Histochem Cell Biol (2004) 122:553–561
DOI 10.1007/s00418-004-0720-y

ORIGINAL PAPER

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Expression of TRPC homologs in endothelial cells and smooth muscle layers of human arteries

Accepted: 6 October 2004 / Published online: 5 November 2004
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Abstract TRPC channels are a group of Ca^{2+}-permeable nonselective cation channels that mediate store-operated and/or agonist-stimulated Ca^{2+} influx in a variety of cell types. In this study, we extensively examined the expression patterns of TRPC homologs in human vascular tissues. RT-PCR amplified cDNA fragments of TRPC1 (505 bp), TRPC3 (372 bp), TRPC4 (499 bp), TRPC5 (325 bp), TRPC6 (509 bp), and TRPC7 (187 bp) from RNA isolated from cultured human coronary artery endothelial cells. In situ hybridization yielded strong labeling of TRPC1,3–6 in the endothelial and smooth muscle cells of human coronary and cerebral arteries. TRPC7 labeling was exclusively found in endothelial cells but not in smooth muscle cells. Results from immunohistochemical staining were consistent with those from in situ hybridization. Similar expression patterns of TRPC homologs were also observed in arterioles and vaso vasora. In conclusion, our study indicates that TRPC homologs are widely expressed in human vessels of all calibers, including medium-sized coronary arteries and cerebral arteries, smaller-sized resistance arteries, and vaso vasora. These results suggest a ubiquitous role of TRPC homologs in regulating blood supply to different regions and in controlling arterial blood pressure.

Keywords Gene expression · Ca^{2+} channel · Immunohistochemistry · Endothelium · Smooth muscle

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Introduction

TRPC, or canonical TRP channels, is a group of mammalian Ca^{2+}-permeable channels sharing homology with *Drosophila* TRP channel (Minke and Cook 2002). Seven different TRPC homologs have been isolated and, on the basis of sequence homology, can be divided into four subgroups TRPC1, TRPC4/5, TRPC3/6/7, and TRPC2 (Clapham et al. 2000). All TRPC homologs possess the predicted topology of six transmembrane segments (S1–S6) and a pore region between S5 and S6. There are three or four ankyrin repeats at the NH_2-terminal domain, a proline-rich sequence in the COOH-terminal domain, and a highly conserved 25-amino acid segment referred to as the TRP domain (Minke and Cook 2002). The physiological role of TRP channels is diverse. TRPC channels mediate store-operated Ca^{2+} entry as well as store-independent Ca^{2+} influx (Minke and Cook 2002; Venneken et al. 2002; Nilius and Droogman 2003; Kwan et al. 2004). Functionally, it is known that TRPC2 participates in pheromone sensory signaling and sperm fertilization (Jungnickel et al. 2001; Keverne 2002) and that TRPC3 plays a crucial role in brain-derived nerve growth factor-induced neuronal differentiation and plasticity (Montell 2001). In cardiovascular system, TRPC6 is responsible for α_1-adrenoceptor-activated Ca^{2+} entry (Inoue et al. 2001) and it affects vascular permeability (Pocock et al. 2004) and TRPC4 is involved in the regulation of vascular tone and vascular permeability (Freichel et al. 2001; Tiruppathi et al. 2002).

There are numerous studies investigating the expression of TRPC channels in vascular cells (Chang et al. 1997; Garcia and Schilling 1997; Groschner et al. 1998; Kamouchi et al. 1999; Freichel et al. 2001; Inoue et al. 2001; McDaniel et al. 2001; Walker et al. 2001; Xu and Beech 2001; Antoniotti et al. 2002; Inoue and Mori 2002), but almost all of them are limited to using RT-PCR examining the expression of TRPC channels in cultured vascular cells (Chang et al. 1997; Garcia and Schilling 1997; Groschner et al. 1998; Kamouchi et al. 1999; Inoue et al. 2001; Kohler et al. 2001; McDaniel et al. 2001; Walker et al. 2001; Wang et al. 2004) and/or in whole vascular tissues that contain different cell types (Facemire et al. 2004; Lin et al. 2004; Pocock et al. 2004; Wang et al. 2004). There are two immunohistochemical studies available, one demonstrating the expression of TRPC3,6 in the smooth muscle layer of rat resistance arteries (Welsh et al. 2002) and the other showing the expression of TRPC1 in the smooth muscle of mouse resistance arteries (Xu and Beech 2001). The expressional study of TRPC homologs in endothelial cells of intact vessels is lacking, and there is no immunohistochemical or in situ hybridization study on the expression of TRPC in human vessels. Therefore, the overall expression patterns of different TRPC homologs in different-sized vessels are not known, especially in human. In the present study we used RT-PCR, in situ hybridization, and immunohistochemistry to examine the expression of TRPC1,3–6 in human vascular tissues and found the expression of all these TRPC homologs in vascular smooth muscle cells and endothelial cells in different human vascular beds and in different-sized arteries. TRPC7 was found to be expressed in endothelial cells using RT-PCR and in situ hybridization. No immunohistochemical experiments were performed for TRPC7 because commercial antibody was not available. TRPC2 is a pseudogene which does not express in human (Minke and Cook 2002) and, therefore, it was not studied.

Materials and methods

Cell culture

A coronary artery endothelial cell line (HCAEC 5282) was purchased from Bio Whittaker, USA. The cells were cultured in EGM-MV medium in a humidified atmosphere with temperature set at 37°C and constant CO_2 (5%) supplied. Culture medium was changed every 2 days until the cells became approximately 80–100% confluent. Confluent cell monolayers were passaged using 0.25% trypsin in phosphate-buffered saline (PBS) containing 2.5 mmol/l EDTA.

RT-PCR

Total RNA was extracted from HCAEC 5282 using the acid guanidinium thiocyanate method. mRNA was transcribed into first strand cDNA using oligo-dT primers and M-MLV reverse transcriptase (Gibco-BRL). Reverse transcription was performed at 42°C for 50 min, followed by a final extension time of 15 min at 72°C. PCR were performed with Taq DNA polymerase (Gibco-BRL). The primers for PCR were designed based on the sequence of human TRPC genes in GenBank database [access number: NM_003304, NM_003305, NM_016179, NM_012471, NM_004621, BI826927 (a TRPC7 Est clone containing exon 10, exon 11, and 3′ untranslated region)]. The primer pairs (forward/reverse) were: TRPC1, TTCTGTGGATTATTGGGATGA (in exon 7)/CAGAACAAAGCAAAGCAGGTG (in exon 11); TRPC3, ATGCTGCTTTTACCACTGTAG (in exon 6)/TCCTTCTGCATTTGGGAAA (in exon 8); TRPC4, GGCGGACTTCAGGACTACAT (in exon 6)/GCTGTGCTTTGACATTGGTC (in exon 8); TRPC5: CTCTCAAGAAACTGGGTCTCCTATTC (in exon 11)/GTTTCAAATACATCCTCTGAGGAGTC (in exon 11); TRPC6, GGCAAAACAAATGAAGCC (in exon 7)/CCCAACCTGTTTTTTGTCAA (in exon 11); TRPC7, TCAACAACTCAGCGA (in exon 11)/TTCGTGTCCTAGAGGAG (in exon 11). PCR of 100 μl contained 2 μl first strand cDNA, 20 mmol/l TRIS-HCl (pH 8.4), 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 0.2 mmol/l dNTP, 1.0 μmol/l primers, and 2.5 U Taq DNA polymerase (Gibco-BRL). Fifty cycles (94°C for 1 min, 58°C for 1 min, 72°C for 1 min) for TRPC1,3,4,6, or 40 cycles for TRPC5 (94°C for 1 min, 55°C for 1 min, 72°C for 1 min) and TRPC7 (94°C for 1 min, 53°C for 1 min, 72°C for 1 min) were performed with a Robocycler (Stratagene, USA). Control reactions without the first strand cDNA were included for each PCR amplification experiment. For TRPC5 or TRPC7, we performed an additional negative control that applied the same samples and mimicked the same experimental procedures except that the step of reverse transcription was omitted. The authenticity of amplified PCR products were verified by an ABI PRISM DNA sequencing system (Perkin Elmer).

In situ hybridization

The amplified products of TRPC homologs were cloned into pPCR-ScriptAmp vector (Stratagene) and then in vitro transcribed into digoxigenin (DIG)-labeled RNA probes using a DIG-labeling kit (Roche Biochemicals). The strand complementary to TRPC genes was used to detect the positive signal. Corresponding sense strands and an irrelevant 211-base riboprobe generated from an intron region of CNG1α (cyclic nucleotide-gated cation channel) gene (Cheng et al. 2002) were used as controls to monitor possible nonspecific binding of RNA to any cellular structure. Probes were used to hybridize with the sections cut from the human tissues embedded in paraffin. The vascular tissues of normal human adult were from autopsy cases with the consent of family members and the approval of the university clinical research ethics committee. Tissues were fixed overnight with 4% paraformaldehyde in PBS. We examined the tissues from three deceased subjects whose bodies were immediately transferred to a cold room and kept at 4°C. One subject was a 16-year-old male who died of X-linked adrenoleukodystrophy, which is a genetic disease with dysfunction of adrenal gland and loss of myelin sheath insulating the nerve. The second subject was a 59-year-old female who died of cardiac tamponade caused by a dissecting aortic aneurysm. The third subject was a 65-year-old male who died of colon cancer. The postmortem delay for the subjects was approximately 72 h. Some atherosclerotic lesions could be observed in the coronary artery walls of the two older subjects. There were no differences with regard to the TRPC expression in vessel samples taken from all three subjects. The tissues were dehydrated through graded ethanol, cleared with xylene, and embedded in paraffin, and 5-μm-thick sections were prepared. After dewaxing and hydration, the sections were washed briefly with diethylpyrocarbonate-treated water followed by PBS for 10 min. They were then digested with proteinase K (20 μg/ml) at 37°C for 10 min. Hybridizations were performed at 52°C in a hybridization buffer containing 4×SSC, 10% dextran sulfate, 1×Denhardt's solution, 5 mmol/l EDTA, 0.1% CHAPS, 50% deionized formamide, 200 μl/ml herring sperm DNA, and 200 ng/ml DIG-labeled probe. The slides were then washed four times, 15 min each, in 2×SSC/0.1% SDS and then twice, 15 min each, in 0.2×SSC/0.1% SDS at 42°C. Colorimetric detections were performed using an anti-DIG antibody conjugated to

alkaline phosphatase followed by incubation with NBT/BCIP color substrates using a digoxigenin-nucleic acid detection kit (Roche, Germany). Images were taken in a Leica Imaging System with SPOT software (Diagnostic Instruments, USA).

Immunohistochemistry

Human coronary and cerebral arteries were used for immunohistochemical experiments. Immunoreactivity was tested using avidin-biotin-peroxidase reactions. Tissue cross-sections of 5 μm were rehydrated in a graded alcohol series to 80% ethanol and then preincubated with 0.5%(v/v) H_2O_2 in absolute methanol to inhibit endogenous peroxidase activity. Sections were incubated at room temperature overnight with primary polyclonal antibodies from Alomone Labs: TRPC1 (1:600 dilution), TRPC3 (1:300 dilution), TRPC4 (1:50 dilution), TRPC5 (1:150), or TRPC6 (1:200 dilution). A secondary biotinylated goat anti-rabbit IgG was consequently applied and immunoreactivity was visualized with streptavidin-biotin-peroxidase along with 3,3′-diaminobenzidine (Sigma) as substrate. In control experiments, the primary antibodies were either omitted or were preabsorbed for 2.5 h at room temperature with a 60-fold molar excess of peptide antigens provided by manufacturer. DNAsis software was used to examine the possibility of each TRPC antibody cross-hybridizing with other TRPC homologs. It was found that each TRPC peptide used for raising antibody was only specific to its targeted TRPC isoform. Therefore, it is highly unlikely that any TRPC antibody could cross-hybridize with other TRPC homologs.

Results

RT-PCR-based detection of TRPC homologs in cultured human coronary endothelial cells

RT-PCR was used to examine the expression of TRPC transcripts. Gene-specific primers were designed according to the corresponding human TRPC sequences published in GenBank. Forward and reverse primers for TRPC1,3,4,6 were located in separate exons. RT-PCR amplified the expected products of 505 bp for TRPC1, 372 bp for TRPC3, 499 bp for TRPC4, and 509 bp for TRPC6 from total RNA isolated from human coronary endothelial cells (Fig. 1). These products did not result from genomic DNA contamination since PCR amplification from genomic DNA should result in the products of much larger molecular sizes (4–19 kb). For TRPC5 and TRPC7, the forward and reverse primers were located within a single exon. Therefore, it was not possible to differentiate the real RT-PCR product from the false amplification product generated from genomic DNA contamination. For this reason, a PCR experiment without the reverse transcription step was included as the control for genomic DNA contamination. Forty cycles of RT-PCR yielded a 325-bp TRPC5 product and a 187-bp TRPC7 product, indicating the expression of TRPC5 and TRPC7 in human coronary endothelial cells. These products were absent in the control experiment without reverse transcription, excluding the possibility of the product being a false product amplified from genomic DNA contamination. The authenticity of all amplification products including TRPC1,3–7 was confirmed by DNA sequencing.

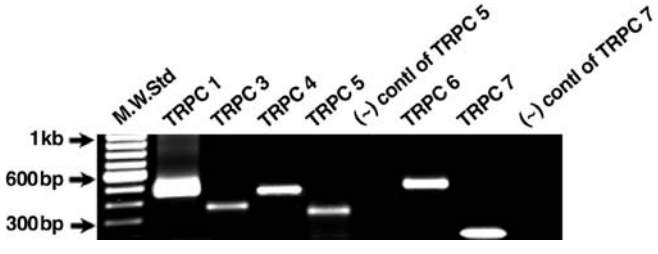

Fig. 1 RT-PCR-based detection of TRPC homologs in human coronary artery endothelial cell HCAEC5282. Targeted products (505 bp for TRPC1, 372 bp for TRPC3, 499 bp for TRPC4, 325 bp for TRPC5, 509 bp for TRPC6, and 187 bp for TRPC7) were observed in ethidium bromide-stained agarose gel. The negative control for TRPC5 or TRPC7 applied the same RNA sample and experimental procedures but without the reverse transcription step

Detection of TRPC homologs in human coronary and cerebral arteries by in situ hybridization and immunohistochemical studies

In situ hybridization was used to explore the cellular localization of TRPC homologs in vascular tissues. We chose to study the expression in human cerebral and coronary arteries due to the well-known physiological and pathological importance of these vessels. Tissue sections from these vessels were hybridized with either antisense or control riboprobes. Hybridizations of blood vessel sections with antisense riboprobes yielded strong labeling in the endothelial layer of cerebral and coronary arteries for TRPC1 (Fig. 2A, H), TRPC3 (Fig. 2B, I), TRPC4 (Fig. 2C, J), TRPC5 (Fig. 2D, K), TRPC6 (Fig. 2E, L), and TRPC7 (Fig. 2F, M). Some smooth muscle cells in the tunica media layer, characterized by their long spindle shape, were also positively stained (Fig. 2A–E, H–L), indicating the expression of TRPC1,3–6 in vascular smooth muscle cells. No hybridization signal was observed in control experiments (Fig. 2G, N). Note that, unlike that of other TRPC homologs, the expression of TRPC7 was only observed in endothelial cells (Fig. 2F, M), but not in smooth muscle layers. Some staining was observed in the outermost tunica adventitia layer for all TRPC homologs (Fig. 2A–F), but we were not able to determine the cell origin of these labeled structures.

The above in situ hybridization results were supported by immunohistochemical investigations using antibodies against TRPC homologs (Fig. 3). Strong positive signals in brown color could be observed in vascular smooth muscle layer as well as in vascular endothelial cells for TRPC1,3–6 homologs (TRPC1, Fig. 3A, C; TRPC3, Fig. 3E, G; TRPC4, Fig. 3I, K; TRPC5, Fig. 3M, O; TRPC6, Fig. 3Q, S). The expression patterns of TRPC homologs in cerebral (Fig. 3A, E, I, M, Q) and coronary (Fig. 3C, G, K, O, S) arteries were very similar. Two sets of control experiments were done, one with antigen preabsorption (Fig. 3B, D for TRPC1; Fig. 3F, H for TRPC3; Fig. 3J, L for TRPC4; Fig. 3N, P for TRPC5; Fig. 3R, T

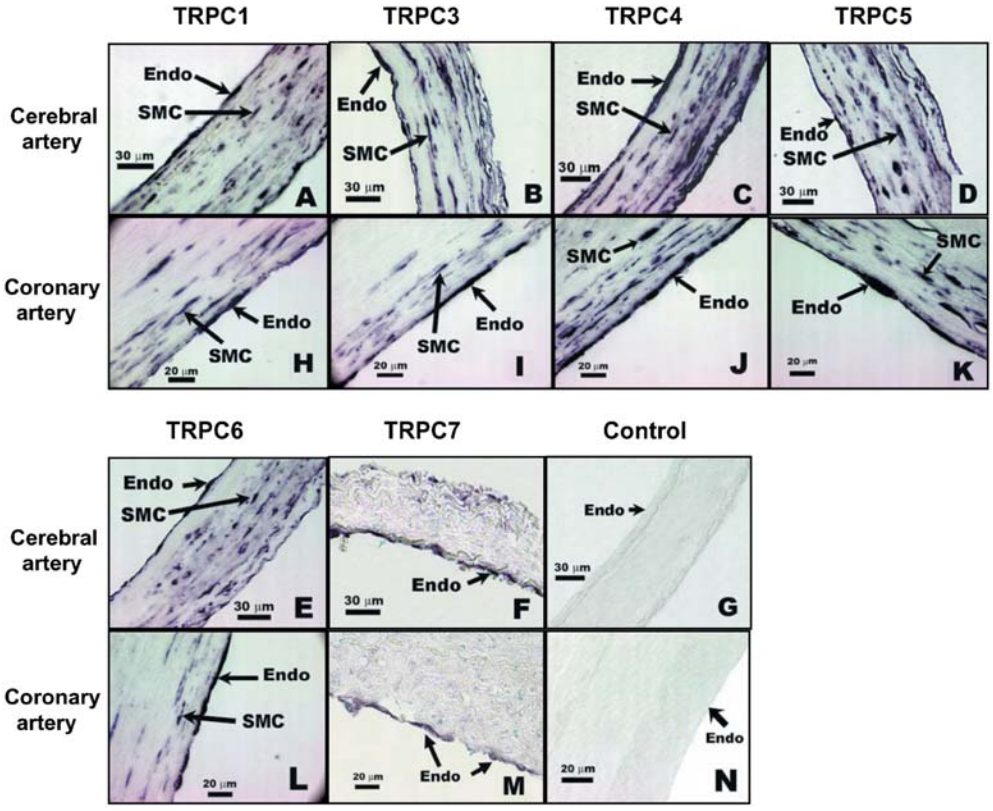

Fig. 2A–N Detection of TRPC mRNAs in human coronary and cerebral arteries by in situ hybridization. **A–G** Cerebral arteries. **H–N** Coronary arteries. Expression of TRPC1 (**A**, **H**), TRPC3 (**B**, **I**), TRPC4 (**C**, **J**), TRPC5 (**D**, **K**), and TRPC6 (**E**, **L**) was observed in vascular endothelial layer and some smooth muscle cells. TRPC7 staining was only observed in endothelial cells, and not in smooth muscle cells (**F**, **M**). Controls (**G**, **N**) had no signal

for TRPC6) and the other in the absence of primary anti-TRPC antibodies. There was no detectable signal in the absence of primary antibodies (data not shown). Faint signal was sometimes observed in antigen preabsorption control, presumably due to insufficient preabsorption (Fig. 3D, H, T). Nevertheless, the difference between positives (without preabsorption) and controls (with preabsorption) was very large and obvious (Fig. 3A–T), indicating that the hybridizations were TRPC-specific. Note that the blue color in experiments and in controls was due to hematoxylin counterstaining, which stained the cell nucleus.

Expression of TRPC homologs in human coronary arterioles and vaso vasora

Similar mRNA expression patterns could be observed by in situ hybridization in small-sized coronary arterioles (approximately 50–150 μm in diameter) that belong to resistance arteries (Fig. 4A for TRPC1; Fig. 4B for TRPC3; Fig. 4C for TRPC4; Fig. 4D for TRPC5; Fig. 4E for TRPC6; Fig. 4F for TRPC7) as well as in vaso vasora of coronary arteries (Fig. 4H for TRPC1; Fig. 4I for TRPC3; Fig. 4J for TRPC4; Fig. 4K for TRPC5; Fig. 4L for TRPC6; Fig. 4M for TRPC7). Again, note that there was no TRPC7 staining in the smooth muscle layer of small-sized arteriole (Fig. 4F). Vaso vasora supply the nutrition to blood vessels. Diameters of the vaso vasora in pictures ranged from 13 to 45 μm. Immunohistochemical studies confirmed the expression of TRPC homologs in the endothelial and smooth muscle layers of coronary arterioles (Fig. 5A for TRPC1; Fig. 5E for TRPC3; Fig. 5I for TRPC4; Fig. 5M for TRPC5; Fig. 5Q for TRPC6) and vaso vasora (Fig. 5C for TRPC1; Fig. 5G for TRPC3; Fig. 5K for TRPC4; Fig. 5O for TRPC5; Fig. 5S for TRPC6). Two sets of control experiments were done, one with antigen preabsorption (TRPC1, Fig. 5B, D; TRPC3, Fig. 5F, H; TRPC4, Fig. 5J, L; TRPC5, Fig. 5N, P; TRPC6, Fig. 5R, T) and the other in the absence of primary antibodies. No signal was observed in the absence of primary antibodies (data not shown). Faint signal was sometimes observed in antigen preabsorption control, presumably due to insufficient preabsorption (Fig. 5F, H, R). Nevertheless, the difference between positives (without preabsorption) and controls (with preabsorption) was very large and obvious (Fig. 5A–T), indicating that the hybridizations were TRPC-specific. Similar expression patterns of TRPC homologs were also found in small-sized arterioles and vaso vasora in cerebral vascular beds as well as in different-sized veins in both coronary and cerebral vascular beds (data not shown).

Although only one set of figures was shown for in situ hybridization (Figs. 2, 4) and immunohistochemistry (Figs. 3, 5), both in situ experiments and immunohistochemical experiments were actually repeated six times, i.e., two separate experiments for each human subject with a total of three human subjects. In each experiment, there were two repetitive hybridization slides for each different antibody. The results from different experiments

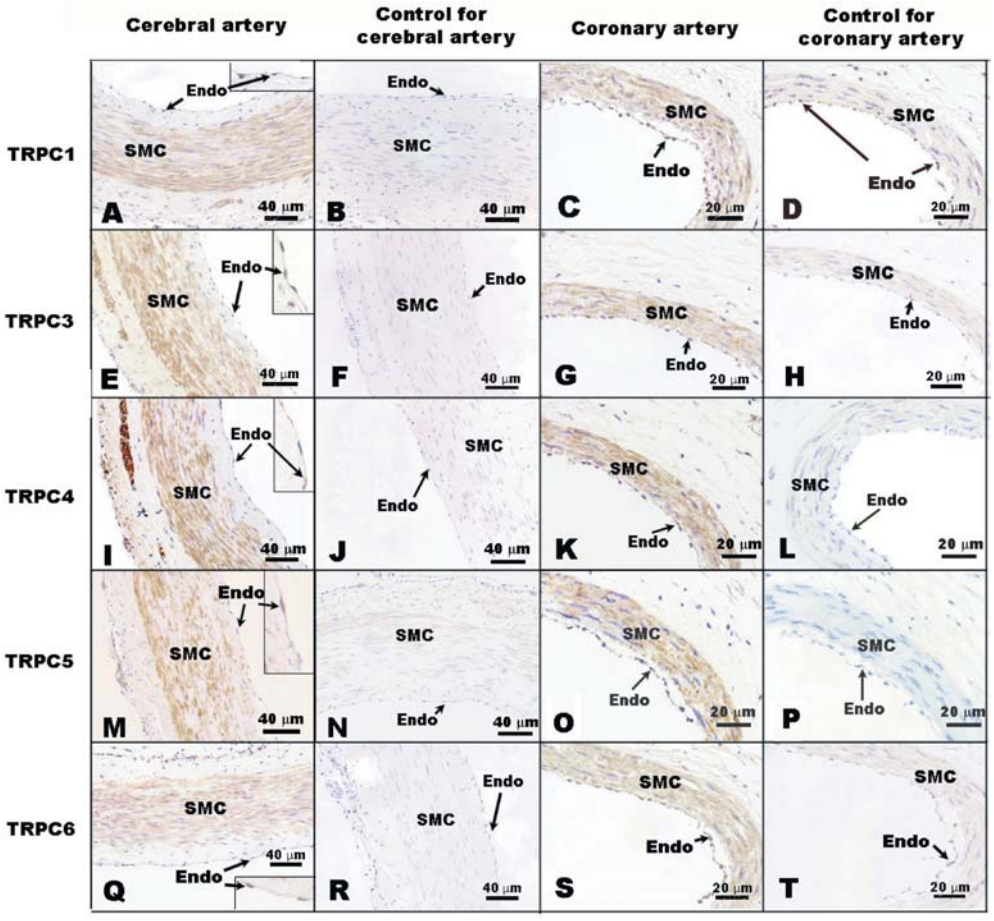

Fig. 3A–T Detection of TRPC proteins in human coronary and cerebral arteries by immunohistochemical studies. **A–D** TRPC1. **E–H** TRPC3. **I–L** TRPC4. **M–P** TRPC5. **Q–T** TRPC6. Positive signals in brown color could be visualized in the endothelial and smooth muscle layers of cerebral (**A**, **E**, **I**, **M**, **Q**) and coronary arteries (**C**, **G**, **K**, **O**, **S**). In control experiments, the primary antibodies were preadsorbed by TRPC1 peptide (**B**, **D**), TRPC3 peptide (**F**, **H**), TRPC4 peptide (**J**, **L**), TRPC5 peptide (**N**, **P**), or TRPC6 (**R**, **T**). Another set of control experiments was performed in the absence of the primary antibodies (data not shown). No positive signal could be observed in both sets of control experiments. Note that the blue color was due to hematoxylin counterstaining which stained cell nuclei. *Insets* in **A**, **E**, **I**, **M**, and **Q** are high magnification pictures which show positive staining in endothelial cells

were consistent. No difference was observed regarding the expression of TRPC homologs, either at mRNA or protein level, in the samples taken from different human subjects.

Efforts were also made to compare the expression level of TRPC homologs between different-sized vessels by laying multiple tissue sections containing different-sized vessels on a single glass slide, followed by hybridization processes. This procedure could overcome the possible variations due to different tissue sections being exposed to different hybridization conditions. Our results showed no difference in TRPC expression between the medium-sized conduit arteries and the small-sized resistance arteries.

Discussion

Cytosolic Ca^{2+} concentration in vascular endothelial cells acts as a second messenger to regulate many diverse processes such as angiogenesis, cell growth, and leukocyte migration (Nilius and Droogman 2001). Ca^{2+} influx may stimulate the activity of a constitutive nitric oxide synthase, which catalyzes the production of nitric oxide (NO) (Luckhoff et al. 1988; Govers and Rabelink 2001). Ca^{2+} entry may also activate phospholipase A_2 (PLA_2), a key enzyme that controls the production of prostacyclin (PGI_2) and endothelium-derived hyperpolarizing factor (EDHF). NO, PGI_2, and EDHF are released from endothelial cells and then diffuse to nearby smooth muscle cells to regulate vascular tone (Busse et al. 2002). Like many other nonexcitable cells, the predominant Ca^{2+} entry pathway in vascular endothelial cells is the store-operated one, which is presumably mediated by TRP channels (Nilius and Droogman 2003). TRPC channels also play important roles in Ca^{2+} influx in vascular smooth muscle cells (Inoue et al. 2001; McDaniel et al. 2001; Xu and Beech 2001; Jung et al. 2002; Welsh et al. 2002; Lin et al. 2004; Wang et al. 2004). Despite the functional importance of TRPC channels in vascular tissues, there are only a few immunohistochemical studies (Xu and Beech 2001; Welsh et al. 2002) and no in situ hybridization studies regarding the expression patterns of TRPC homologs in intact vessels in animals. The data for TRPC-related in situ hybridization or immunohistochemical studies in human tissues are absent. In the present study, we examined the expression patterns of TRP1,3–7 using immunohistochemical and in situ hybridization techniques, and found the expression of TRPC1,3–6 homologs in vascular endothelial cells and vascular smooth muscle cells in all the vessels we studied, including medium-sized coronary and cerebral arteries, small-sized arterioles, and vaso vasora. The expression of TRPC7 was found exclusively in endothelial cells but not in smooth muscle layers. Medium-sized arteries, includ-

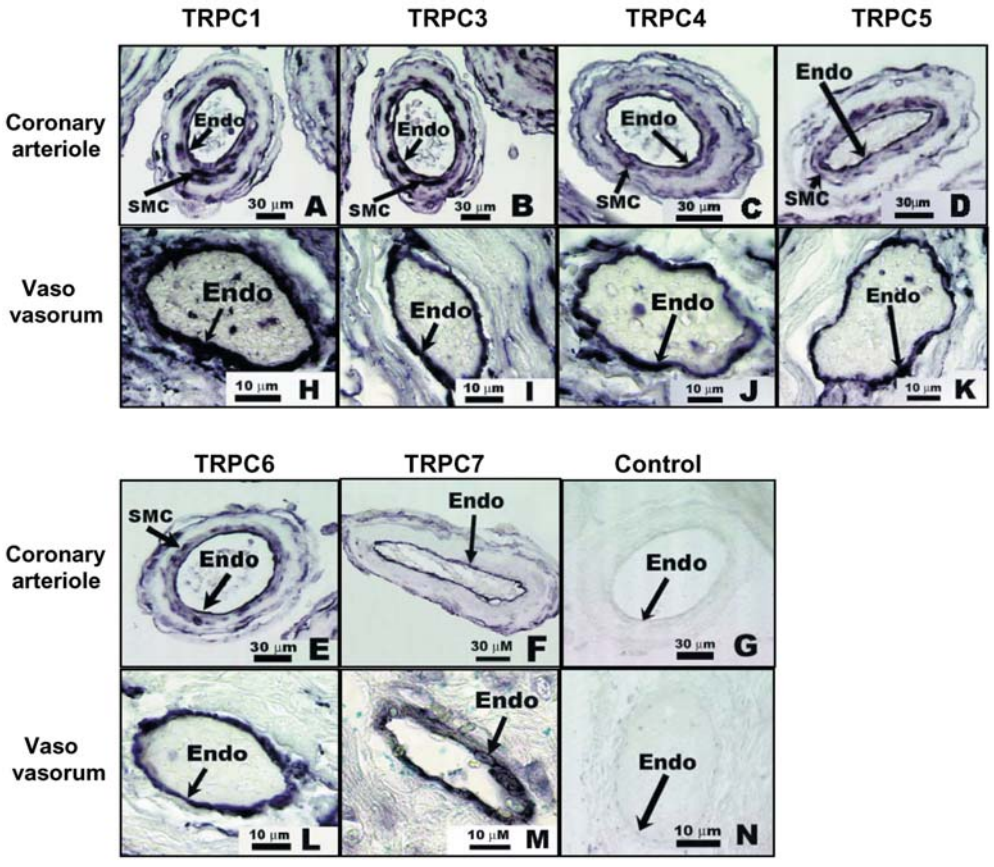

Fig. 4A–N Detection of TRPC mRNAs in human coronary arterioles and vaso vasora by in situ hybridization. **A–G** Coronary arterioles. **H–N** Vaso vasora of coronary arteries. Expression of TRPC1 (**A**, **H**), TRPC3 (**B**, **I**), TRPC4 (**C**, **J**), TRPC5 (**D**, **K**), and TRPC6 (**E**, **L**) was observed in vascular endothelial layer and some smooth muscle cells. TRPC7 staining was only observed in endothelial cells (**F**, **M**), but not in smooth muscle cells (**F**). Controls (**G**, **N**) had no signal

ing coronary arteries and cerebral arteries, control the distribution of blood to different organs, thereby regulating the blood supply to such vital organs as heart and brain. Arterioles, including cerebral and coronary arterioles, belong to resistance arteries that are the principle sites for peripheral resistance. Widespread expression of TRPC channels suggests a ubiquitous role of TRPC channels in mediating Ca^{2+} influx in endothelial and smooth muscle cells and implies a possible involvement of TRPC channels in regulating blood supply to different organs and controlling arterial blood pressure.

There is inconsistency in the literature regarding which specific subtypes of TRPC channels are expressed in vascular endothelial cells. For example, Garcia and Schilling (1997) detected the expression of TRPC1,3–6 in bovine aortic endothelial cells using RT-PCR, whereas similar experiments from other groups failed to observe the expression of TRPC4 in bovine pulmonary endothelial cells (Kamouchi et al. 1999) or TRPC4,6 in human mesenteric artery endothelial cells (Kohler et al. 2001). One possibility for this discrepancy is that endothelial cells isolated from different species and/or different vessels may express different TRPC homologs. But in some cases, different expression patterns were reported even in the same cell type (Garcia and Schilling 1997; Antoniotti et al. 2002). For example, with the use of RT-PCR, Garcia et al. demonstrated the expression of TRPC3 in bovine endothelial cells (Garcia and Schilling 1997), but a similar study by Antoniotti et al. failed to detect the expression of TRPC3 in the same cell type (Antoniotti et al. 2002). Caution should be taken in interpreting these available data. Firstly, the expression of TRPC channels may change in different culture conditions and/or during serial passage of cultured cells. Secondly, all available expressional data were obtained almost exclusively by the highly sensitive RT-PCR method. The correct data interpretation from these experiments would depend on the preparation of samples, the choice of appropriate primers, the right PCR conditions, and whether appropriate procedures were performed to avoid false RT-PCR signals generated from genomic DNA. In the present study, we have used several independent methods including RT-PCR, in situ hybridization, and immunocytochemistry to study the expression of the TRPC homologs in vascular tissues. In situ hybridization and immunohistochemical studies were performed on sections cut from intact human vessels in autopsy samples, thereby eliminating the possible errors that could result from cell culture conditions. Our data from intact vessel sections agreed well with those from RT-PCR, showing the expression of all TRPC members in human vascular endothelial cells and TRPC1,3–6 in vascular smooth muscle cells. In human tissues, our immunostaining experiments also demonstrated the expression of TRPC1,3–6 homologs in cultured vascular cells of animal origins, including rat aortic endothelial cells (self-prepared), rat smooth muscle cells (A7r5, ATCC), and bovine pulmonary endothelial cells (CCL-209; ATCC) (data not shown). Note that the only

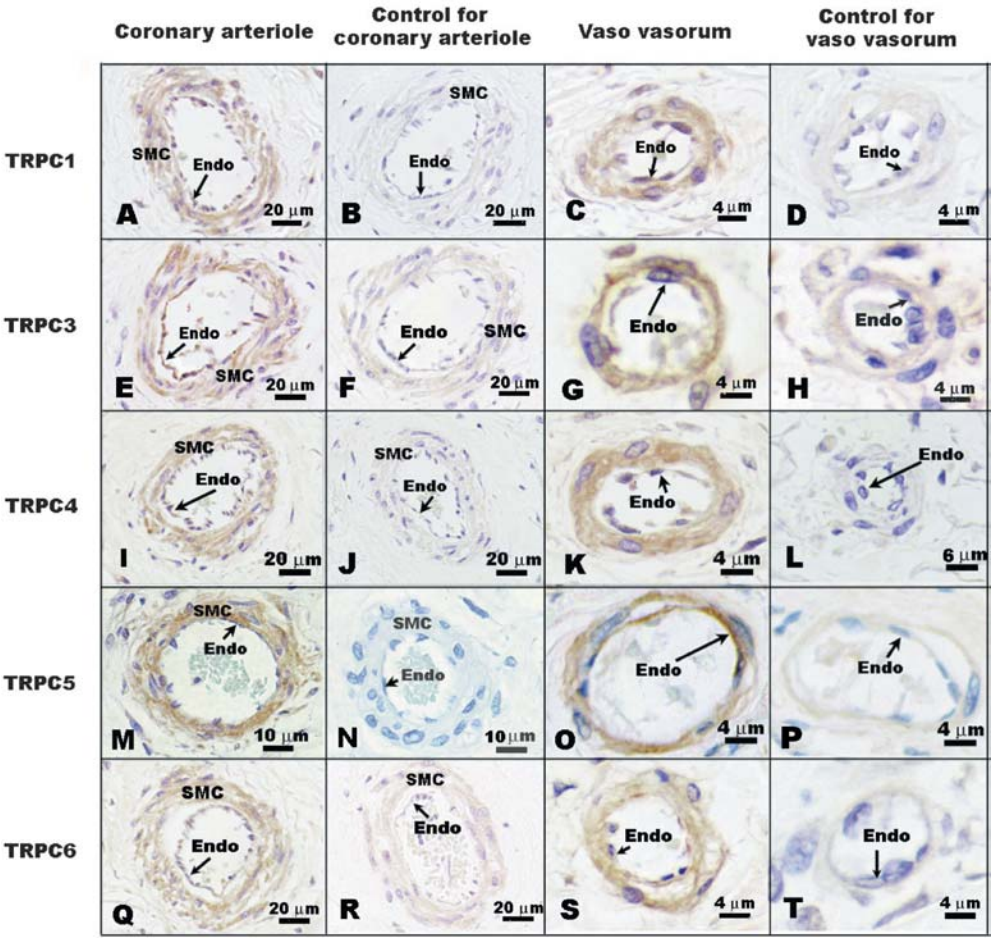

Fig. 5A–T Detection of TRPC proteins in human coronary arterioles and vaso vasora by immunohistochemical studies. **A–D** TRPC1. **E–H** TRPC3. **I–L** TRPC4. **M–P** TRPC5. **Q–T** TRPC6. Positive signals in brown color could be visualized in the endothelial and smooth muscle layers of coronary arterioles (**A**, **E**, **I**, **M**, **Q**) and vaso vasora (**C**, **G**, **K**, **O**, **S**). In control experiments, the primary antibodies were preadsorbed by TRPC1 peptide (**B**, **D**), TRPC3 peptide (**F**, **H**), TRPC4 peptide (**J**, **L**), TRPC5 peptide (**N**, **P**), or TRPC6 (**R**, **T**). Another set of control experiments was performed in the absence of the primary antibodies (data not shown). No positive signal could be observed in both sets of control experiments. Note that the blue color was due to hematoxylin counterstaining which stained cell nuclei

exception was TRPC7, which was exclusively expressed in vascular endothelial cells but not in smooth muscle cells. The expression of TRPC7 in endothelial cells may confer endothelial cells with unique functional properties, because TRPC7, compared to other TRPC homologs, displays different channel parameters such as a higher sensitivity to phospholipase C-linked agonist and unique pore properties (Okada et al. 1999). Our data on TRPC7 expression also appeared to agree with two previous studies, which reported the lack of TRPC7 expression in isolated blood vessels using the RT-PCR method (Facemire et al. 2004; Wang et al. 2004). Because the majority of cells in intact vessels are vascular smooth muscle cells, the failure in detecting TRPC7 expression using RNA isolated from intact vessels was consistent with the notion that TRPC7 was not expressed in vascular smooth muscle cells.

It is generally believed that functional TRPC channels are made of four TRPC subunits (Birnbaumer et al. 1996). These four subunits may consist of the same TRPC subtypes (homomultimeric) or different TRPC subtypes (heteromultimeric). Several studies have already established the ability of TRPC homologs to coassemble to form heteromultimers (Lintschinger et al. 2000; Strubing et al. 2001; Hoffmann et al. 2002). Likely coassembly could occur at least between TRPC1 and TPPC3 (Lintschinger et al. 2000), TRPC1 and TRPC4,5 (Strubing et al. 2001), and between the members within the same TRPC subgroups, for example, TRPC4,5 or TRP3,6,7 (Hoffmann et al. 2002). The results from our study demonstrated the expression of six different homologs of TRPC in human vascular endothelial cells and five homologs in smooth muscle cells. The coexistence of different TRPC homologs in a single cell type, together with the ability of different TRPC homologs to coassemble, suggests the possible existence of multiple forms of TRPC heterotetramers in vascular cells, which may confer the vascular cells with a variety of different Ca^{2+}-permeable channels with different properties. Unfortunately, in the present study we were not able to compare the relative expression of different TRPC homologs in the same cell type in intact vessels, as different antibodies might have different affinity to their respective antigens and the in situ hybridization riboprobes for different TRPC homologs were of different molecular sizes. Therefore, it would not be possible to compare the relative expression level based on the hybridization intensity generated by different riboprobes/antibodies. Several previous publications are available in which the quantitative RT-PCR technique was used to investigate the relative mRNA expression levels of different TRPC homologs in intact vascular tissues including arteries such as

rat aorta, rat and canine renal artery, and rat and canine pulmonary artery (Walker et al. 2001; Facemire et al. 2004). However, bear in mind that these results could not provide information on relative expression of TRPC homologs in the same cell type, because the RNA used for assay was isolated from intact vessels that contained multiple cell types. Apparently, further studies are needed to reveal the relative amount of TRPC mRNAs/proteins in the same cell types in intact vessels.

In conclusion, we have extensively studied the expression of TRPC homologs (TRPC1,3–7) in human vascular tissues. TRPC1,3–6 are coexpressed in vascular endothelial cells and vascular smooth muscle cells of human vessels with different diameters, whereas TRPC7 is exclusively expressed in vascular endothelial cells. The widespread distribution of multiple TRPC homologs suggests an important general role of TRPC channels in regulating Ca^{2+} influx, thereby influencing vascular tone in human vessels.

Acknowledgements This study was supported by Hong Kong Research Grant Council (CUHK4174/02 M) and France/Hong Kong Joint Research Scheme (F-HK11/01T).

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