Heteromultimeric TRPC6-TRPC7 Channels Contribute to Arginine Vasopressin-Induced Cation Current of A7r5 Vascular Smooth Muscle Cells

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Abstract—The molecular identity of receptor-operated, nonselective cation channels (ROCs) of vascular smooth muscle (VSM) cells is not known for certain. Mammalian homologues of the Drosophila canonical transient receptor potential channels (TRPCs) are possible candidates. This study tested the hypothesis that heteromultimeric TRPC channels contribute to ROC current of A7r5 VSM cells activated by [Arg⁸]-vasopressin. A7r5 cells expressed transcripts encoding TRPC1, TRPC4β, TRPC6, and TRPC7. TRPC4, TRPC6, and TRPC7 protein expression was confirmed by immunoblotting and association of TRPC6 with TRPC7, but not TRPC4β, was detected by communoprecipitation. The amplitude of arginine vasopressin (AVP)-induced ROC current was suppressed by dominant-negative mutant TRPC6 (TRPC6DN) but not TRPC5 (TRPC5DN) mutant subunit expression. These data indicate a role for TRPC6- and/or TRPC7-containing channels and rule a more complex subunit composition including TRPC1 and TRPC4. Increasing extracellular Ca²⁺ concentration ([Ca²⁺]₀) from 0.05 to 1 mmol/L suppressed currents owing to native, TRPC7, and heteromultimeric TRPC6-TRPC7 channels, but not TRPC6 current, which was slightly enhanced. The relative changes in native and heteromultimeric TRPC6-TRPC7 current amplitudes for [Ca²⁺]₀, between ~0.01 and 1 mmol/L were identical, but the changes in homomultimeric TRPC6 and TRPC7 currents were significantly less and greater, respectively, compared with the native channels. Taken together, the data provide biochemical and functional evidence supporting the view that heteromultimeric TRPC6-TRPC7 channels contribute to receptor-activated, nonselective cation channels of A7r5 VSM cells. (Circ Res. 2006;98:1520-1527.)

Key Words: TRPC6 ■ TRPC7 ■ receptor-operated cation channel ■ vascular smooth muscle

Activation of G protein-coupled receptors (GPCRs) of vascular smooth muscle (VSM) cells by a variety of vasoconstrictor agonists causes an elevation in [Ca²⁺]₀, and contraction owing to depolarization and Ca²⁺ influx from the extracellular space, Ca²⁺ release from internal stores and Ca²⁺ sensitization of contractile filaments.¹⁻⁴ The depolarization and influx of Ca²⁺ evoked by GPCRs is attributable in part to the activation of receptor-operated, nonselective cation channels (ROCs) by a signaling pathway involving phospholipase and diacylglycerol (reviewed previously³⁻⁶). The molecular basis of VSM ROCs is not known with certainty, but accumulating evidence suggests that transient receptor potential channel (TRPC) family subunits (TRPC1 to TRPC7)⁷⁻⁹ are likely involved.³⁻⁶ Moreover, ROCs owing to heterologous expression of TRPC3, TRPC6, or TRPC7 are activated by a similar mechanism involving phospholipase and diacylglycerol.¹⁰⁻¹² Understanding the molecular basis of VSM ROCs is clearly warranted in light of their important role in control of VSM excitability and contractility⁴⁻⁶ and evidence of changes in TRPC expression associated with abnormal contractility and/or VSM cell proliferation.¹³⁻¹⁵

Identifying the contribution of TRPC subunits to VSM ROCs has been compromised by a lack of specific/selective pharmacological blockers. For this reason, alternative strategies involving antibodies, anti-sense or small interfering RNAs (siRNAs) were used to indicate roles for: (1) TRPC1 as store-operated channels¹⁶; (2) TRPC3 as ROCs³⁷; (3) TRPC6 as ROCs and/or mechanosensitive channels¹¹,¹⁸⁻²⁰; (4) TRPV2 as mechanosensitive channels²¹; (5) TRPM4 as mechanosensitive channels²²; and (6) TRPV4 in the stimulation of focal Ca²⁺ release (“sparks”) from sarcoplasmic reticulum Ca²⁺ stores.²³ In some cases, the biophysical and/or pharmacological properties of heterologously expressed TRPC and native VSM ROCs have been compared. It is significant, however, that the properties of homomultimeric TRPC are similar, but not necessarily identical, to those of native ROCs.⁴⁻⁶,¹¹,¹²,¹⁴,¹⁸⁻²⁰. For example, antisense and siRNA experiments indicate that TRPC6 is an important
component of α1-adrenergic receptor– and arginine vasopressin (AVP) V1α receptor–induced ROC currents of portal vein11 and A7r5 cultured VSM cells,18,19 respectively, as well as cation current activated by diacylglycerol in rat cerebral myocytes.20,22 However, the Ca2⁺-sensitivity of these channels is markedly different; specifically, increasing [Ca2⁺]o from 0.05 to 2 mmol/L suppressed native ROC currents11,18,25 but increased in TRPC6 current.12 Additionally, as noted by Beech et al,4 the current-voltage (I-V) relations of TRPC6-containing cation channels of portal vein,11 cerebral,20,24 and pulmonary arterial14 myocytes and A7r5 VSM cells18 are not identical. The molecular basis of these differences in functional properties is presently unknown.

Previous results indicate that multiple types of TRPC subunits are expressed at varied levels by VSM cells of different vessels in a species-dependent manner.3,4,6,13,14,18,19,27 Thus, VSM ROCs may have a subunit composition that varies in a vessel-dependent fashion, different agonists may activate ROCs composed of different subunits, and/or a given agonist may activate multiple ROCs with varied subunit composition.3,26 VSM ROC could also result from the heteromultimeric assembly of more than 1 type of TRPC subunit.4–6 Coassociation of different voltage-gated K⁺ channel subunits is an established principle, and it occurs in many cell types, including VSM cells.28,29 Expression of heteromultimeric TRPC6-containing channels with unique properties provides a viable explanation for the lack of complete functional identity of homomultimeric TRPC6 and native ROCs, as well as the varied properties of TRPC6-containing ROCs in different VSM cells.4–6 TRPC3 and TRPC6 antisense oligonucleotides both inhibit ROC currents of rat prostate smooth muscle cells,30 providing indirect evidence for the presence of heteromultimeric channels. However, direct biochemical evidence of TRPC heteromultimerization, such as that shown for heterologous expression systems and brain tissues,31–34 has not been obtained for VSM cells.

Here we tested the hypothesis that heteromultimeric TRPC channels contribute to ROCs of A7r5 VSM cells. This aortic smooth muscle cell line was specifically used because it permitted a detailed analysis of the biochemical and electrophysiological properties of the ROCs that would otherwise not be possible using native cells because of the low level of channel expression and relatively low affinity of currently available subunit-specific antibodies. TRPC expression and heteromultimerization was assessed by RT-PCR, immunoblotting, and coimmunoprecipitation. The participation of TRPC-containing ROCs was addressed using a dominant-negative strategy,32,34 and the sensitivity of the native ROCs and homo- and heteromultimeric TRPC to [Ca2⁺]o, was compared. Our results provide the first direct evidence that heteromultimeric TRPC channels can contribute to ROCs activated by GPCRs in VSM cells.

**Materials and Methods**

Human embryonic kidney 293 (HEK293; passage 5 to 17) and A7r5 VSM cells (passage 10 to 22) (American Type Culture Collection, Manassas, Va) were used. Both cell types were maintained in DMEM (Invitrogen [Gibco-BRL], Burlington, Canada) supplemented with 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). HEK293 cells grown on glass coverslips were transfected with cDNAs encoding 0.5 μg of angiotensin AT1 receptor, 0.5 μg of green fluorescent protein (GFP), and varied amounts of TRPC subunit cDNAs using FuGENE 6 (Roche Diagnostics Canada, Laval) and used within 24 to 48 hours. Eighty percent confluent cultures of A7r5 cells were transfected with cDNAs encoding 0.5 μg of GFP, 1.5 μg of TRPC subunit cDNAs, or equal amounts of empty vector pcDNA3 (mock transfection). The A7r5 cells were plated onto coverslips 48 hours after transfection and used within 24 hours. The methods for whole-cell voltage clamp, immunocytochemistry, immunoprecipitation, immunoblotting, and/or molecular biology were as previously described28–29 and/or as indicated in the online data supplement. All primers used are included in the online data supplement available at http://circres.ahajournals.org.

**Results**

**TRPC Transcript Expression**

TRPC message expression was analyzed by RT-PCR using subunit-specific primers and mRNA from A7r5 cells and rat brain; the latter used to confirm primer function and reaction integrity (Figure 1A). TRPC1, TRPC4, TRPC6, and TRPC7 amplicons were detected in A7r5 cell mRNA (Figure 1B) and confirmed by sequencing. TRPC3 and TRPC5 expression was not detected but was consistently apparent for rat brain (Figure 1A). Alternate primer sets for TRPC3, TRPC4α/ TRPC4β, and TRPC7 were also used. TRPC3 (data not shown) and TRPC4α (Figure 1C) were not detected, but amplicons for TRPC4β (245 base pairs [bp]; Figure 1C) and TRPC7 (649 bp; Figure 1D) were identified (additional = 300 and 400 bp amplicons in Figure 1C did not correspond to any known gene).
TRPC Protein Expression and Coassembly
We probed for TRPC1 protein expression using anti-TRPC1 and detected α-145 kDa protein (data not shown), as noted previously.35 The presence of TRPC4β, TRPC6, and TRPC7 in lysates of A7r5 cells was also detected. Anti-c-myc immunoreactive bands were evident in HEK293 cells expressing full-length c-myc–tagged TRPC4e and the smaller TRPC4β between ∼105 and 100 kDa (Figure 2A). An immunoreactive band of ∼100 kDa and consistent with TRPC4β was detected with anti-TRPC4 in A7r5 (Figure 2A) and HEK293 (not shown) cell lysates. Anti-TRPC6 detected multiple bands between ∼100 to 130 kDa in lysates of HEK293 cells expressing TRPC6, likely corresponding to monomeric and glycosylated subunit,36 as well as protein at ∼125 kDa in A7r5 cell lysates (Figure 2B). These bands were not evident in lysates of non- or mock-transfected (empty vector) HEK293 cells (not shown). Anti-TRPC7 failed to detect protein in mock-transfected HEK293 cells or cells transfected with TRPC6, but multiple immunoreactive bands between ∼100 to ∼125 kDa were identified in lysates of cells expressing TRPC6 and TRPC7 (Figure 2C). An immunoreactive band of ∼100 kDa was detected in A7r5 cell lysates using anti-TRPC7 (Figure 2C).

Figure 2D shows the results of communoprecipitation experiments using anti-TRPC6 followed by immunoblotting with antibodies against TRPC6, TRPC4, or TRPC7. TRPC6 immunoreactive protein was detected between ∼110 and 130 kDa in anti-TRPC6 immunoprecipitates of HEK293 cells expressing TRPC6 (Figure 2Da). No evidence of immunoreactive protein corresponding to TRPC4 at ∼100 kDa was identified by anti-TRPC4 in anti-TRPC6 immunoprecipitates of A7r5 cells, but nonspecific bands were apparent at ∼72 to 80 kDa and ∼130 kDa (closed circles; Figure 2Db). TRPC7 immunoreactive protein was detected at ∼100 kDa in anti-TRPC6 immunoprecipitates (Figure 2Dc) corresponding to the ∼100 kDa band present in immunoblot of A7r5 and TRPC7-expressing HEK293 cells, indicating the association of these subunits in A7r5 cells (Figure 2C). Nonspecific bands similar to those in Figure 2Db were also evident in TRPC7 immunoblots of anti-TRPC6 immunoprecipitates.

Dominant-Negative Suppression of AVP-Induced ROC Current
The contribution of TRPC channels to AVP-induced ROC current of A7r5 cells was assessed using dominant-negative pore mutants of TRPC6 (TRPC6DN) and TRPC5 (TRPC5DN) with C- and N-terminal c-myc and hemagglutinin (HA) tags, respectively. The mutants were tested for specificity in HEK293 cells (see online data supplement). Anti-c-myc immunofluorescence was evident in GFP-positive A7r5 cells transfected with TRPC6DN but not mock-transfected cells (Figure 3A). AVP caused a marked increase in N-methyl-D-glutamine (NMGD)-sensitive cation current at −60 mV in GFP-positive A7r5 cells expressing empty vector (Figure 3B) but consistently less current in GFP-positive cells of sister culture dishes expressing TRPC6DN (Figure 3D) and 3G; 18.2±4.8 [n=15] versus 5.4±2.4 pA/pF [n=16]; P<0.02). In contrast, AVP-induced current amplitude was not different in GFP-positive cells of sister dishes transfected with empty vector or TRPC5DN (Figure 3F and 3G; 5.3±1.7 [n=15] versus 3.0±1.4 pA/pF [n=11]; P=0.15). The I-V relationship of AVP-induced current in mock-transfected (n=14) and in 9 TRPC6DN-transfected A7r5 cells (with >1.0 pA/pF current) was doubly rectifying (Figure 3C and 3E). Increasing [Ca2+]o from 0.05 to 1 mmol/L reduced current in mock- and TRPC6DN-transfected cells, particularly between −100 and 0 mV (Figure 3B through 3E). The relative change in current at
Effect of \([\text{Ca}^{2+}]_o\) on Native and Recombinant Nonselective Cation Currents

The effect of increasing \([\text{Ca}^{2+}]_o\) on native ROCs was compared with that of homo- and heteromultimeric TRPC6 and TRPC7 channels. TRPC3 and TRPC3-TRPC6 channels were also considered because of the expression of TRPC3 (rather than TRPC7) with TRPC6 in some vessels.\(^4\)\(^6\)\(^7\) Ang II was used as the agonist for the recombinant channel experiments because of availability of cDNAs encoding the AT₁ receptor and justified by the lack of a difference in AVP- and Ang II-induced ROC currents of A7r5 cells (see online data supplement) and previous reports that similar A7r5 cell ROC currents are activated by serotonin, PDGF, AlF₄⁻, and OAG.\(^5\)\(^9\) Ang II–induced ROC currents were observed in HEK293 cells expressing TRPC3, TRPC6, and TRPC7 alone or in combination (Figure 4B through 4F). The I-V relations for homo- and heteromultimeric TRPC combinations all doubly rectified (Figure 4B through 4F), consistent with previous studies.\(^1\)

Increasing \([\text{Ca}^{2+}]_o\) caused a rapid inhibition of native current (Figure 4A), as well as currents owing to TRPC7 and TRPC3 (Figure 4C and 4D) and heteromultimeric TRPC3-TRPC6 and TRPC6-TRPC7 channels (Figure 4E and 4F). In contrast, TRPC6 current amplitude increased immediately on the change in \([\text{Ca}^{2+}]_o\), and subsequently declined to a maintained level that was not different from that recorded before the solution change (Figure 4B). The differing effects of \([\text{Ca}^{2+}]_o\) on current amplitude were most apparent between -100 and 0 mV (Figure 4G).

A more detailed analysis of the effects of \([\text{Ca}^{2+}]_o\) on current amplitude was performed for native A7r5 ROCs, TRPC6, TRPC7, and TRPC6-TRPC7 channels using the approach of Helliwell and Large.\(^25\) Currents were evoked in 1 mmol/L \([\text{Ca}^{2+}]_o\), before switching to nominally \([\text{Ca}^{2+}]_o\)–free, 0.05, 0.1, or 0.3 mmol/L \([\text{Ca}^{2+}]_o\) solutions (Figure 5). TRPC7 currents showed the greatest increase in peak inward current when \([\text{Ca}^{2+}]_o\) was lowered from 1 mmol/L (Figure 5). In contrast, TRPC6 current was only slightly enhanced by decreasing \([\text{Ca}^{2+}]_o\), and consistently exhibited a slight increase in amplitude on subsequent reexposure to 1 mmol/L \([\text{Ca}^{2+}]_o\). The mean changes in current amplitude at each \([\text{Ca}^{2+}]_o\) were determined by normalizing peak amplitude after the change in \([\text{Ca}^{2+}]_o\), to the value in 1 mmol/L \([\text{Ca}^{2+}]_o\), and plotted as a function of \([\text{Ca}^{2+}]_o\) (Figure 6). The relative change in amplitude of homomultimeric TRPC6 and TRPC7 currents were less and greater, respectively, compared with that of the native ROCs (Figure 6). In contrast, the change heteromultimeric TRPC6-TRPC7 current mimicked that of the native A7r5 cell ROCs (Figure 6).

Discussion

This study provides the first direct evidence that heteromultimeric TRPC channels can contribute to ROCs of VSM cells. At least 4 distinct types of cation channels are present in smooth muscle cells, including store-operated, ROC, stretch-activated, and tonically active cation channels, but the molecular identity of each conductance is a matter of debate.\(^4\)\(^6\)\(^37\) These channels are thought to contribute to several physiological functions of VSM cells, including agonist- and stretch-induced depolarization, maintenance of SR \(\text{Ca}^{2+}\) store filling, and mediating \(\text{Ca}^{2+}\) influx in response to ligands and physical stimuli (eg, stretch).\(^4\)\(^6\)\(^3\) Understanding the molecular basis of VSM ROCs is of particular interest in light of the important contribution of these channels to the actions of vasoconstrictors,\(^1\)\(^6\) eg, \(\alpha_1\)-adrenoceptor agonists, Ang II, and endothelin-1, that are well known to contribute to vascular pathology and the recognition that alterations in TRP channel expression, including TRPC subunits, is associated with abnormal control of vascular contractility and proliferation.\(^1\)\(^3\)\(^1\)\(^5\) Multiple TRPC subunits, as well as members of TRPV and TRPM families, have been shown to be expressed by VSM cells in a tissue- and species-specific pattern.\(^4\)\(^6\)\(^1\)\(^3\)\(^1\)\(^2\)\(^7\) For example, A7r5 cells were found to express TRPC1 and TRPC6\(^1\)\(^8\) and, more recently, TRPC3, TRPC4, TRPC5 and/or

Figure 3. A, Lack of c-myc immunofluorescence in GFP-positive A7r5 cell transfected with cDNAs encoding GFP and empty vector (A7r5 Mock) but present in cell expressing tagged-TRPC6\(^1\) (A7r5 TRPC6DN-myc). B, Representative AVP-induced ROC current of mock-transfected A7r5 cell. C, Representative current of mock-transfected A7r5 cell between -100 and +100 mV at times (○; ●) indicated in B. D, Representative AVP-induced ROC current in A7r5 cell transfected with TRPC6\(^1\)D. E, Representative current of TRPC6\(^1\)D-transfected A7r5 cell between -100 and +100 mV at times (○; ●) in D (left) and mean fractional current amplitude at -100 and +100 mV in 1.0 ○/100 nm Ang II, and +100 mV at times (○; ●) in D (left) and mean fractional current amplitude at -100 and +100 mV in 1.0 ○/100 nm Ang II and medially current density (at -60 mV) in A7r5 cells expressing empty vector (n=9) A7r5 cells (right). F, Representative AVP-induced current of TRPC5\(^1\)D-transfected A7r5 cell. G, Mean AVP-induced current density (at -60 mV) in A7r5 cells expressing empty vector (n=15) or TRPC5\(^1\)D (n=11) and empty vector (n=15) or TRPC6\(^1\)D (n=16). Note current suppression by TRPC6\(^1\)D (P<0.05) but not TRPC5\(^1\)D (P=0.15).
TRPC7, depending on the primers used and/or specific strain of A7r5 cells studied,\textsuperscript{19,38} a pattern of expression that is apparent in several arteries and veins.\textsuperscript{4} Here we show the expression of transcripts encoding the short TRPC4 splice variant of TRPC4, as well as TRPC1, TRPC6, and TRPC7, in A7r5 cell mRNA extracts, and the presence of these proteins was confirmed by immunoblotting. Additionally, we provide biochemical and electrophysiological evidence of coassembly of TRPC6 and TRPC7 and functional identity of native ROC and TRPC6-TRPC7 heteromultimeric channels in terms of Ca\textsuperscript{2+} sensitivity, respectively. These observations support the view that VSM ROC current may be the result of expression and association of multiple TRPC subunits.

The evidence that TRPC6 and TRPC7 coassemble and contribute to the AVP-induced ROCs of A7r5 cells may be summarized as follows. First, TRPC6 and TRPC7 message and protein expression were detected by RT-PCR and immunoblotting using subunit-specific primers and antibodies with a specificity demonstrated in previous studies\textsuperscript{33} and were confirmed here using HEK293 cells expressing TRPC6 and/or TRPC7. Second, direct biochemical evidence of TRPC subunit coassembly was obtained in experiments in which anti-TRPC7 immunoreactive protein was coimmunoprecipitated from lysates of A7r5 cells using anti-TRPC6, similar to the association previously identified to occur in synaptosomes derived from adult rat brain.\textsuperscript{33} Third, a dominant-negative strategy demonstrated the involvement of TRPC subunits in the response of A7r5 cells to AVP. The strategy is based on the assumption that mutant subunits with the conserved leucine-phenylalanine-tryptophan channel pore motif replaced with an alanine triplet (AAA) coassemble with wild-type subunits and suppress cation permeation through the resultant heteromultimeric channels.\textsuperscript{32} Using this approach, Hofmann et al.\textsuperscript{32} concluded that the TRPC family divides into 2 subgroupings capable of heteromultimerization: TRPC3, TRPC6, and TRPC7 versus TRPC1, TRPC4, and TRPC5. Here we found a significant dominant-negative suppression of AVP-induced A7r5 cell ROC current by TRPC6\textsuperscript{DN} but not TRPC5\textsuperscript{DN}. In the absence of TRPC3 expression, this result indicates the potential involvement of homo- and/or heteromultimeric TRPC6 and TRPC7 channels. Recent observations indicate that TRPC coassembly may be more complex than that described by Hofmann et al.,\textsuperscript{32} with interactions between members of the subgroups being possible.\textsuperscript{34} For example, TRPC3 or TRPC6 in combination with TRPC1, TRPC4, or TRPC5 were identified in lysates of embryonic rat brain and a critical role for TRPC1 as a mediator of cross-grouping interactions was indicated.\textsuperscript{34} In

Figure 4. Representative effects of increasing [Ca\textsuperscript{2+}]\textsubscript{o} from 0.05 to 1 mmol/L on AVP- or Ang II-induced ROC currents at 0 mV (top) and between 100 to +100 mV at times indicated (\(\Delta\), \(\triangle\)) (bottom) in an A7r5 cell (A) and HEK293 cells expressing TRPC6 (B), TRPC3 (C), TRPC7 (D), TRPC6 and TRPC7 (E), or TRPC6 and TRPC3 (F). G, Mean fractional (1.0 \(\Delta\)/0.05 \(\triangle\) mmol/L [Ca\textsuperscript{2+}]\textsubscript{o}) current amplitude at 100 and +100 mV in A7r5 (n=9) and HEK293 cells expressing TRPC3 (n=3), TRPC6 (n=5), TRPC7 (n=10), TRPC3-TRPC6 (n=4), or TRPC6-TRPC7 (n=8).
light of these findings and the expression of TRPC1 and TRPC4/H9252 by the A7r5 cells used here, the possibility that A7r5 cell ROCs may have a more complex subunit composition was considered. We probed for an association of TRPC4/H9252 with TRPC6 but were unable to detect the presence of TRPC4 protein in anti-TRPC6 immunoprecipitates. It is possible that the channel density and/or antibody affinity was too low to permit detection or that the antigenic sites on the channel protein were inaccessible when in a heteromultimeric complex.33 However, we also failed to observe a suppression of ROC current in A7r5 cells expressing TRPC5 DN and suppression of TRPC1 expression by A7r5 cells using an anti-sense oligonucleotide approach has no effect on AVP-induced ROC currents (L.I. Brueggman and K.L. Byron, personal communication). Taken together, these data imply that channels containing TRPC6, TRPC7, and TRPC1 alone or with TRPC4 likely do not contribute to AVP-induced ROCs of A7r5 cells.

The fourth line of evidence supporting the view that heteromultimeric ROCs are activated by AVP in A7r5 cells was obtained in experiments that compared the effects of [Ca^{2+}]_o on A7r5 cell ROCs versus homo- and heteromultimeric TRPC channels. Previous studies using TRPC6 anti-sense oligonucleotides or siRNAs imply that TRPC6 is a component of portal vein, cerebral arterial, and A7r5 VSM cell ROCs, as these approaches resulted in a suppression of agonist- and/or OAG-induced induced ROC currents.11,18,19,20,24 However, the previously reported 1.5 increase in TRPC6 current amplitude caused by increasing [Ca^{2+}]_o from between 0.2 to 0.5 to between 1 to 2 mmol/L11,12 contrasts markedly with the rapid inhibition observed for native VSM ROC currents of portal vein and A7r5 cells evoked by α-adrenoceptor agonists and AVP, respectively.11,18,25 This difference in behavior cannot be attributed to variations in the [Ca^{2+}]_o sensitivity of the mechanism of channel activation, as identical responses have been reported for TRPC6, TRPC7, and native currents activated by a diverse set of compounds.6,11,12,18 Rather, the results of this study indicate that the difference in functional identity between TRPC6 and these VSM ROCs can be attributed to the presence of multiple pore-forming subunit(s) in the channel complex. Despite sharing considerable sequence identity with TRPC6 subunits, TRPC3/H9253 and TRPC7/H9251/H11015 channels are inhibited by changing [Ca^{2+}]_o from nominally Ca^{2+} free to 1 mmol/L, confirmed here by stepping [Ca^{2+}]_o from 0.05 to 1 mmol/L and from 1 mmol/L to between nominally Ca^{2+} free and 0.3 mmol/L. Moreover, we show for the first time that heteromultimeric TRPC3-TRPC6 and TRPC6-TRPC7 chan-

![Figure 5](image-url)  
**Figure 5.** Representative effect of decreasing [Ca^{2+}]_o from 1 mmol/L to nominally Ca^{2+} free, 0.05, 0.1, or 0.3 mmol/L on ROC current (at −60 mV) of A7r5 cell (A) or HEK293 cells expressing TRPC6 and TRPC7 (B), TRPC6 (C), or TRPC7 (D).

![Figure 6](image-url)  
**Figure 6.** Mean relative change in ROC current amplitude in A7r5 cells (n=3 to 6) and HEK293 cells expressing TRPC6/ TRPC7, TRPC6, or TRPC7 (n=3 to 5) at [Ca^{2+}]_o between nominally Ca^{2+} free to 0.3 mmol/L (○) normalized to amplitude in 1 mmol/L [Ca^{2+}], (▲) based on experiments as shown in Figure 5.
TRPC1, TRPC4, TRPC6, and TRPC7 mRNAs were previously shown to be expressed together in canine pulmonary and renal arteries, and TRPC6 and TRPC7 were both detected in rat thoracic and cerebral arteries. These findings are consistent with our data showing the presence of TRPC6 and TRPC7 transcripts in mRNA derived from isolated rat cerebral myocytes, an approach that precludes false-positive identification of subunit expression attributable to contamination by message derived from endothelial cells, blood cells, and/or fibroblasts present in the vessel wall. Taken together, these findings provide molecular evidence consistent with the view that TRPC6- and TRPC7-containing ROCs may also be expressed by native VSM cells within some vessels. However, based on the reported variability in properties of native VSM ROC currents, TRPC6-TRPC7 channels may not be the only ROC channel type expressed by VSM cells; other homomultimeric (eg, uridine triphosphate-activated TRPC3 channels13) and heteromultimeric combinations of TRPC subunits (eg, TRPC1, TRPC3, and TRPC614) could also be present. In summary, this report provides molecular, biochemical, and electrophysiological evidence that multiple TRPC subunits are expressed by A7r5 VSM cells, that TRPC6 and TRPC7 coassemble in this cell type, and that heteromultimeric TRPC6-TRPC7 channels share a functional identity with AVP-induced ROCs of A7r5 cells with respect to their I-V relation and inhibition by [Ca2+]o.

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Disclosures

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ONLINE SUPPLEMENTARY METHODS

Electrophysiological measurements: Transfected HEK293 and A7r5 cells on glass cover slips were placed in a 300 μl constant flow chamber containing bathing solution (at 20-22°C) attached to the stage of a Diaphot-TMD epifluorescence inverted microscope (Nikon Canada, Mississauga, Canada). Whole-cell currents due to native and heterologously expressed cation channels were measured using heat-polished glass pipettes (tip resistance of 1~2 MΩ) prepared with a Sutter P-87 puller (Sutter Instrument Co., Novato, USA) and MF-83 microforge (Narishige Scientific Instrument Laboratory, Tokyo, Japan). The standard extracellular solution contained (in mM) 140 NaCl, 5 CsCl, 0.1 MgCl₂, 0.05 CaCl₂, 10 glucose, and 10 N-[hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES) (pH 7.4 with NaOH). N-methyl-D-glucamine (NMDG)-containing bath solution (Na⁺ and Cs⁺ replaced with NMDG and Ca²⁺ omitted) was used at the end of each experiment to assess leak current density; on average leak current density of A7r5 cells was 0.10 ± 0.03 pA/pF in 0.05 mM (n = 10 cells) and 0.11 ± 0.01 pA/pF in 1.0 mM Ca²⁺-containing bath solution and on average ≤ 0.2 pA/pF (n = 25 cells) in HEK293 cells both Ca²⁺ solutions. Nicardipine (1 μM) was present in all external solutions to block voltage-dependent Ca²⁺ channels. For experiments involving TRPC5 channels, [Ca²⁺]₀ was increased to 2 mM to facilitate the recording of current induced by Ang II. The pipette solution contained (in mM) 130 CsOH, 110 aspartic acid, 15 CsCl, 1 MgCl₂, 3.6 CaCl₂, 10 ethyleneglycol-bis (β-aminoethyl ether)-N, N, N’, N’-tetraacetic acid (EGTA), and 10 HEPES (pH 7.2 with CsOH) with a calculated [Ca²⁺] in 100 nM. Under these experimental conditions, the reversal potentials for cation and Cl⁻ currents would be expected at 0 and -46 mV, respectively. The pipette EGTA was replaced in a small set of control experiments with 1,2-bis(o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid
(BAPTA), but there was no difference in the results obtained. Whole-cell voltage-clamp recordings were obtained using an Axopatch 200A amplifier (Axon Instruments, Union City, USA) and a Digidata 1200 A-D convertor (Axon Instruments), filtered at 1 kHz, sampled at 1 kHz. Series resistance was not compensated and leakage current was not subtracted in this study. The liquid junction potential between pipette and bath solutions was minimized using an agar bridge. The whole-cell current was monitored at holding potential of –60 mV and ramp depolarizations of 2 s duration from -100 to +100 mV were applied every 16 s. Whole cell current records were analysed using pCLAMP 8.1 software (Axon Instruments).

**Immunocytochemistry:** Immunocytochemistry was performed using 72 hr post-transfection A7r5 cells. Cells were washed with phosphate-buffered saline (PBS), fixed for 20 min with 3.7% formaldehyde and permeabilized for 5 min with 0.1% Triton X-100. Cells were washed with PBS, blocked for 1 hr with 1% bovine serum albumin (BSA)/PBS, exposed to primary antibody in 0.5% BSA/PBS for 2 hrs before 0.5% BSA/PBS and labeled for 1 hr with anti-mouse IgG-conjugated Cy3 secondary antibody (Jackson Immunoresearch Laboratories, West Grove, USA). Unbound secondary antibody was removed before visualization.

**Western blotting and Immunoprecipitation:** HEK293 cells and A7r5 cells were gently washed twice in cold PBS, scraped into 300 μL of extraction buffer containing: 20 mM Tris-HCl (pH 7.5), 138 mM NaCl, 3 mM KCl, 1 mM EGTA, 2 mM [ethylene dinitrilo]tetraacetic acid (EDTA), 1 mM benzamidine, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 5 μg/mL pepstatin A, 0.2 mM pepabloc SC, 1 mM dithiothreitol and 1% Triton X-100, and incubated for 30 min on ice. The cell lysates were then centrifuged at 16,000 × g for 5 min and the protein content of the supernatant assayed (BCA Protein Assay Reagent, Pierce Biotechnology,
Rockford, USA). Proteins were heated for 5 min at 95°C in SDS sample buffer containing: β-mercaptoethanol. Proteins were separated by SDS-PAGE (7.5-15% gels), followed by transfer to 0.45 µm nitrocellulose membranes using standard techniques. The membranes were blocked with 5% non-fat dried milk (NFDM) in 0.1% Tween-20 PBS (PBST). Membranes were labelled with primary antibodies in 1% NFDM PBST and washed with PBST prior to labeling with 1:10000 anti-rabbit or anti-mouse IgG (H+L)-conjugated Alexa Fluor®680 (Invitrogen) or anti-rabbit IgG (H+L)-conjugated IRDye800 (RockLand Immunochemicals, Gilbertsville, USA). Unbound secondary antibody was washed from membranes prior to scanning with an Odyssey Infrared Imager (Li-Cor Biotechnology, Lincoln, USA). For the immunoprecipitation experiments, all reactions were performed while tumbling at 4°C. HEK293 cells and A7r5 cell protein extracts were pre-cleared for 1 hr with Protein A Sepharose CL-4B beads (Pharmacia) and then incubated for 3 hr with 5 µg of antibody. Antibody-protein complexes were captured by the addition of Protein A Sepharose and incubated overnight to facilitate binding. Immunoprecipitated complexes were eluted from the beads using SDS sample buffer prior to SDS-PAGE and immunoblotting. Blots were visualized as described above, or using SuperSignal® West Femto chemiluminescent substrate (Pierce Biotechnology) with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG 2° antibody prior to transfer to film.

**Molecular Biology: a) RT-PCR:** Total RNA was prepared from A7r5 cells and rat brain using an RNAeasy Kit (Qiagen, Mississauga, Canada). First-strand cDNA was prepared from the RNA preparations using SuperTaq Plus and 500 µg/µL oligo(dT) primers were used to reverse transcribe the RNA sample. The cDNA reverse transcription product was
amplified with primers designed for conserved regions of TRPC1-TRPC7 based upon the DNA sequences of multiple species. The amplified products (18 µL) were separated by electrophoresis on a 1% agarose/1X TAE (Tris, acetic acid and EDTA) gel and the DNA bands were visualized with ethidium bromide. All amplicons detected were subsequently sequenced to confirm their identity. The forward and reverse primers employed were respectively as follows (5’-3’):

<table>
<thead>
<tr>
<th>Channel</th>
<th>Length (b.p.)</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPC1</td>
<td>486</td>
<td>CCTTCACGATTGGACTGACACA</td>
<td>CTGACCTTGCCCTTTGGAGGTG</td>
</tr>
<tr>
<td>TRPC2</td>
<td>486</td>
<td>CAGTTTCACCCGATTGGCGTAT</td>
<td>CTTTGGGGATGGCAGGATGTTA</td>
</tr>
<tr>
<td>TRPC3 set 1</td>
<td>314</td>
<td>TGACT participant a</td>
<td>TCTGAAGTCTTTCTCCTCCTGC</td>
</tr>
<tr>
<td>TRPC3 set 2</td>
<td>513</td>
<td>GGGCAGGTGACGACTTCTATG</td>
<td>ATGGCAAGTGGACCCGACTC</td>
</tr>
<tr>
<td>TRPC4 all isoforms</td>
<td>414</td>
<td>TCTGCAGATATCTCTGGGAAGAATGC</td>
<td>AAGCTTTGGTTCAGCAAATTTCCACTC</td>
</tr>
<tr>
<td>TRPC4α 500 b.p. vs TRPC4β 245 b.p.</td>
<td>500 vs 245</td>
<td>GAAGCGGTACGTGGGAGCAGCCAT</td>
<td>CTCTCAAGTGCTCTGCGCC</td>
</tr>
<tr>
<td>TRPC5</td>
<td>443</td>
<td>ACCTCTCATCAGAACCATGCCA</td>
<td>TGCATGAGCAAGTCAAGGCGCT</td>
</tr>
<tr>
<td>TRPC6</td>
<td>582</td>
<td>GGTTTACGGCGACGCAGACCAT</td>
<td>GATCGGTAGGGTCCCACCTTATC</td>
</tr>
<tr>
<td>TRPC7 set 1</td>
<td>137</td>
<td>GAAGTACGACACAAAGTTCATCG</td>
<td>TGCCTCTCCTCGATTTCC</td>
</tr>
</tbody>
</table>
TRPC7 set 2, 649 b.p.: GAAGTACGACCACAAGTTCATCG
TCAGGTGGTCTTTGTCAAT

ET-1 Outer set 445 b.p. GAGCTGAGAAGGAAG
GGTCTTGATGCTGTT

ET-1 Nested set 328 b.p. TGTGTCTACTTCTGC
GCCTCAACCTTCTT

Note that positive control experiments using brain mRNA and the ET-1 primer sets were as previously reported by Plane et al.¹

b) Dominant negative TRPC construct: Dominant-negative mouse TRPC5 and human TRPC6 mutant subunits were generated by PCR-mediated substitution mutagenesis replacing the three conserved amino acids LFW with alanine residues (AAA) within the respective pore regions of wild-type channels (amino acids 575-577 in mouse TRPC5 and 678-680 in human TRPC6).² The identity of the mutations was confirmed by sequencing.

Drugs, chemicals and cDNA constructs: Nicardipine was obtained from Sigma-Aldrich (Oakville, Canada). Ang II and AVP were purchased from Calbiochem-Novabiochem (Boston, USA). cDNA encoding the Ang II AT₁ receptor in pRc vector was a kind gift of Dr. K. Burns (University of Ottawa, Canada). Human TRPC1, TRPC3 and TRPC6 cDNAs in pcDNA3 vector were obtained from Dr L. Birnbaumer (UCLA, USA). Mouse TRPC5 and TRPC7 cDNAs in pCIneo vector were obtained from Dr Y. Mori (University of Kyoto, Japan). Anti-TRPC4 and anti-TRPC6 were purchased from Chemicon (Temecula, USA), a second anti-TRPC6 and anti-TRPC7 were very appreciated gifts of Dr. W.P. Schilling (MetroHealth Medical Center, Cleveland, USA). Anti-c-myc and anti-HA were purchased from Oncogene (Boston, USA) and Invitrogen Canada (Burlington, Canada), respectively.

Statistical analysis: Data were expressed as the mean ± S.E.M. based on the number of
observations. Statistical significance of differences in mean values was assessed by unpaired Student’s t test for single comparisons and one-way analysis of variance followed by Bonferroni’s or Dunnett’s post-hoc test for multiple comparisons.

ONLINE SUPPLEMENTARY RESULTS

Online Figure 1 shows cation currents and immunoblot analysis of wild-type and mutant subunit expression by HEK293 cells. No cation currents were observed following mock transfection of HEK293 cells (Online Fig. 1A). Cells transfected with wild-type TRPC6 and AT1 receptor cDNAs exhibited a marked increase in NMDG-sensitive cation with a typical doubly rectifying current-voltage (I-V) relation after exposure to Ang II (Online Fig. 1A & E). In contrast, cells co-expressing cDNAs encoding TRPC6DN and the AT1 receptor did not exhibit cation current in response to Ang II and there was no difference in I-V relation from that recorded using cells transfected with empty vector and AT1 receptor cDNAs (n = 6; Online Fig. 1 A & E). The expression of c-myc immunoreactive bands of appropriate molecular weight for TRPC6 (~125 kDa) was apparent only in cells expressing the myc-tagged TRPC6DN, but not in untransfected, mock transfected or cells expressing TRPC6 or non-tagged TRPC6DN (Online Fig. 1B). Similar results were obtained for the HA-tagged TRPC5DN; external [Ca2+]o was raised to 2 mM from 0.05 mM to facilitate the recording of Ang II-induced TRPC5 current, as this subunit requires extracellular Ca2+ (i.e. TRPC5 currents were not observed in 5 of 5 cells with standard extracellular solution, data not shown). The TRPC5 current displayed a doubly rectifying I-V relation, and the current was potentiated by increasing external Ca2+ (Online Fig. 1C & F). The presence of TRPC5 protein was confirmed using anti-HA that revealed the presence of immunoreactivity with an apparent molecular weight consistent with that of TRPC5 (~100 kDa; Online Fig. 1D).
In contrast, cation current was not evoked in cells expressing TRPC5\textsuperscript{DN}-HA despite the presence of anti-HA immunoreactive protein (Online Fig. 1C & D). This indicates that expression of the dominant-negative constructs does not yield functional channels.

The specificity of the dominant-negative constructs was tested using cells transfected with cDNAs encoding the AT\textsubscript{1} receptor and wild-type TRPC5 or TRPC6 in combination with empty vector or with TRPC5\textsuperscript{DN} or TRPC6\textsuperscript{DN} at a cDNA ratio of 1:3 (wild-type versus empty vector or dominant negative). Online Figures 2A and C show that empty vector and TRPC6\textsuperscript{DN} did not affect the activation of TRPC5\textsubscript{wt} current by Ang II, but co-expression with TRPC5\textsuperscript{DN} suppressed the wild-type current. Conversely, online figures 2B and D show that empty vector and TRPC5\textsuperscript{DN} did not affect the activation of TRPC6\textsubscript{wt} current by Ang II, but co-expression with TRPC6\textsuperscript{DN} suppressed the wild-type current.

Online supplementary Figure 3 shows cation current of an A7r5 cell evoked by 100 nmol/L angiotensin II (Ang II). Note the doubly rectifying I-V relation of the current was identical to that of ROC current evoked by AVP.

### ONLINE REFERENCES


### ONLINE FIGURE LEGENDS

**Online Figure 1:** A. Lack of change in NMDG-sensitive cation current at -60 mV in HEK293 cells expressing AT\textsubscript{1} receptors and mock-transfected with empty vector (Mock)
or transfected with dominant-negative pore mutant TRPC6\textsuperscript{DN} cDNAs (TRPC6\textsuperscript{DN}) versus marked increase in current in cells transfected with wild-type TRPC6 (TRPC6\textsuperscript{wt}) during exposure to Ang II. B. Immunoblots showing the presence of c-myc immunoreactive bands with an apparent molecular weight consistent with that of TRPC6 only in lystate of HEK293 cells expressing C-terminal c-myc-tagged TRPC6\textsuperscript{DN}, but not in cells transfected with non-tagged TRPC6\textsuperscript{DN}, wild-type TRPC6 (TRPC6\textsuperscript{wt}), empty vector (Mock), or untransfected cells (Untransfected). C. Activation of NMDG-sensitive and Ca\textsuperscript{2+}-sensitive current in HEK293 cells transfected with HA-tagged wild-type TRPC5 (TRPC5\textsuperscript{wt-HA}), but not in cells transfected with HA-tagged dominant-negative pore mutant TRPC5\textsuperscript{DN} cDNAs (TRPC5\textsuperscript{DN-HA}) during treatment with Ang II. D. Anti-HA immunoblots of lysates of HEK293 cells expressing wild-type (left lane) and dominant-negative mutant (right lane) HA-tagged TRPC5 subunits. E. I-V relation of cation current in HEK293 cells transfected with vector alone (Mock), wild-type (TRPC6\textsuperscript{wt}) and mutant (TRPC6\textsuperscript{DN}) TRPC6 subunits. Symbols indicate corresponding points in the traces of panel A at which 2 s voltage ramp protocols between -100 and +100 mV were applied. F. I-V relation of cation current in HEK293 cells transfected with vector alone (Mock), wild-type (TRPC5\textsuperscript{wt}) and mutant (TRPC5\textsuperscript{DN}) TRPC5 subunits. Symbols indicate corresponding points in the traces of panel C at which 2 s voltage ramp protocols between -100 and +100 mV were applied.

Online Figure 2: A. NMDG-sensitive cation current was apparent at -60 mV in HEK293 cells expressing AT\textsubscript{1} receptor and wild-type TRPC5 (TRPC5\textsuperscript{wt}) alone or with dominant-negative TRPC6\textsuperscript{DN} (TRPC5\textsuperscript{wt}+TRPC6\textsuperscript{DN}) cDNAs, but not AT\textsubscript{1} receptor and wild-type plus dominant-negative TRPC5\textsuperscript{DN} cDNAs (TRPC5\textsuperscript{wt}+TRPC5\textsuperscript{DN}) during exposure to Ang II. B. NMDG-sensitive cation current was apparent at -60 mV in HEK293 cells expressing AT\textsubscript{1}
receptor and wild-type TRPC6 (TRPC6wt) alone or with dominant-negative TRPC5<sub>DN</sub> (TRPC6wt+TRPC5DN) cDNAs, but not AT<sub>1</sub> receptor and wild-type TRPC6 plus dominant-negative TRPC6<sub>DN</sub> cDNAs (TRPC6wt+TRPC6DN) during exposure to Ang II. C. Average current density (pA/pF) at -60 mV evoked by Ang II in HEK293 cells expressing wild-type TRPC5 alone (n = 10 cells) or with TRPC5<sub>DN</sub> (TRPC5wt / TRPC5DN; n = 11 cells) (top) and wild-type TRPC5 alone (n = 14 cells) or with TRPC6<sub>DN</sub> (TRPC5wt / TRPC6DN; n = 14 cells) (bottom). Note the significant difference (P < 0.05) in current density in the presence of TRPC5<sub>DN</sub>, but not TRPC6<sub>DN</sub>. D. Average current density (pA/pF) at -60 mV evoked by Ang II in HEK293 cells expressing wild-type TRPC6 alone (n = 8 cells) or with TRPC5<sub>DN</sub> (TRPC5wt / TRPC5DN; n = 8 cells) (top) and wild-type TRPC6 alone (n = 13 cells) or with TRPC6<sub>DN</sub> (TRPC6wt / TRPC6DN; n = 13 cells) (bottom). Note the significant decrease in current density (P < 0.05) in the presence of TRPC6<sub>DN</sub>, but not TRPC5<sub>DN</sub>.

**Online Figure 3:** A. Representative reversible activation of cation current in A7r5 cell by 100 nmol/L angiotensin II (Ang II) treatment. B. Representative currents evoked by application of voltage ramp protocols between -100 and +100 mV before (△) and after (◆, current indicated in red) application of Ang II at times indicated in panel A. Note the doubly-rectifying nature of the current in presence of Ang II is identical to that evoked by AVP (Fig. 3 in article).
A TRPC5wt
Ang II 100 nM NMDG

TRPC5wt + TRPC5DN
Ang II 100 nM NMDG

TRPC5wt + TRPC6DN
Ang II 100 nM NMDG

B TRPC6wt
Ang II 100 nM NMDG

TRPC6wt + TRPC5DN
Ang II 100 nM NMDG

TRPC6wt + TRPC6DN
Ang II 100 nM NMDG

C TRPC5wt / TRPC5DN

D TRPC6wt / TRPC5DN

TRPC5wt / TRPC6DN

TRPC6wt / TRPC6DN

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