_2. Cells were seeded in 100 mm culture dishes and used when they just reached confluence for RNA preparation, and harvested 2–3 days post-confluence for $[Ca^{2+}]_i$ measurements. Media for plated cells were fully replenished semi-weekly.

In studies of hormone regulation, cells were cultured under conditions deprived of steroidal agents. This entailed use of media devoid of phenol red, and fetal bovine serum stripped by charcoal. Cells were plated in normal medium/serum for 24 h before switching to the

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steroid-free medium/serum. Each tested hormone was then supplemented to a final concentration of 1 μM, and replenished every other day thereafter. Hormone-treated cells were harvested for both RNA preparation and $[Ca^{2+}]_i$ measurements on the sixth day following the initial plating.

2.2. RT-PCR

One microgram of total RNA was reverse-transcribed into first-strand cDNA by using random hexameric primers (0.067 μg) and MMLV reverse-transcriptase (Pharmacia). The synthesized cDNA products were then used directly as templates for PCR amplification with the following cycling conditions: 10 min at 94°C, followed by up to 40 cycles of 1 min at 94°C/1 min at 50–55°C/2 min at 72°C, and ending with an additional 10 min at 72°C. These conditions provided specific amplification of duplex products which were absent in control reactions devoid of cDNA-template input. Reaction product(s) were directly cloned into a TA vector (pCR2.1; Invitrogen) and transformed into DH5α bacterial cells (Life Technologies). Recombinant plasmids were sequenced by the dideoxy chain-termination method using Sequenase II in the presence of 7-deaza-dGTP (US Biochemicals). For semi-quantitative comparisons, PCR-derived products were electrophoretically fractionated through 1.5% agarose gels and then capillary-transferred onto Hybond-N membranes (Amersham). Transferred DNA fragments were immobilized onto the membrane by UV crosslinking, then solution-hybridized at 65°C, with 6×SSC (0.9 M NaCl, 0.09 M Na-citrate), 5×Denhardt's (0.1% (w/v) each of polyvinylpyrrolidone, bovine serum albumin and Ficoll 400), 0.5% SDS and radiolabelled probe. Each probe incorporated [α-^{32}P]dCTP (3000 Ci/mmol; New England Nuclear) by random-priming using the Prime-It Labelling Kit (Stratagene). Post-hybridization washes were conducted at 65°C in 0.2×SSC, 0.1% SDS. The membrane was subjected to autoradiography in the presence of intensifying screens (DuPont) and, upon visualization of hybridization results, the membrane region corresponding to each radioactive signal was excised and quantitated by liquid scintillation spectrometry

The entire coding region of the *trp-1* cDNA was segregated into three separate domains (the N-terminal domain, the hydrophobic or transmembrane domain, and the C-terminal domain), and each one was individually amplified by RT-PCR. The primers used for this purpose were: N-terminal domain, 5′-cctggcctgccccttcatg-3′ and 5′-ctggccaaaagatgcctactgtc-3′; hydrophobic domain, 5′-gttttgacagtaggcatcttttgg-3′ and 5′-ggtaagcacaatcacaaccac-3′; C-terminal domain, 5′-taaaagctttcagttgatagcaaatc-3′ and 5′-cgctcgatgatttagaaaatgg-3′. For the purpose of semi-quantitative PCR, another pair of primers was used that bracketed the splice region in the N-terminal domain: 5′-gaacataaattgcgtagatg-3′ and 5′-cgatgagcagctaaaatgacag-3′. Additional primers were also designed to amplify select regions of other *trp* cDNAs: *trp-3*, 5′-tgacttccgttgtgctcaaatatg and 5′-ccttctgaagccttctccttctgc-3′; *trp-4*, 5′-cctggacatcttgaagtttctgttc-3′ and 5′-tatatccgcatggtcagcaataag-3′; *trp-5*, 5′-atctactgcctagtactactggct-3′ and 5′-cagcatgatcggcaatgagctg-3′.

2.3. Measurement of $[Ca^{2+}]_i$

Confluent monolayers of bovine aortic endothelial cells (BAECs) were harvested by trypsinization and pelleted by centrifugation. The cells were washed with HEPES-buffered saline (140 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 15 mM HEPES, 10 mM glucose, 0.1% BSA, pH 7.4 at 37°C), resuspended at a concentration of approximately 10^7 cells/ml, and supplemented with fura-2/AM (Molecular Probes) to a concentration of 25 μM. The cell suspension was incubated with the fluorescent dye at 37°C for 30 min, diluted with HEPES-buffered saline such that the final dye concentration was 3.33 μM and then incubated for an additional 30 min at 37°C. The dye-loaded cells were diluted to 2×10^6 cells/ml and then centrifuged, washed and resuspended twice into nominally Ca^{2+}-free saline (HEPES-buffered saline devoid of $CaCl_2$ and supplemented with 0.3 mM EGTA). Aliquots (2 ml) of the cell suspension were loaded into quartz cuvettes and inserted into the temperature-regulated (37°C) sample chamber of a spectrofluorometer (SLM, Model 8100). The cell suspension was stirred continuously and exposed to light excitation alternating between 340 nm and 380 nm; emissions were measured at 510 nm at 1-s intervals and recorded as the ratio ('*R*') of fluorescence intensities at 340 nm and 380 nm. Ligands, $CaCl_2$ and $BaCl_2$ were added to the stirred cell suspensions at the indicated times; both Ca^{2+} and Ba^{2+} were replenished such that the extracellular free concentration of each divalent cation was 1.8 mM. Calibration of cellular fura-2 content was achieved by sequential addition of Triton and EGTA to final concentrations of 0.1% and 10 mM, respectively, and the attained R_{max} and R_{min} values were used in the calculation of $[Ca^{2+}]_i$ by the method of Grynkiewicz et al. [19]. A K_d value of 224 nM was used for Ca^{2+} binding to fura-2 at 37°C.

3. Results

3.1. Coexpression of trp genes

We initially assessed the repertoire of vertebrate *trp* genes which are expressed in cultured BAECs. Based upon the available human cDNA sequences for *trp-1* [10,11], we targeted each of its three encoded domains separately for PCR amplification. In this manner, we obtained from BAECs the cDNA sequences that correspond to the N-terminal domain, the hydrophobic (or transmembrane) domain, as well as the C-terminal domain of bovine *trp-1*. The sequence encoding each domain overlapped with that of the adjacent one(s) in order to ensure capture of the entire protein-coding region. The deduced primary sequence of bovine *trp-1* protein, as shown in alignment with human and mouse *trp-1* sequences (Fig. 1), is highly conserved with few variations in amino acid residues. Within the N-terminal domain, we observed a 34-residue insertion which was absent in the initial forms of cloned human *trp-1* but purportedly was present in its splicing variant [10,11]. This prompted our subsequent use of additional PCR primers, which flank the junction of this splicing-mediated insertion, to assess whether BAECs also express the shorter isoform of *trp-1* which lacks said insertion. Indeed, these primers provided specific amplification from BAECs of two products, 180 bp and 282 bp in length respectively. Nucleotide sequencing analyses indicated that these two products differed only in the presence of coding sequence for 34 residues, and were otherwise identical. Thus, BAECs coexpress two isoforms of *trp-1*: *trp-1a*, which contains added coding sequence for 34 residues, and *trp-1b*, which lacks said coding sequence for 34 residues but preserved the same reading frame as the other isoform. It should be noted that the human homolog of *trp-1b* was named TRPC1A in a previous report [12].

A recent study in a mouse insulinoma cell line detected the coexpression of four *trp-1* splice variants at the transcript level [15] which arise by variable splicing of two immediately adjacent exons, the more distal of which corresponds precisely to the sequence deviation between *trp-1a* and *trp-1b* in BAECs and human tissues as previously reported [11]. Alternative usage of the more proximal exon (155 bp in length), led to generation of transcripts which were frame-shifted in their protein-coding region and thereby resulted in prematurely terminated translation of *trp-1*. It is unclear whether such truncated forms have any cellular impact on *trp-1* functions. Because the proximal primer which we designed to assess the coexistence of *trp-1a* and *-1b* variants resides within the more proximal exon, we cannot rule out the possible presence in BAECs of additional *trp-1* transcript isoforms which encode truncated forms of this protein.

In assessing the possible BAEC expression of additional *trps*, we designed selective primers for PCR amplification based upon available cDNA sequences of said genes. By this approach, we obtained a cDNA fragment which bears significantly high homology with known *trp-3* sequences (Fig. 2). Additionally, we have performed preliminary se-

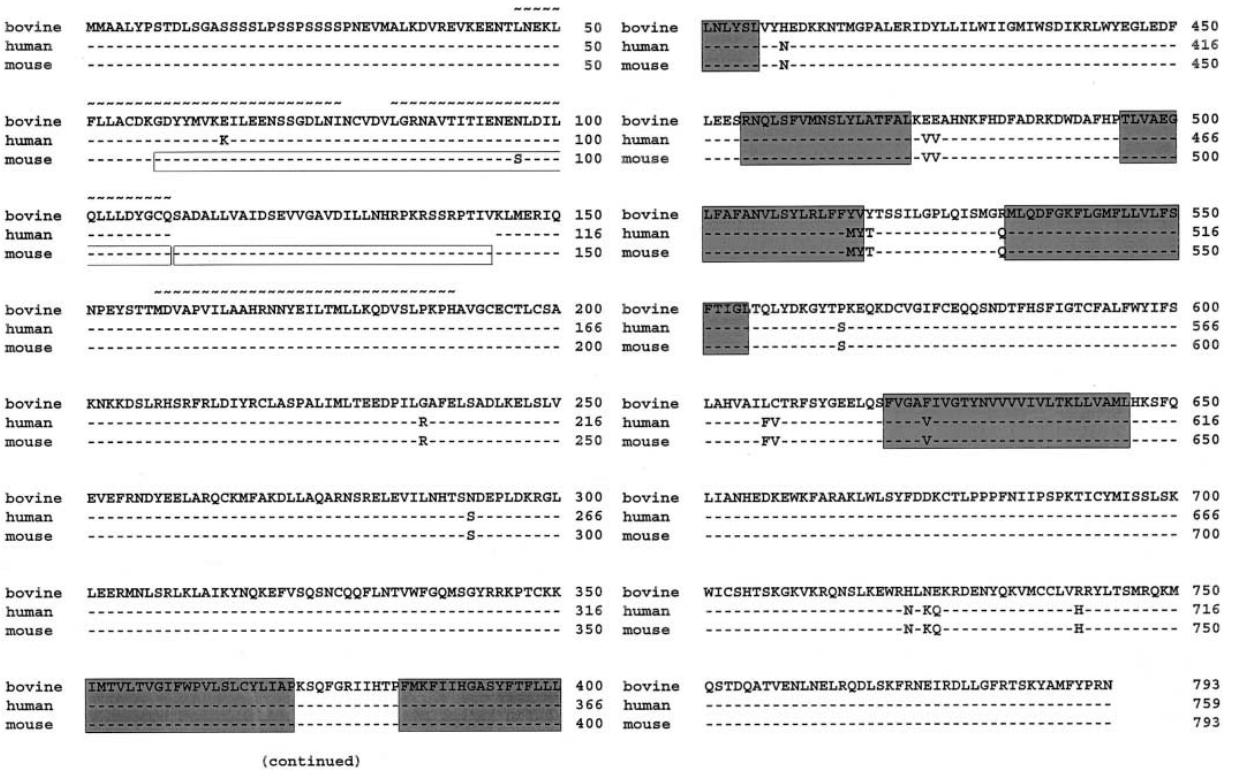

Fig. 1. Alignment of bovine, human and mouse *trp-1* protein sequences. The bovine sequence, *trp-1a*, is deduced from the BAEC-derived cDNA sequence (GenBank accession no. AF012900), while human and mouse sequences are as previously reported [10,11,15]. Note that the human sequence is the homolog of bovine *trp-1b* (GenBank accession no. AF012901, which is truncated by 34 residues relative to *trp-1a*), while the mouse sequence is the *trp-1a* homolog. Conservation of each amino acid residue in the bovine sequence is indicated by '–' in the corresponding position of both human and mouse proteins. Positions of the six presumptive transmembrane domains are indicated by the shaded boxes over all three sequences. Positions of ankyrin-like repeats in the N-terminal domain are indicated by '∼' over the bovine sequence. Positions of alternatively spliced exons, which give rise to the two additional isoforms of *trp-1* transcripts in mouse insulinoma cells [15], are indicated by boxes in the mouse sequence.

quencing analyses on PCR-derived products which bear respective resemblances to mouse *trp-4* and *trp-5*, and in each case with significant levels of sequence conservation at both the nucleotide and protein levels. Thus, BAECs concomitantly express transcripts encoding multiple members of the vertebrate *trp* family, including two splice-variants of one such member.

3.2. Hormonal regulation of trp expression

We next examined the possibility that vertebrate *trp* genes might be transcriptionally regulated by exogenous hormones. This study was conducted by first modifying the culture medium used for maintaining BAECs in vitro such that the cells were deprived of continuous exposure to steroidal agents. Thus, phenol red was removed from the formulation of commercially attained medium and supplemental calf serum was charcoal-stripped. Use of culture conditions modified in this manner had minimal effects on cell growth, cell viability, or apparent morphology. Subsequently, we resupplemented cells grown in the modified fashion with the following hormonal agents individually: β-estradiol; progesterone; dexamethasone; vitamin D; *trans*-retinoic acid; thyroid hormone (3,3′,5-triiodo-L-thyronine). Cells grown in the continuous presence of each of these agents were harvested and processed for RT-PCR of each of the detected *trp* genes, under amplification conditions that provided near-linear yield of each amplicon. This approach enabled comparisons of the relative changes in expression of each *trp* gene in response to varying hormonal stimuli. As control for this analysis, we used the expression levels of cyclophilin as the normalizing basis for comparison.

The results of this analysis, as shown in Fig. 3, revealed that the expression of *trp-1a* was relatively insensitive to hormonal stimuli, exhibiting possibly slight elevation in response to *trans*-retinoic acid and thyroid hormone. Detected levels of *trp-1b* increased notably following administration of all tested hormones except β-estradiol. Responses to *trans*-retinoic acid and thyroid hormone were more pronounced than those to progesterone, dexamethasone or vitamin D. The expression levels of both variants were unaffected by modifications in the culture medium. Changes in the relative levels of *trp-3* exactly mirrored those of the shorter variant of *trp-1* and equally insensitive to changes in the culture medium. *Trp-4*,

Fig. 2. Alignment of bovine, human and mouse *trp-3* protein sequences. The partial bovine sequence is deduced from the BAEC-derived cDNA (GenBank accession no. AF012902), while the partial human and mouse sequences are as previously reported [14]. Conservation of each amino acid residue in the bovine sequence is indicated by '–' in the corresponding position of both human and mouse proteins. Positions of the sixth presumptive transmembrane domain are indicated by the shaded box over all three sequences.

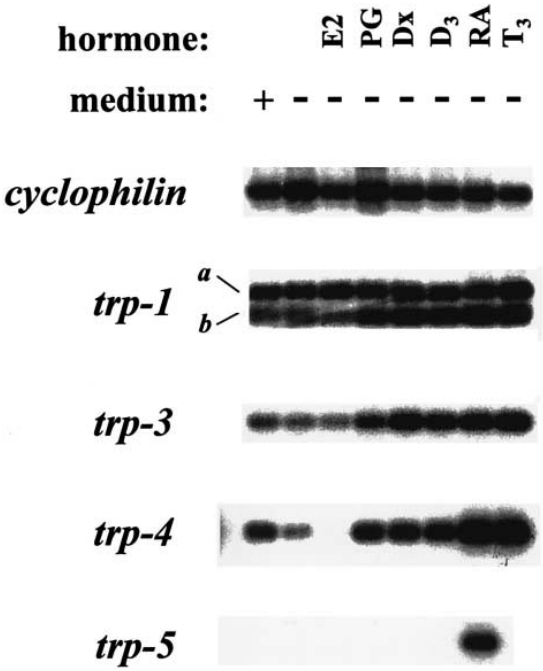

Fig. 3. Hormonal effects on *trp* gene expression. BAECs were cultured in normal medium ('+'), in the modified, steroid-deprived medium ('–'), or the modified medium supplemented with the indicated hormones (at 1 μM). Cells were harvested and the extracted cellular RNAs were used for RT-PCR of each of the indicated *trp* genes. Enzymatic amplifications were conducted under semi-quantitative conditions, and product levels were visualized following Southern blotting/hybridization and autoradiography. PCR reactions selective for cyclophilin were used as normalizing basis for quantitative comparisons. Amplicons representing both isoforms of *trp-1* were simultaneously amplified and distinguishable based upon their size differences (180 and 282 bp). The tested hormones (each administered at 1 μM), were: E2, β-estradiol; PG, progesterone; Dx, dexamethasone; D_3, vitamin D_3; RA, *trans*-retinoic acid; T_3, 3,3′,5-triiodo-L-thyronine. Autoradiographs are representative of five experiments.

however, revealed more dramatic changes in its expression in response to hormonal administration. Its transcript level was discernably reduced by modifications introduced in the culture medium, becoming undetectably low following β-estradiol administration and notably elevated following *trans*-retinoic acid, as well as thyroid hormone administration. The other tested hormones had relatively little effect on *trp-4* transcript levels. Expression levels of *trp-5* were undetectable in all instances except following *trans*-retinoic acid administration, indicating substantial up-regulation of expression in specific response to retinoidal stimulation.

Each autoradiographically discerned signal was directly quantitated by liquid scintillation spectrometry and then sequentially normalized by its corresponding cyclophilin as well as hormonally non-stimulated levels. This approach provided semi-quantitative comparisons of the relative changes in expression of each *trp* in response to hormonal stimulation (Fig. 4). The *trp-1b* variant exhibited a slightly larger range of change in transcript expression than the *trp-1a* variant; the shorter variant was maximally up-regulated close to four-fold in response to thyroid hormone. *Trp-3* expression was up-regulated by over four-fold in response to several hormonal agents. *Trp-4* exhibited dynamic changes, with dramatic down-regulation (to undetectable levels), in response to β-estradiol, and over eight-fold stimulation in response to both *trans*-retinoic acid and thyroid hormone. The most dramatic change in expression, however, was the up-regulation of *trp-5* (by about 60-fold), following *trans*-retinoic acid administration. Thus, expression of these *trp* genes is dynamically responsive to stimulation by exogenous hormones, and each gene manifests a relatively unique pattern of response to the hormonal agents tested herein.

3.3. Hormonal effects on capacitative calcium entry

The vertebrate *trp* genes are believed to encode channel proteins that contribute to the CCE pathway [12,14]. Since

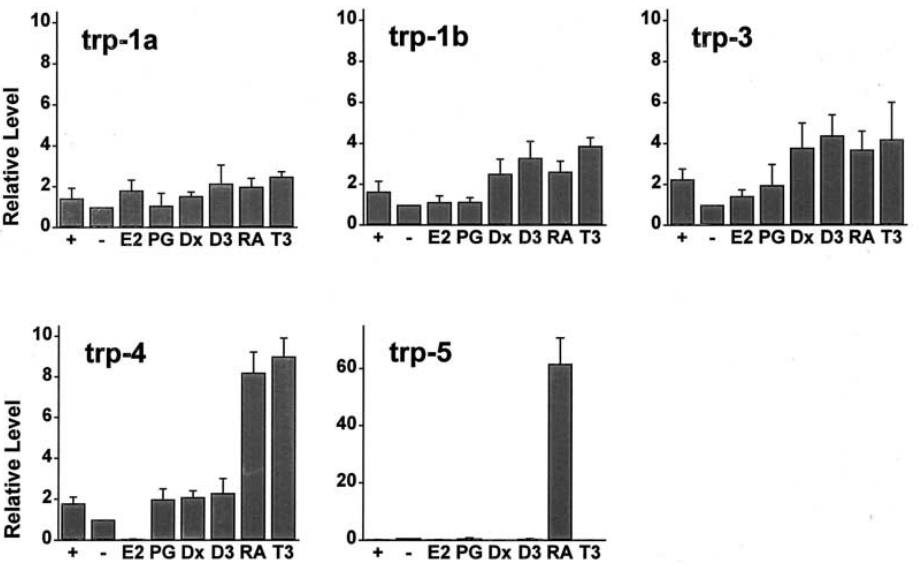

Fig. 4. Semi-quantitative analyses of *trp* expression in BAECs following hormonal stimulation. Hybridization signals as visualized in Fig. 3 were individually excised from the blotting membrane and subjected to liquid scintillation spectrometry. The results were first normalized by the corresponding cyclophilin level, and expressed as a ratio of the corresponding level of each *trp* gene/isoform detected in cells cultured with the modified or '–' medium. The tested hormones were as indicated in the legend to Fig. 3. Each bar is mean ± S.D. ($n = 5$).

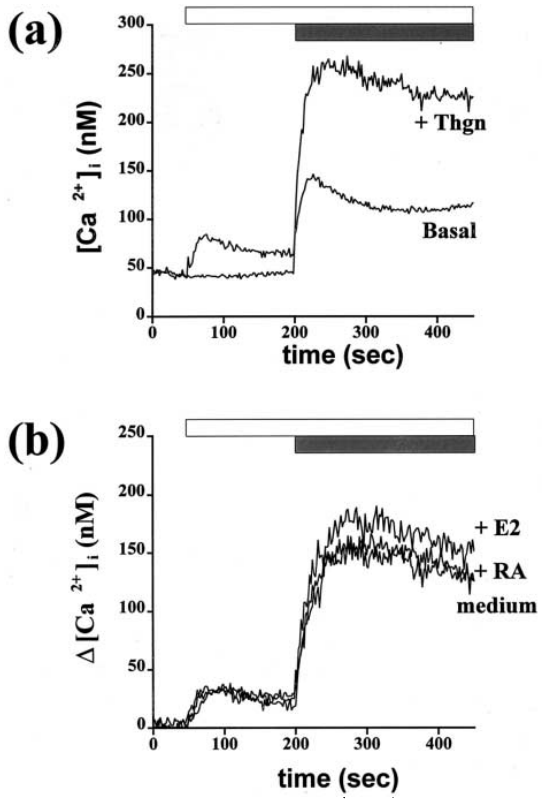

Fig. 5. Hormonal effects on CCE activity. BAECs were cultured in the presence/absence of β-estradiol and *trans*-retinoic acid, and subsequently assessed for CCE activity. a: Cells cultured in the modified medium were loaded with fura-2 and incubated in HEPES-buffered saline devoid of Ca^{2+} (and supplemented with 0.3 mM EGTA). Extracellular Ca^{2+} was replenished to a final concentration of 1.8 mM starting at t = 200 s (as indicated by the gray bar at the top), either with ('+Thgn') or without ('Basal') prior stimulation by thapsigargin (10 nM) starting at t = 50 s (as indicated by the open bar at the top). b: Thapsigargin-activated CCE activity, discerned as the difference in $[Ca^{2+}]_i$ profiles shown in (a), for BAECs cultured in the modified medium ('medium'), or in the modified medium supplemented with 1 μM of either β-estradiol ('E2') or *trans*-retinoic acid ('RA'). Note that both internal release and Ca^{2+} influx components of CCE were not significantly altered following exogenous hormone presence. All traces are representative of three independent experiments.

exogenous hormones imparted dynamic effects on the expression of several members of this gene family in BAECs, we subsequently assessed the effects of hormonal agents on cellular CCE activity in these cells. Our experimental paradigm entailed incubation of fura-2-loaded BAECs initially in HEPES-buffered saline devoid of Ca^{2+} and supplemented with 0.3 mM EGTA. Application of thapsigargin, a selective inhibitor of endoplasmic Ca^{2+}-ATPases, to the cells resulted in depletion of internal Ca^{2+} stores which caused rapid and sustained increase in $[Ca^{2+}]_i$. Readdition of external Ca^{2+} (final free concentration of 1.8 mM) led to a rapid and sustained increase in $[Ca^{2+}]_i$ (Fig. 5a). According to the CCE hypothesis, this latter increase in $[Ca^{2+}]_i$ is attributed to plasmalemmal Ca^{2+} influx pathways which are activated by depletion of the internal Ca^{2+} stores. In support of the existence of additional influx activity, cells manifested $[Ca^{2+}]_i$ elevation upon external Ca^{2+} replenishment with much smaller magnitude when not previously stimulated by thapsigargin (Fig. 5a). The difference between the two profiles shown in Fig. 5a represents temporal separation of the two functional CCE components activated by thapsigargin: stimulus-dependent release of internal Ca^{2+} stores and store depletion-activated Ca^{2+} influx.

This experimental paradigm was applied to BAECs cultured in the modified medium (devoid of phenol red and serum-derived steroids), both in the individual presence and absence of β-estradiol and *trans*-retinoic acid. These two hormones elicited the most dramatic changes in the expression of *trp* transcripts. Continuous administration of these hormones in culture led to no appreciable change in basal $[Ca^{2+}]_i$ levels in BAECs. Following administration of *trans*-retinoic acid, both the store release and the Ca^{2+} influx components of CCE were virtually indistinguishable from those without hormone administration (Fig. 5b). Administration of β-estradiol led to essentially no changes as well (Fig. 5b). It should be pointed out that basal Ca^{2+} influx activities, resulting from external Ca^{2+} replenishment without prior thapsigargin stimulation, remained essentially invariant following these hormone administrations. Thus, despite dramatic effects on *trp* expression, exogenous hormones seem to have little effect on cellular CCE activities.

4. Discussion

The present findings indicate the concomitant expression, in BAECs, of at least four members of the vertebrate *trp* gene family, as well as at least two splicing variants for one such gene. Such coexistence of multiple *trp* genes within a single cell-type might be a generalized phenomenon, particularly since recent reports described the coexpression of several *trp*s and their splicing variants in a megakaryocytic cell line as well as an insulinoma cell line [15,16]. Expression of *trp-1* in BAECs is consistent with the apparently wide tissue distribution of this gene [10]. Detection of *trp-3* in BAECs, however, was unexpected since it exhibits a much more restricted pattern of tissue distribution when assessed by Northern analysis [14].

The transcription-regulatory mechanisms that govern the expression of vertebrate *trp* genes have yet to be delineated. In order to gain initial insights into such regulatory mechanisms, we assessed the possibility that exogenous hormones might alter the expression of the discerned *trp* genes in BAECs. Indeed, our findings suggest that the expression of these genes is dynamically responsive to the tested hormones. Most notably, β-estradiol significantly down-regulated *trp-4* transcripts while *trans*-retinoic acid dramatically up-regulated *trp-5* transcripts. Additionally, progesterone, dexamethasone, *trans*-retinoic acid and thyroid hormone all seemed to have generally stimulatory effects on BAEC *trp* genes. It remains to be established whether such observations are attributable to direct hormonal effects on gene transcription, and whether such changes in *trp* expression underlie hormonal effects on the vascular endothelium in vivo.

Several lines of evidence have collectively suggested that vertebrate *trp* proteins are structural components of CCE-activated cation channels. The observed effects of exogenous hormones on BAEC *trp* expression led to our assessments of possible hormonal impact on endothelial CCE. In this regard, we compared two thapsigargin-activated components of CCE in cells with or without stimulation by β-estradiol and *trans*-retinoic acid, respectively. Choice of these two agents

was predicated by their pronounced effects on *trp-4* and *trp-5*. Surprisingly, both the store release and plasmalemmal influx components of CCE were insensitive to exogenous hormone presence. Beyond down-regulating *trp-4*, β-estradiol rendered modest up-regulations of *trp-1a* and *trp-3* expression; *trans*-retinoic acid up-regulated these latter genes much more than β-estradiol and yet rendered no functional change. Thus, hormone-induced changes in *trp* expression do not produce corresponding changes in CCE activities.

Several explanations can be posed to account for the present lack of correlation between hormone-induced *trp* expression and CCE. First, each tested hormone produces changes in expression of several genes concomitantly in BAECs, and their aggregate functional effects render no discernable change in cellular CCE. In this regard, the relevant genes that counterbalance the induced changes in *trp* expression must be functionally involved in the CCE cascade distal to store release. Second, the manner in which *trp*-encoded channel proteins are assembled into functional channel complexes that mediate CCE-activated Ca^{2+} influx remains to be identified. A recent report described the coassembly of *trp-1* and *trp-3* proteins into both homomeric and heteromeric complexes in vitro, akin to their *Drosophila* relatives [20]. Multimeric assembly might be a generalized capability of *trp* proteins, which would not only provide significant structural and functional diversity, but could also diminish the overall importance of any one *trp* to cellular CCE. Third, the CCE-activated influx pathway requires structural components, beyond *trp*-encoded channel proteins, which are either differentially sensitive or refractory to regulation by exogenous hormones. This possibility is supported by the recent finding that Drosophila *trp* interacts with accessory proteins in vitro, and that such accessory proteins might in turn impart regulatory effects on *trp*-relevant functions [21]. As such, simple alterations in bovine *trp* expression levels (which presumably reflect corresponding changes in the cellular levels of the encoded proteins) are inadequate to impart CCE changes in BAECs.

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