Expression and relative abundance of short transient receptor potential channels in the rat renal microcirculation

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Facemire, Carie S., Peter J. Mohler, and William J. Arendshorst. Expression and relative abundance of short transient receptor potential channels in the rat renal microcirculation. Am J Physiol Renal Physiol 286: F546–F551, 2004. First published December 16, 2004; 10.1152/ajprenal.00338.2003.—In the resistance vessels of the renal microcirculation, store- and/or receptor-operated calcium entry contribute to the rise in vascular smooth muscle cell (VSMC) intracellular calcium concentration in response to vasoconstrictor hormones. Short transient receptor potential (TRPC) channels are widely expressed in mammalian tissues and are proposed mediators of voltage-independent cation entry in multiple cell types, including VSMCs. The seven members of the TRPC gene family (TRPC1–7) encode subunit proteins that are thought to form homo- and heterotetrameric channels that are differentially regulated depending on their subunit composition. In the present study, we demonstrate the relative abundance of TRPC mRNA and protein in freshly isolated rat renal resistance vessels, glomeruli, and aorta. TRPC1, 3, 4, 5, and 6 mRNA and protein were detected in both renal resistance vessels and aorta, whereas TRPC2 and TRPC7 mRNA were not expressed. TRPC1, 3, 5, and 6 protein was present in glomeruli. TRPC3 and TRPC6 protein levels were significantly greater in the renal resistance vessels, about six- to eightfold higher than in aorta. These data suggest that TRPC3 and TRPC6 may play a role in mediating voltage-independent calcium entry in renal resistance vessels that is functionally distinct from that in aorta.

Although L-type voltage-dependent calcium channels have been the focus of much study on the regulation of vascular reactivity, voltage-independent cation channels represent an important component of calcium entry in cells of the systemic and renal vasculature as well. In the preglomerular resistance vessels (interlobular arteries and afferent arterioles), non-L-type calcium entry contributes to vascular smooth muscle cell (VSMC) calcium responses to norepinephrine (6, 27) and vasopressin (8), vasoactive hormones that regulate renal blood flow and glomerular filtration rate. Store-operated calcium influx has been shown to negatively regulate renin secretion by juxtaglomerular cells (28). In VSMCs of conduit arteries such as aorta, calcium entry through store- and/or receptor-operated channels (SOCs, ROCs) is activated by norepinephrine and endothelin-1 (10, 30, 38). Calcium entry through these SOCs and ROCs also modulates vascular endothelial and glomerular mesangial cell function (20, 24, 26). These receptor-mediated calcium entry pathways have been incompletely characterized due to a lack of pharmacological agents able to discriminate between the function of SOCs and ROCs. Identification of the molecular components of these cation channels in renal resistance vessels would permit genetic manipulation and more detailed investigation into the regulation of these important calcium entry pathways.

The transient receptor potential (TRP) superfamily comprises a diverse group of ion channel proteins that are expressed in many tissues and cell types. Drosophila TRP, the first identified member of this gene family, functions as a light-sensitive, calcium-permeable nonselective cation channel essential to phototransduction (22, 40). Studies in Drosophila indicate that TRP is the target of a phosphoinositide signaling cascade downstream of a G protein-coupled receptor, suggesting that Drosophila phototransduction may be analogous to the widespread process of receptor-mediated calcium entry. These studies prompted efforts to identify vertebrate TRP homologs, which have recently been implicated in mediating diverse processes including olfaction, vasorelaxation, and responses to mechanical, chemical, and osmotic stimuli (22).

Members of the mammalian short (canonical) TRP (TRPC) family of channels, those most closely related to Drosophila TRP, have recently been implicated in mediating store- and receptor-operated calcium entry in various cell types, including vascular endothelial and smooth muscle cells (14, 24, 32, 36). These channels appear to be widely expressed in smooth muscles, and evidence suggests that the specific complement of TRPC proteins present may govern the mechanisms by which these channels are regulated in a given cell type (22, 40). TRPCs have been shown to mediate agonist-induced voltage-independent calcium entry in native VSMCs (14, 36) as well as myogenic tone in isolated resistance arteries (35). Aortic and lung vascular endothelial cells of TRPC4 knockout mice display significantly reduced store-operated calcium entry, confirming the role of TRPC4 in endothelial nitric oxide-mediated regulation of vascular tone and thrombin-induced increases in microvascular permeability (9, 32). Based on a growing body of evidence confirming the role of TRPCs in the regulation of endothelial and VSMC function, we investigated the presence of these channels in the renal microcirculation, as they represent exciting new targets in the study and treatment of renal and cardiovascular disease. In this study, we report the expression and relative abundance of the seven TRPC gene products in freshly isolated renal and nonrenal vascular tissues.

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MATERIALS AND METHODS

Tissue isolation. The iron oxide sieving technique, previously described in our laboratory (5, 15), was used to isolate preglomerular resistance vessels and glomeruli from 5-wk-old male Sprague-Dawley rats maintained in the Chapel Hill breeding colony. All procedures were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and in compliance with the guidelines and practices of the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee. Two rats were used for each preparation. The isolation of preglomerular vessels and glomeruli was performed essentially as described previously (5, 15), with the following exceptions. After the tissue was passed through a 100-μm sieve, the preglomerular vessels were further purified with another magnet separation and treated with collagenase (type I, Worthington Biochemical, Lakewood, NJ; 0.14–0.20 mg/ml in PBS) for 30 min at 35–37°C. The preparation was then passed through a 22-gauge needle to further separate vessels from tubule fragments, and a magnet was used to isolate the final preparation of preglomerular resistance vessels, consisting of short segments of interlobular arteries and afferent arterioles. Preglomerular resistance vessels and glomeruli were rinsed in PBS by centrifugation and resuspended in TRIReagent (Sigma, St. Louis, MO) or RNALater (Ambion, Austin, TX) for extraction of RNA or in Laemmli sample buffer (50 mM Tris-HCl, 100 mM dithiothreitol, 2% SDS, 10% glycerol) for SDS-PAGE.

Segments of thoracic aorta and brain (cerebrum) were obtained from rats immediately after removal of the kidneys. Aortic segments were quickly cleaned of excess connective tissue and then cut lengthwise. The luminal surface of the vessel was gently scraped with forceps to remove endothelium. Aorta and brain tissue were stored in RNAlater for extraction of RNA or ice-cold PBS. Aortic segments and brain tissue were stored in TRIReagent or RNAlater (Sigma, St. Louis, MO) or DNA-free (Ambion) to remove genomic DNA contamination. Total RNA was extracted from freshly isolated preglomerular resistance vessels, aorta, and brain using TRIReagent (Sigma) according to the manufacturer’s protocol. RNA was DNase treated using DNA-free (Ambion) to remove genomic DNA contamination. RNA yield was quantified by UV spectrophotometry, and integrity was verified by 1% agarose gel electrophoresis and staining with ethidium bromide. Only RNA with A260/280 > 1.7 and displaying no significant degradation was used for reverse transcription.

cDNAs were synthesized from 3 μg of total RNA using random hexamers and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). “No RT” samples lacking reverse transcriptase were prepared during each RT reaction for use as negative controls during PCR. cDNA templates and no RT samples were amplified by PCR for 35 cycles of 94°C for 1 min, 50–59°C for 1 min, and 72°C for 2 min; this was followed by a final extension step at 72°C for 10 min. PCR products were visualized by 1.5% agarose gel electrophoresis and stained with ethidium bromide.

Real-time quantitative PCR. Real-time quantitative PCR was performed using the fluorogenic 5′-exonuclease assay (12). TRPC primers and dual-labeled probes (5′-FAM, 3′-TAMRA) were designed from the coding regions of the TRPC genes using Primer3 software (Whitehead Institute, Massachusetts Institute of Technology) and prepared by the oligonucleotide synthesis core facility at the University of North Carolina at Chapel Hill (Table 1). PCR reactions were performed in duplicate on a RotorGene 3000 multiplexing system (Corbett Research, Sydney, Australia). cDNA and negative control (no RT, water) templates (1 μl) were added to a 20-μl multiplex PCR reaction mixture consisting of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM MgCl₂, 200 μM dNTPs, 1 U of Platinum Taq DNA polymerase (Invitrogen), and 0.2 nmol of each primer. cDNA templates and no RT samples were amplified by PCR for 35 cycles of 94°C for 1 min, 50–59°C for 1 min, and 72°C for 2 min; this was followed by a final extension step at 72°C for 10 min. PCR products were visualized by 1.5% agarose gel electrophoresis and stained with ethidium bromide.

Table 1. Oligonucleotide sequences of primers and dual-labeled probes used for real-time RT-PCR

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GenBank accession numbers represent sequences used in primer/probe design.

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with affinity-purified antibodies against specific TRPC proteins: rabbit anti-TRPC1 (90 kDa, antibody against QLYKDGYTSKEQKDC (residues 557–571); Alomone Labs, Jerusalem, Israel; 1:500), rabbit anti-TRPC3 (107 kDa, antibody against residues HKLSEKLNPSV-LRC (822–835); Alomone; 1:500), rabbit anti-TRPC4 (95–100 kDa, antibody against residues KEKHAHEEDSSIDYL (943–958); Alomone; 1:500), rabbit anti-TRPC5 (100 kDa, antibody against residues HKWGDGQEEQVTTRL; Alomone; 1:300), or rabbit anti-TRPC6 (105 kDa, against residues RINESQKULLMDELG (24–38); Alomone; 1:500). Blots were analyzed with antibody against β-actin (Sigma) as a loading control. Blots were washed, incubated with 125I-labeled protein A, washed again, and analyzed by phosphorimaging.

As we observed nonspecific bands using commercial antibodies with TTBS wash buffer, we changed our washing conditions, with the first washes in 150 mM NaCl, 10 mM phosphate buffer, 1 mM EDTA, 0.2% Triton X-100, and 1 mM NaN3. The blots were then washed in 150 mM NaCl, 10 mM phosphate buffer, 1 mM EDTA, 1 mM NaN3, 2 M urea, 0.2 M glycine, and 1% Triton X-100. Only using these conditions were we able to convincingly confirm distinct TRPC polypeptides at the correct molecular weight with little or no nonspecific bands. No bands corresponding to TRPC polypeptides were detected using rabbit nonimmune serum, and specific TRPC bands were absent when antibodies were preincubated with control antigen peptides.

Statistical analysis. Data are presented as means ± SE. SigmaStat (SPSS, Chicago, IL) software was used for statistical analysis. Statistical significance was evaluated by one-way ANOVA and Holm-Sidak post hoc test for multiple comparisons. Kruskal-Wallis one-way ANOVA on ranks was used to analyze data sets with nonnormal distributions. A value of \( P < 0.05 \) was considered significant.

RESULTS

RT-PCR analysis of brain, aorta, and renal preglomerular resistance vessels produced amplicons of the predicted size for each TRPC family member (Fig. 1). Transcripts for all TRPC genes were detected in brain, although TRPC2 was barely detectable. The low level of TRPC2 mRNA in brain was not surprising, because TRPC2 expression has only been demonstrated in a limited number of tissues, including mouse sperm and the rat vomeronasal organ (22). Both aorta and renal preglomerular resistance vessels express mRNA for TRPC1, 3, 4, 5, and 6, whereas TRPC2 and TRPC7 transcripts were not detected. Three splice variants of TRPC6 have been cloned in rat: TRPC6A (full length), TRPC6B (missing NH2-terminal 56 amino acids), and TRPC6C (missing NH2-terminal 56 amino acid and COOH-terminal 68 amino acids) (37). Therefore, the two amplicons observed in preglomerular resistance vessels likely represent different TRPC6 isoforms.

To determine the relative abundance of the five TRPC transcripts expressed in aorta and renal resistance vessels, real-time quantitative RT-PCR was performed using RNA from these freshly isolated tissues. Figure 2A shows that TRPC3 mRNA is at least threefold more abundant than TRPC1, TRPC5, and TRPC6, whereas TRPC4 expression is quite low in preglomerular resistance vessels. In aorta, TRPC1 and TRPC6 mRNAs predominate, showing significantly higher expression levels than TRPC3, 4, and 5 (Fig. 2B). Interestingly, there is no significant difference in mRNA expression of TRPC1, 5, and 6 between preglomerular resistance vessels and aorta, whereas TRPC3 mRNA is nearly seven times more abundant in the renal resistance vessels.

TRPC protein levels in aorta, glomeruli, and preglomerular resistance vessels were assessed by semiquantitative immunoblotting, which yielded bands of the appropriate molecular masses for all antibodies tested (TRPC1, 90 kDa; TRPC3, 107 kDa; TRPC4, 95–100 kDa; TRPC5, 100 kDa; TRPC6, 105 kDa; Fig. 3A, left). All bands were absent in control experiments where antibody was incubated with antigen peptide (Fig. 3A, right). Low levels of all TRPC polypeptides examined were detected in rat aorta (Fig. 3A). Significantly, we observed an approximately eightfold increase in TRPC3 expression in preglomerular resistance vessels compared with aorta (Fig. 3B). TRPC1 and TRPC6 protein levels are also significantly greater in preglomerular resistance vessels than in aorta, unlike the mRNA expression pattern. The pattern of glomerular TRPC expression is similar to that in aorta, with the exception of TRPC5, which is more abundant in glomeruli than in aorta.

DISCUSSION

TRPCs are known to be present in VSMCs of several vascular beds, including pulmonary artery (21, 23, 34), aorta (36), portal vein (14), renal artery (34), cerebral arteries (35), and pial arterioles (36). However, the relative abundances of the seven TRPC family members have not been determined quantitatively in any freshly isolated resistance vessels. The only evidence for TRPC expression in the renal microcirculation consists of immunohistochemical localization of TRPC1 in rat afferent and efferent arterioles; the presence of other
TRPC proteins was not investigated in this study (29). In our study, we present a comprehensive characterization of TRPC expression in freshly isolated resistance and conduit vessels of the renal and systemic circulations as well as in glomeruli. Based on these mRNA and protein expression data, it is clear that TRPC3 is the predominant TRPC gene product expressed in renal preglomerular resistance vessels and that TRPC6 is also abundant. This expression pattern differs markedly from that in aorta, where TRPC1 and TRPC6 predominate, although at lower protein levels than those found in renal resistance vessels.

We cannot exclude the possibility that some TRPC expression observed in the preglomerular resistance vessels is contributed by endothelial cells, because the fresh renal resistance vessel preparation used in these studies contains not only VSMCs but also vascular endothelial cells. However, we can be reasonably certain that the expression pattern observed in pregglomerular resistance vessels is primarily due to VSMC TRPC expression, because there are no reports of elevated TRPC3 or TRPC6 in freshly isolated vascular endothelial cells, where TRPC1 and TRPC4 appear to predominate (1, 11, 18). Our belief that TRPC levels in the preglomerular resistance

Fig. 2. Relative TRPC1, 3, 4, 5, and 6 mRNA expression levels in renal PRVs, aorta, and brain, determined by real-time quantitative RT-PCR. A: relative abundance of TRPC transcripts in PRVs. *P < 0.01 vs. TRPC1, 4, 5, and 6. B: relative comparison of TRPC mRNA levels in brain (positive control, gray bars), aorta (open bars), and renal PRVs (filled bars). TRPC expression was normalized to 18S ribosomal RNA (18S rRNA) expression in the same template. Experiments were repeated using fresh tissue preparations from 4 different sets of animals (n = 4). Data are means ± SE. Statistical analysis was performed using 1-way ANOVA and Holm-Sidak method for multiple comparisons. *P < 0.05 vs. aorta TRPC3, 4, and 5.

Fig. 3. TRPC protein expression in renal and nonrenal vascular tissues. Immunoblotting experiments were performed on cell lysates prepared from freshly isolated rat aorta, glomeruli, and PRVs. A: representative blots for TRPC1, 3, 4, 5, and 6 show bands of 90, 107, 93, 100, and 105 kDa, respectively, for all antibodies tested (left). Bottom, left: β-actin was used as a protein loading control. Right: blots using antibodies in the presence of control antigen peptides. B: relative TRPC protein levels in renal PRVs (filled bars), glomeruli (gray bars), and aorta (open bars), determined by phosphorImaging of 125I-labeled blots. TRPC protein levels were corrected for β-actin and expressed as fold expression relative to aorta. Experiments were repeated using tissues from 3 different sets of animals (n = 3). Data are means ± SE. Statistical analysis was performed using 1-way ANOVA and Holm-Sidak method for multiple comparisons. *P < 0.05 vs. aorta (open bars).
vessel preparation are a reflection of VSMC TRPC expression is supported by immunofluorescence localization of TRPC3 and TRPC6 in cerebral resistance arteries (35). In this study, the pattern of TRPC3 and TRPC6 immunofluorescent labeling is consistent with intact VSMC morphology. The freshly isolated glomerular preparation used in our study also contains multiple cell types, including glomerular mesangial and endothelial cells as well as podocytes. There is functional evidence for the presence of SOC and/or ROCs in cultured mesangial cells (20, 26) and a few studies that suggest non-L-type channels may be activated by vasoactive agonists in podocytes (13, 25). However, compelling evidence for these calcium entry pathways in glomerular endothelial cells is lacking. It is therefore probable that TRPC expression in glomeruli is contributed primarily by mesangial cells and possibly podocytes, where these proteins likely mediate store- and/or receptor-operated calcium entry.

In preglomerular resistance vessels, TRPC1, 3, 4, and 5 protein levels correlate well with the observed mRNA expression pattern. However, TRPC6 protein levels are much greater than in aorta, whereas TRPC6 mRNA levels are similar between the two tissues (Figs. 2B and 3B). Because the primers used for quantitative RT-PCR in the present study amplify three TRPC6 splice variants (TRPC6A, B, and C), but the TRPC6 antibody used does not recognize TRPC6C, the observed mRNA/protein discrepancy could reflect a difference in TRPC6C expression between aorta and renal resistance vessels rather than a difference in total TRPC6 protein abundance. Alternatively, TRPC6 mRNA and/or protein may display increased stability in preglomerular resistance vessels, suggesting that TRPC6 mRNA is subject to posttranscriptional and/or posttranslational modification via an unknown regulatory mechanism.

The abundance of TRPC3 in preglomerular resistance vessels compared with that in aorta suggests an important role for TRPC3 in the renal resistance vessels that may be absent in larger conduit arteries. TRPC3 mRNA has been detected in freshly isolated VSMCs from main renal artery, but its abundance relative to other TRPCs was not determined (34). To date, a functional role for endogenous TRPC3 has been demonstrated only in the brain, where it appears to be involved in brain-derived nerve growth factor-induced neuronal differentiation and plasticity (19). Although there is evidence that TRPC3 expressed in DT40 cells can be activated by store depletion, the majority of data support a receptor-operated mechanism of activation (33). Activation of TRPC3 by diacylglycerol (DAG) has been confirmed by several groups using different in vitro expression systems (33). Several studies also suggest that TRPC3 activity may be regulated through interactions with the inositol 1,4,5-trisphosphate receptor (2, 16, 17, 30) and calmodulin (31, 39).

TRPC6 is also reported to form a ROC that is activated by DAG analogs in vitro (33). Such an activation mechanism appears to be absent for the TRPC6B splice variant, suggesting that a sequence contained in the 56 amino acids missing from the TRPC6B NH2 terminus is responsible for activation by DAG (37). Interestingly, protein kinase C appears to negatively regulate TRPC6 channel function in vitro (37) and is also thought to have a similar action on TRPC3 (33). Inoue et al. (14) recently reported that TRPC6 is a component of the α1-adrenoceptor-activated nonselective cation channel in rabbit portal vein myocytes, which appears to function as a store-independent calcium entry pathway.

Based on the current literature and the unique TRPC expression pattern observed in renal preglomerular resistance vessels, we speculate that TRPC3 and TRPC6 may form functional ROCs in VSMCs of the renal microcirculation. Our findings are significant in that this TRPC expression pattern appears to be specific to either renal vasculature or to small-diameter resistance vessels, or perhaps both. These data provide a basis for further functional studies investigating the physiological regulation of voltage-independent calcium entry pathways in preglomerular VSMCs.

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