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Differential expression and alternative splicing of TRP channel genes in smooth muscles

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Walker, Rebecca L., Joseph R. Hume, and Burton Horowitz. Differential expression and alternative splicing of TRP channel genes in smooth muscles. *Am J Physiol Cell Physiol* 280: C1184–C1192, 2001.—Nonselective cation channels (NSCC) are targets of excitatory agonists in smooth muscle, representing the nonselective cation current I_{cat}. Na^+ influx through NSCC causes depolarizations and activates voltage-dependent Ca^{2+} channels, resulting in contraction. The molecular identity of I_{cat} in smooth muscle has not been elucidated; however, products of the transient receptor potential (TRP) genes have characteristics similar to native I_{cat}. We have determined the levels of TRP transcriptional expression in several murine and canine gastrointestinal and vascular smooth muscles and have analyzed the alternative processing of these transcripts. Of the seven TRP gene family members, transcripts for TRP4, TRP6, and TRP7 were detected in all murine and canine smooth muscle cell preparations. TRP3 was detected only in canine renal artery smooth muscle cells. The full-length cDNAs for TRP4, TRP6, and TRP7, as well as one splice variant of TRP4 and two splice variants of TRP7, were cloned from murine colonic smooth muscle. Quantitative RT-PCR determined the relative amounts of TRP4, TRP6, and TRP7 transcripts, as well as that of the splice variants, in several murine smooth muscles. TRP4 is the most highly expressed, while TRP6 and TRP7 are expressed at a lower level in the same tissues. Splice variants for TRP7, deleted for exons encoding amino acids including transmembrane segment S1, predominated in murine smooth muscles, while the full-length form of the transcript was expressed in canine smooth muscles.

calcium channels; gastrointestinal; vascular; ribonucleic acid expression

IN VISCERAL SMOOTH MUSCLES, muscarinic stimulation results in excitation and muscle cell contraction (15). Receptor activation [predominantly M3 (24)] couples to G_q, which stimulates phospholipase C (PLC). Inositol 1,4,5-trisphosphate (IP_3) is cleaved from phosphatidylinositol 4,5-bisphosphate (PIP_2), which will result in release of Ca^{2+} from IP_3-dependent stores. In addition, muscarinic receptor activation will release $G\beta\gamma$, which has been suggested to activate nonselective cation channels (representing nonselective cation current, I_{cat}) (11), although other modes of activation for this current may exist. Influx of Na^+ and Ca^{2+} through these channels depolarizes membrane potential, activating voltage-sensitive Ca^{2+} channels that are the primary source of Ca^{2+} influx leading to smooth muscle contraction. The Ca^{2+} release due to IP_3 augments the activation of smooth muscle I_{cat}. The properties of I_{cat} have been determined for several smooth muscle preparations. In physiological solutions, removal of external Na^+ reduces I_{cat} by ~90%, suggesting that Na^+ is the primary charge carrier (10). However, permeability ratios for I_{cat} from guinea pig jejunum (19), portal vein (26), guinea pig gastric (14), and canine pulmonary artery myocytes (13) suggest a high relative selectivity of Ca^{2+} over monovalents.

The unitary conductance of I_{cat} recorded from guinea pig jejunum, canine pylorus, and rabbit portal vein has been reported to be ~25–30 pS (11, 25). While the properties of the I_{cat} have been examined, a molecular candidate encoding I_{cat} has not been determined.

Transient receptor potential (TRP) gene products encode nonselective cation channels with properties similar to I_{cat}. First cloned from *Drosophila* and found to be involved in visual signal transduction (20, 22), TRP channels play a prominent role in store-operated Ca^{2+} entry (1, 20, 22). To date, seven members of the TRP family have been cloned, and their expression has been detected within a variety of mammalian species and tissues. In addition to contributing to capacitative calcium entry, TRP channels have also been associated with receptor-operated Ca^{2+} influx stimulated by a G_q-coupled mechanism (9), possibly through direct interaction with diacylglycerol (8, 18). The topological and multimeric structures of TRP channels are similar to those of other cation-selective channels (see Ref. 1). That is, they form from monomeric subunits with six transmembrane segments, a cation-selective pore structure between transmembrane segments 5 and 6, and associate into functional tetramers by either combining four monomers or combining with other TRP family members to form heterotetramers (6, 29).

Because TRP channels are excellent candidates for I_{cat} in smooth muscles, we determined the expression of members of the TRP family in visceral and vascular smooth muscle preparations. We report the identification of TRP4, TRP6, and TRP7 transcripts in isolated smooth muscle cell preparations and the cloning of all

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three forms from murine smooth muscle. Splice variants were detected for TRP4 and TRP7 expressed in smooth muscle cells from the gastrointestinal (GI) tract and vascular muscles. Quantitative RT-PCR was used to determine the relative expression levels of these TRP channels and splice variants in several smooth muscles.

MATERIALS AND METHODS

Dissection of smooth muscles and smooth muscle cell preparations. Mongrel dogs of either sex were overdosed with pentobarbital sodium (100 mg/kg), and incisions were made along the abdomen. Strips of small bowel and proximal colon were removed and placed into Krebs solution containing (in mM) 120.35 NaCl, 5.9 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 15.5 $NaHCO_3$, 1.2 Na_2HPO_4, and 11.5 glucose. Segments of tissue were pinned in a dissecting dish with the mucosa facing upward. The mucosa and submucosa were removed by sharp dissection. Small portions of the circular smooth muscle tissue were placed into a Ca^{2+}-free Hanks' solution containing (in mM) 125 NaCl, 5.36 KCl, 15.5 NaOH, 0.336 Na_2HCO_3, 0.44 KH_2PO_4, 10 glucose, 2.9 sucrose, and 11 HEPES. Strips of tissue were incubated in a Ca^{2+}-free Hanks' solution containing 230 units of collagenase (Worthington Biochemical), 2 mg of fatty acid-free bovine serum albumin (BSA FFA; Sigma), 2 mg of trypsin inhibitor (Sigma), and 0.11 mg of ATP (Sigma). Incubation in this enzyme was carried out at 37°C for 8–12 min. The tissues were washed with Ca^{2+}-free Hanks' solution, and gentle trituration resulted in the isolation of individual myocytes. Cells were transferred to the stage of a phase-contrast microscope and allowed to adhere to the glass coverslip bottom for 5 min. Smooth muscle cells were differentiated by their characteristic morphology. Single cells were collected through applied suction by aspirating them into a wide-bore patch-clamp pipette (borosilicate glass; Sutter Instruments). Approximately 60 smooth muscle cells were collected, flash-frozen in liquid nitrogen, and stored at −80°C until use. BALB/c mice were killed by cervical dislocation, and incisions were made along the abdomen. Segments of the small bowel and proximal colon were isolated, and cells were collected as described above.

Pulmonary and renal canine smooth muscle isolation. Mongrel dogs were euthanized as described in *Dissection of smooth muscles and smooth muscle cell preparations*, and segments of renal and pulmonary arteries were isolated. The main pulmonary and renal arteries were flushed with physiological saline solution (PSS) containing (in mM) 125 NaCl, 5.36 KCl, 0.336 Na_2HPO_4, 0.44 K_2HPO_4, 11 HEPES, 1.2 $MgCl_2$, 0.05 $CaCl_2$, 10 glucose, and 2.9 sucrose, with pH 7.4 adjusted with 10 mM Tris and osmolarity of 300 mosM (sucrose). The solution was continuously bubbled with 100% O_2 during dissections. Once isolated, arteries were cleaned by removal of bulk connective tissue and then were cut into small squares to prepare for digestion. Pulmonary arteries were digested in a solution containing (in mg/6 ml) 2 mg of collagenase type XI (Sigma), 0.4 mg of elastase type III, and 2 mg of BSA free fatty acids (FFA). Digestion took place for 16–18 h at 4°C. The tissues were then washed in cold Ca^{2+}-free PSS solution and slowly triturated with fire-polished Pasteur pipettes to isolate individual myocytes. Renal arteries were digested in PSS solution containing (in mg/2.5 ml) 5 mg of collagenase type XI, 0.4 mg of elastase type IX, and 2 mg of BSA FFA. Tissues were digested for 18–23 min at 34°C, and cells were isolated. Individual myocytes from renal and pulmonary artery were collected as described above.

Total RNA isolation and RT-PCR. Total RNA was prepared from tissue and isolated smooth muscle cells with the use of a SNAP Total RNA isolation kit (Invitrogen, San Diego, CA) per manufacturer's instructions, including the use of polyinosinic acid (20 μg) as an RNA carrier. Total RNA was also isolated from heart and brain tissue by using this method. First-strand cDNA was prepared from the RNA preparations by using the Superscript II Reverse Transcriptase kit (GIBCO BRL, Gaithersburg, MD); 500 μg/μl oligo(dT) primers were used to reverse transcribe the RNA sample. The cDNA reverse transcription product was amplified with channel-specific primers by PCR. The amplification profile for these primer pairs were as follows: 95°C for 10 min to activate the Amplitaq polymerase (PE Biosystems), 95°C for 15 s, and 60°C for 1 min, each of 40 cycles. The amplified products (5 μl) were separated by electrophoresis on a 4% agarose/1× TAE (Tris, acetic acid, EDTA) gel, and the DNA bands were visualized by ethidium bromide staining. For the RT control, a cDNA reaction was used as template for which the reverse transcriptase was not added, controlling for genomic DNA contamination in the source RNA. The NTC (no-template control) was a PCR amplification for which the template was not added, controlling for nonspecific amplification and spurious primer-dimer fragments. These negative controls were subjected to a second round of amplification to assure specificity of the reactions and the quality of the reagents.

Primer design. The following PCR primers were used: TRP1 (GenBank accession no. U73625): sense nt 1794–1816 and antisense nt 2290–2310, amplicon = 516 bp; TRP2 (AF111107): sense nt 1531–1550 and antisense nt 1881–1900, amplicon = 369 bp; TRP3 (U47050): sense nt 1483–1502 and antisense nt 1795–1814, amplicon = 331; TRP4 (U50922): sense nt 2535–2553 and antisense nt 2781–2800, amplicon = 265; TRP5 (AF029983): sense nt 675–694 and antisense nt 1075–1094, amplicon = 419; TRP6 (U49069): sense nt 738–758 and antisense nt 1128–1148, amplicon = 410; TRP7 (AF139923): sense nt 2397–2417 and antisense nt 2637–2657, amplicon = 260; β-actin (V01217): sense nt 2384–2402 and antisense nt 3071–3091, amplicon = 498 bp; TRP4 span (U50922): sense nt 2467–2487 and antisense nt 2863–2883, amplicons = 420 or 167 bp depending on splice variant present; TRP7 span (AF139923): sense nt 747–769 and antisense nt 1278–1299, amplicons = 550, 385, or 203 bp depending on splice variant present.

Specific primers also were designed for quantitative PCR to determine relative levels of expression of TRP splice variants in smooth muscle, as follows: TRP4-all splice forms (GenBank accession no. AF019663): sense nt 1667–1686 and antisense nt 1742–1760, amplicon = 93 bp, will amplify both forms of TRP4; TRP4-wild type (AF019663): sense nt 2518–2538 and antisense nt 2599–2620, amplicon = 102 bp, will amplify only the full-length form of TRP4 and not the alternatively spliced variant because the primers were designed to hybridize to the deleted exon in the splice variant; TRP7-all splice forms (AF139923): sense nt 1516–1535 and antisense nt 1614–1632, amplicon = 116, will amplify all three forms of TRP7; TRP7-wild type (AF139923): sense nt 999–1019 and antisense nt 1118–1139, amplicon = 140 bp, will amplify only the full length form of TRP7 because the primers were designed to bind within the deleted exons of the alternatively spliced forms; TRP7-splice 2 (AF139923): nt 845–865 and antisense nt 968–988, amplicon = 144 bp, will amplify the wild-type form of TRP7 as well as splice 2 because it was designed to bind within the deleted exon defining splice 1. PCR products generated from each pair of primers were extracted and sequenced to confirm the specificity of the primers.

Quantitative RT-PCR. Real-time quantitative PCR was performed with the use of Syber Green chemistry on an ABI 5700 sequence detector (PE Biosystems). Regression analysis

of the mean values of eight multiplex RT-PCRs for the $\log_{10}$ diluted cDNA was used to generate standard curves. Unknown quantities relative to the standard curve for a particular set of primers were calculated, yielding transcriptional quantitation of TRP gene products relative to the endogenous standard (β-actin). The reproducibility of the assay was tested by analysis of variance (ANOVA) comparing repeat runs of samples, and mean values generated at individual time points were compared by Student's *t*-test.

Cloning of TRP channels from murine colonic smooth muscle. TRP6 was cloned from murine colonic smooth muscle by performing PCR in the presence of the following gene-specific primers: TRP6 (GenBank accession no. U49069), sense nt 289–310 and antisense 3067–3088. PCR was performed under the following protocol: 94°C for 2 min; 30 cycles of 94°C for 45 s; 63°C for 90 s, and 72°C for 3 min; followed by 72°C for 10 min and holding at 4°C. Full-length fragments were ligated into PCR2.1 vector constructs (Invitrogen) and transformed with the use of a TA cloning kit (Invitrogen). TRP4 was cloned from murine colonic smooth muscle by performing PCR in the presence of the following gene-specific primers: TRP4 (AF019663), sense nt 184–200 and antisense 3101–3118. PCR was performed under the following protocol: 94°C for 5 min; 30 cycles of 94°C for 1 min, 52.4°C for 1 min, and 72°C for 2 min; followed by 72°C for 7 min and holding at 4°C. Wild-type TRP4 fragments, including a splice variant (splice 1), were ligated into pcDNA3.1 mammalian vector (Invitrogen) and transformed as described above. TRP7 was cloned from murine colonic smooth muscle by performing PCR in the presence of the following gene-specific primers: TRP7 (AF139923), sense nt 91–108 and antisense 2673–2692. PCR was performed under the following protocol: 3 cycles of 94°C for 20 s, 54°C for 40 s, and 72°C for 3 min; 3 cycles of 94°C for 20 s, 51°C for 40 s, and 72°C for 3 min; 3 cycles of 94°C for 20 s, 48°C for 40 s, and 72°C for 3 min; and 25 cycles of 94°C for 20 s, 50°C for 40 s, and 72°C for 3 min; followed by 72°C for 7 min and then holding at 4°C. Wild-type TRP7 fragments, as well as two different splice variants, were ligated into pcDNA 3.1 and transformed as described above.

RESULTS

Expression of TRP family members in smooth muscles. The expression of members of the TRP family in canine and murine smooth muscle cells was determined by performing RT-PCR on total RNA isolated from smooth muscle cell preparations. Qualitative RT-PCR was performed on individually selected smooth muscle cells to avoid the contaminating effects of other cell types as reported previously (3, 4). PCR products were generated through the use of gene-specific primers for TRP 1–7. Canine and murine brain-derived cDNA was used as a positive control for the various TRP primers to test their ability to produce the correct amplicon (Fig. 1). β-Actin primers were used to confirm that the products generated were representative of RNA (498-bp band) and not contaminated with genomic DNA (intron containing 708-bp band) because these primers were designed to span an intron as well as two exons. PCR reactions were also performed on aliquots of RNA in which reverse transcriptase was not added during the cDNA synthesis step. If DNA contamination was noted with either β-actin or in the RT lane, these samples were discarded. Detectable amplicons for TRP4 (270 bp), TRP6 (410 bp), and TRP7 (260 bp) were observed in all canine and murine smooth muscle cell preparations (Fig. 1). TRP3 expression was detected only in canine renal artery. The presence of other TRP transcripts was not detected even with 30 additional cycles of PCR amplification.

TRP channel splice variants expressed in smooth muscle cells. Full-length cDNA cloning for TRP4 and TRP7 identified several alternatively spliced transcripts differentially expressed in smooth muscles. The full-length sequences for TRP4, TRP6, and TRP7

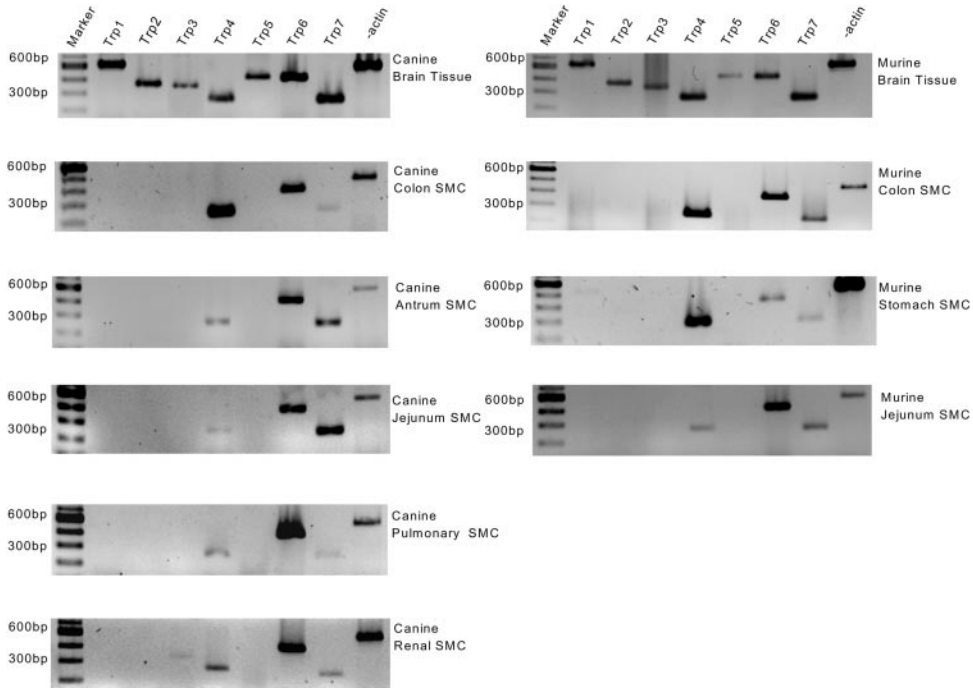


Fig. 1. RT-PCR detection of transient receptor potential (TRP) channel family members in canine (*left*) and murine (*right*) smooth muscle cells (SMC). PCR products were generated through the use of gene-specific primers for TRP1–TRP7. A 100-bp molecular weight marker was used to estimate the size of the amplicons. Primers were tested on canine and mouse brain and sequenced to confirm their identity and determine species specificity. Amplicons for TRP4 (265 bp), TRP6 (410 bp), and TRP7 (260 bp) were observed in all smooth muscle cell preparations. An amplicon for TRP3 was detected in canine renal artery. RT-PCR performed in the presence of β-actin gene-specific primers demonstrates that the products are representative of RNA (498 bp, see MATERIALS AND METHODS).

cDNAs were 99% identical to murine sequences previously reported (GenBank accession no: TRP4, AF019663; TRP6, U49069; and TRP7, AF139923). Alternatively spliced transcripts have been reported for TRP7 (18). However, alternatively spliced transcripts have not been previously published for TRP4. Figure 2*A* describes the positions on a linear depiction of the nucleotide sequence for the alternative exons recovered for these gene transcripts. Primers designed to span the alternatively spliced exons were used in RT-PCR reactions with RNA from smooth muscle cells as template. An analysis of the splice variants for TRP4 and TRP7 from several canine and murine smooth muscles is shown in Fig. 2*B*. The expression of specific splice products is dependent in a qualitative and quantitative manner on the source of smooth muscle RNA. This is a consistent pattern ($n = 3$) and was analyzed further by real-time quantitative PCR procedures (see *Quantitative determination of TRP transcripts in smooth muscles*). TRP7 amplification of canine smooth muscle cells consistently detected the full-length splice form, while murine smooth muscle cells did not. However, when murine colonic muscle was used as the source of RNA, the full-length amplicon was recovered (see Fig. 3*C*). This is presumably due to non-smooth muscle cell types in the tissue preparation. Therefore, TRP7 expression in murine smooth muscle cells is restricted to splice forms 1 and 2.

A depiction of the topology for TRP channels is shown in Fig. 3*A* with the alternatively spliced regions for TRP4 and TRP7 outlined. The amino acid sequences for the resulting amplicons are shown in Fig. 3, *B* and *C*. These sequences are derived from murine colonic myocytes; however, amplification of canine GI and vascular smooth muscle RNA results in similar alternatively spliced transcripts with similar sequences. TRP4-splice 1 is deleted for a 252-bp exon encoding 84 amino acids. Deletion of this exon retains the reading frame of the transcript and does not insert a premature stop codon. TRP7-splice 1 deletes two exons and 345 bp encoding 115 amino acids including predicted transmembrane segment S1. TRP7-splice 2 retains the upstream exon but deletes the downstream exon. This arrangement deletes 165 bp encoding 55 amino acids in the same region of the channel protein. A splice variant that deletes only the upstream exon (retaining the downstream exon) was not detected in any RT-PCR reactions performed either for full-length cloning or for spanning primer analysis. It is therefore assumed that this splice product is not transcribed.

Quantitative determination of TRP transcripts in smooth muscles. The ABI 5700 genetic analyzer (PE Biosystems) was used for accurate quantitation of steady-state transcript levels by RT-PCR. An analysis of TRP6 with qualitative methods indicated no alternative splice variants. Therefore, primers were designed that were specific for TRP6 and used in quantitative analysis. Total RNA was prepared from murine colon, jejunum, and antrum and from canine pulmonary and renal arteries. The RNA was prepared from ~25–50 mg of tissue with mucosa removed from the GI tissues and the endothelium removed from the vascular muscles, as described in MATERIALS AND METHODS. However, these preparations contain smooth muscle

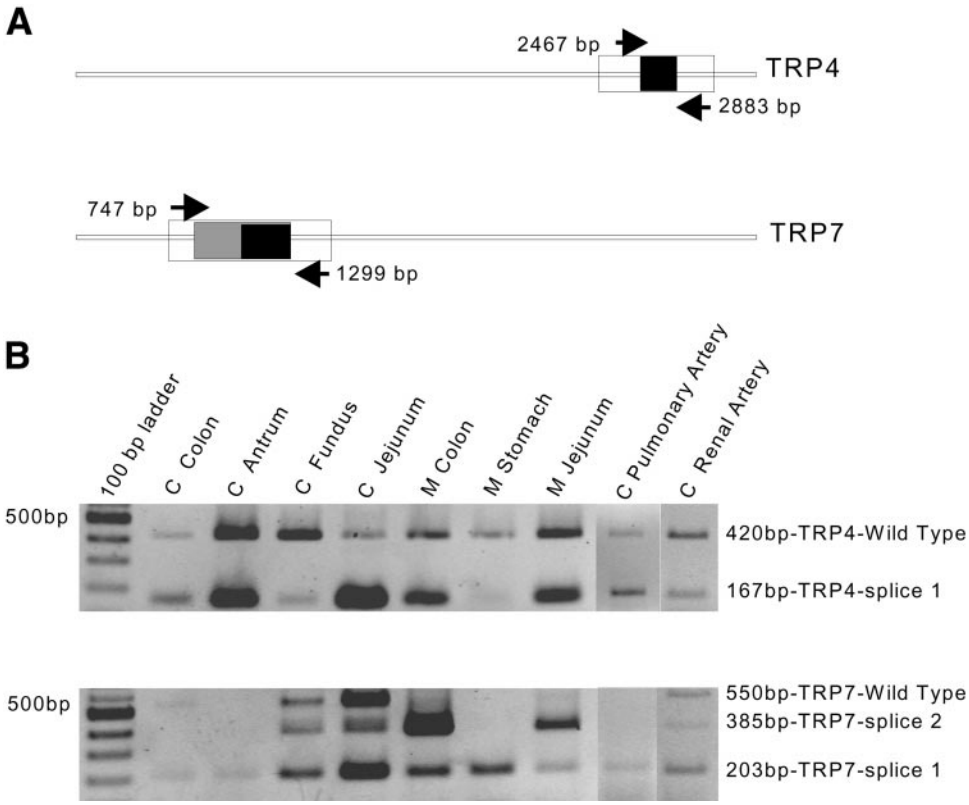
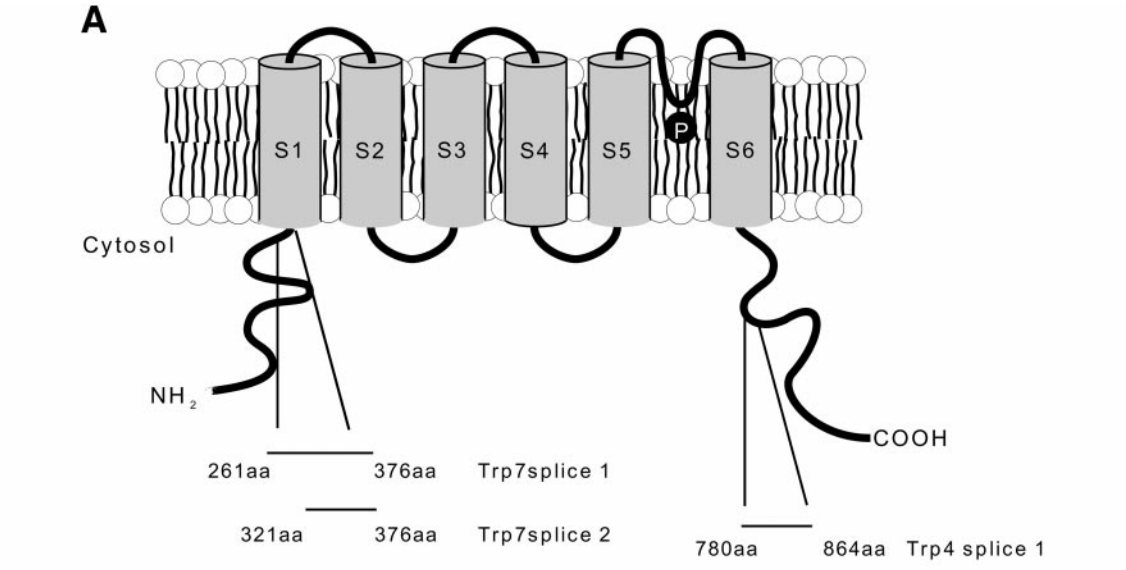


Fig. 2. RT-PCR detection of TRP channel splice variants in canine and murine gastrointestinal and vascular myocytes. *A*: linear depiction of TRP4 (*top*) and TRP7 (*bottom*) cDNA sequence indicating the primers used to detect splice variants. Primers for TRP4 were designed to hybridize at the base pairs indicated. These primers span an exon that is deleted (solid box) in an alternatively spliced form of the TRP4 transcript, resulting in a 167-bp amplicon. Primers for TRP7 were designed to hybridize at the base pairs indicated. These primers span a 2-exon region that is alternatively spliced in TRP7 transcripts, deleting either 348 or 168 bp (shaded/solid box). A 100-bp marker was used to estimate the size of the fragment. *B*: amplicons for TRP4-wild type (420 bp) and TRP4-splice 1 (167 bp) were detected in all myocytes tested (*top*); amplicons for TRP7-wild type (550 bp) and TRP7-splice 1 (385 bp) were detected in canine fundus, jejunum, and pulmonary and renal arteries (*bottom*); amplicons for TRP7-splice 2 (203 bp) were detected in all myocytes tested. C, canine; M, murine.



B

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TRP4 Splice 1       RRHHQYQEVM XNLVKRYVAA MIREAKTEEG LTEENVKELK QDISSFRFEV  750
TRP4 Wild Type      RRHHQYQEVM RNLVKRYVAA MIREAKTEEG LTEENVKELK QDISSFRFEV

TRP4 Splice 1       LGLLRGSKLS TIQSANAASS AYSDEKSQSK ---------- ----------  800
TRP4 Wild Type      LGLLRGSKLS TIQSANAASS ADSDEKSQSK GNGKDKRKNL SLFDLTTLIH

TRP4 Splice 1       ---------- ---------- ---------- ---------- ----------  850
TRP4 Wild Type      PRSAAIASER HNLSNGSALV VQEPPREKQR KVNFVADIKN FGLFHRRSKQ

TRP4 Splice 1       ---------- ----EEITRQ QAAGALERNI ELESKGLASL GDRSIPGLNE  900
TRP4 Wild Type      NAAEQNANQI FSVSEEITRQ QAAGALERNI ELESKGLASL GDRSIPGLNE

TRP4 Splice 1       QCVLVDHRER NTDTLGLQVG KRVCSTFKSE KVVVEDTVPI IPKEKHAHEE  950
TRP4 Wild Type      QCVLVDHRER NTDTLGLQVG KRVCSTFKSE KVVVEDTVPI IPKEKHAHEE

TRP4 Splice 1       DSSIDYDLSP TDTAAHEDYV TTRL*
TRP4 Wild Type      DSSIDYDLSP TDTAAHEDYV TTRL*
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C

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TRP7 Wild Type      NECTEKQRKD SFSHSRSRMN AYKGLASAAY LSLSSEDPVL TALELSNELA  250
TRP7 Splice 1       NECTEKQRKD SFSHSRSRMN AYKGLASAAY LSLSSEDPVL TALELSNELA
TRP7 Splice 2       NECTEKQRKD SFSHSRSRMN AYKGLASAAY LSLSSEDPVL TALELSNELA

TRP7 Wild Type      RLANIETEFK NDYRKLSMQC KDFVVGVLDL CRDTEEVEAI LNGDVNLQVW  300
TRP7 Splice 1       RLANIETEFK ---------- ---------- ---------- ----------
TRP7 Splice 2       RLANIETEFK NDYRKLSMQC KDFVVGVLDL CRDTEEVEAI LNGDVNLQVW

TRP7 Wild Type      SDHHRPSLSR IKLAIKYEVK KFVAHPNCQQ QLLTMWYENL SGLRQQSIAV  350
TRP7 Splice 1       ---------- ---------- ---------- ---------- ----------
TRP7 Splice 2       SDHHRPGLSR IKLAIKYEVK ---------- ---------- ----------

TRP7 Wild Type      KFLAVFGVSI GLPFLAIAYW IAPCSKLGQT LRSPFMKFVA HAVSFTIFLG  400
TRP7 Splice 1       ---------- ---------- ------LGQT LRSPFMKFVA HAVSFTIFLG
TRP7 Splice 2       ---------- ---------- ------LGQT LRSPFMKFVA HAVSFTIFLG

TRP7 Wild Type      LLVVNASDRF EGVKTLPNET FTDYPKQIFR VKTTQFSWTE MLIMKWVLGM  450
TRP7 Splice 1       LLVVNASDRF EGVKTLPNET FTDYPKQIFR VKTTQFSWTE MLIMKWVLGM
TRP7 Splice 2       LLVVNASDRF EGVKTLPNET FTDYPKQIFR VKTTQFSWTE MLIMKWVLGM
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Fig. 3. Amino acid sequence of TRP4 and TRP7 cloned from murine smooth muscle. *A*: predicted topology for TRP channels (see Ref. 1). The alternative exons deleted in smooth muscle for TRP4 and TRP7 are outlined with amino acid (aa) numbers (*bottom*). The amino acid sequences for the alternative spliced regions are shown in *B* and *C*.

cells and other minor cell types (e.g., interstitial cells of Cajal, macrophages, and fibroblasts) that will contribute to the quantitative measurement. RNA was reverse transcribed to cDNA, and steady-state transcripts were determined relative to an endogenous control housekeeping gene (β-actin). Therefore, the data are expressed as TRP/β-actin. The relative transcriptional expression of TRP6 is shown in Fig. 4 for several murine and canine smooth muscles. Note that the scale for all the TRP expression data is kept consistent to allow comparison of relative expression levels between TRP forms, and all expression data are expressed as means ± SE. TRP6 expression relative to β-actin (arbitrary units) was 0.018 ± 0.0063 for murine colon, 0.0061 ± 0.0018 for murine jejunum, 0.0075 ± 0.0029 for murine antrum, 0.038 ± 0.014 for canine pulmonary artery, and 0.023 ± 0.0033 for canine renal artery ($n = 3$ for all these data).

TRP4 expression in smooth muscle cells resulted in two alternatively spliced forms of the transcript (see Fig. 2). Primers were designed to quantitatively determine the levels of each spliced transcript (Fig. 5). Figure 5*A* displays the positions of sequences homologous to the primers on a linear depiction of the TRP4 sequence. The first set of primers binds to a region of the transcript common to both splice forms detected in smooth muscle cells and will amplify all splice forms. TRP4 expression relative to β-actin (arbitrary units) was 0.07 ± 0.013 for murine colon, 0.055 ± 0.002 for murine jejunum, 0.026 ± 0.0041 for murine antrum, 0.075 ± 0.01 for canine pulmonary artery, and 0.054 ± 0.0067 for canine renal artery. Expression of TRP4 was greater than that of TRP6 or TRP7 in all the smooth muscles examined ($n = 3$, $P < 0.05$). The second set of primers (Fig. 5*A*, TRP4-wild type) will only bind to transcripts containing the full-length TRP4 transcripts. This quantitation is listed in Fig. 5*B* (TRP4-wild type). TRP4-wild type expression relative to β-actin (arbitrary units) was 0.0026 ± 0.001 for murine colon, 0.0053 ± 0.0018 for murine jejunum, 0.021 ± 0.0047 for murine antrum, 0.024 ± 0.0067 for canine pulmonary artery, and 0.0075 ± 0.0018 for canine renal artery. The expression level of the alternatively spliced form of TRP4, deleted for an exon between nt 2467 and 2883, was determined by subtracting individual values for paired samples of TRP4-all splice forms and TRP4-wild type. This calculation assumes that there are no other splice variants in smooth muscles, which is indicated from the studies on smooth muscle cells (see Fig. 2 and full-length cloning). TRP4-splice expression relative to β-actin (arbitrary units) was 0.06 ± 0.012 for murine colon, 0.039 ± 0.0035 for murine jejunum, 0.005 ± 0.0025 for murine antrum, 0.051 ± 0.0034 for canine pulmonary artery, and 0.045 ± 0.0054 for canine renal artery. There was quantitatively more TRP4-splice transcript expressed in all smooth muscles, except murine antrum, than TRP4-wild type ($n = 3$, $P > 0.05$).

A similar strategy was used to determine the relative levels of TRP7-splice form expression in smooth muscles. Cloning of TRP7 from murine colonic muscle resulted in three alternatively spliced products (see Fig. 3, *B* and *C*). Three sets of quantitative primers were used to calculate the relative expression levels of the three forms in smooth muscles (Fig. 6). The first set of primers was designed to hybridize to a region of the transcript common to all the splice forms (TRP7-all splice forms). TRP7 expression relative to β-actin (arbitrary units) was 0.013 ± 0.0018 for murine colon, 0.016 ± 0.0042 for murine jejunum, 0.013 ± 0.003 for murine antrum, 0.031 ± 0.0053 for canine pulmonary artery, and 0.033 ± 0.0011 for canine renal artery.

Primers were designed to hybridize within the upstream exon (deleted only in splice 1 form, forward) and the downstream exon (deleted in splice 1 and splice 2 forms, reverse). These primers will only amplify full-length transcripts (TRP7-wild type). TRP7-wild type expression relative to β-actin (arbitrary units) was 0.00026 ± 0.00019 for murine colon, 0.000047 ± 0.000006 for murine jejunum, 0.000095 ± 0.000006 for murine antrum, 0.003 ± 0.0012 for canine pulmonary artery, and 0.0069 ± 0.0014 for canine renal artery.

Primers were also designed to hybridize to a common region (forward) and sequence within the deleted upstream exon of the splice 1 form. Amplicons derived from this amplification will represent only splice 2 and full-length transcripts (TRP7-splice 2 primers). Therefore, subtracting individual values for paired samples of TRP7-all splice forms and TRP7-wild type + splice 2 will yield values for the splice 1 form. TRP7-splice 1 expression relative to β-actin (arbitrary units) was 0.0063 ± 0.002 for murine colon, 0.011 ± 0.0029 for murine jejunum, 0.00081 ± 0.00055 for murine antrum, 0.021 ± 0.0042 for canine pulmonary artery, and 0.025 ± 0.015 for canine renal artery.

The corollary to this calculation for TRP7-splice 1 will result in quantitation of TRP7-splice 2. Subtracting individual values for paired samples of TRP7-all splice forms and TRP7-wild type + splice 1 will yield values for the splice 2 form. TRP7-splice 2 expression relative to β-actin

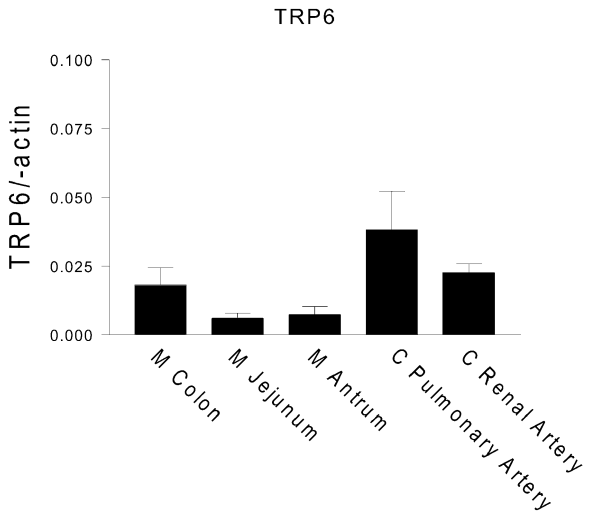

Fig. 4. Quantitative RT-PCR for TRP6 expression relative to β-actin. Values are shown for TRP6 steady-state transcripts relative to β-actin in the same preparation. Results are expressed as means ± SE.

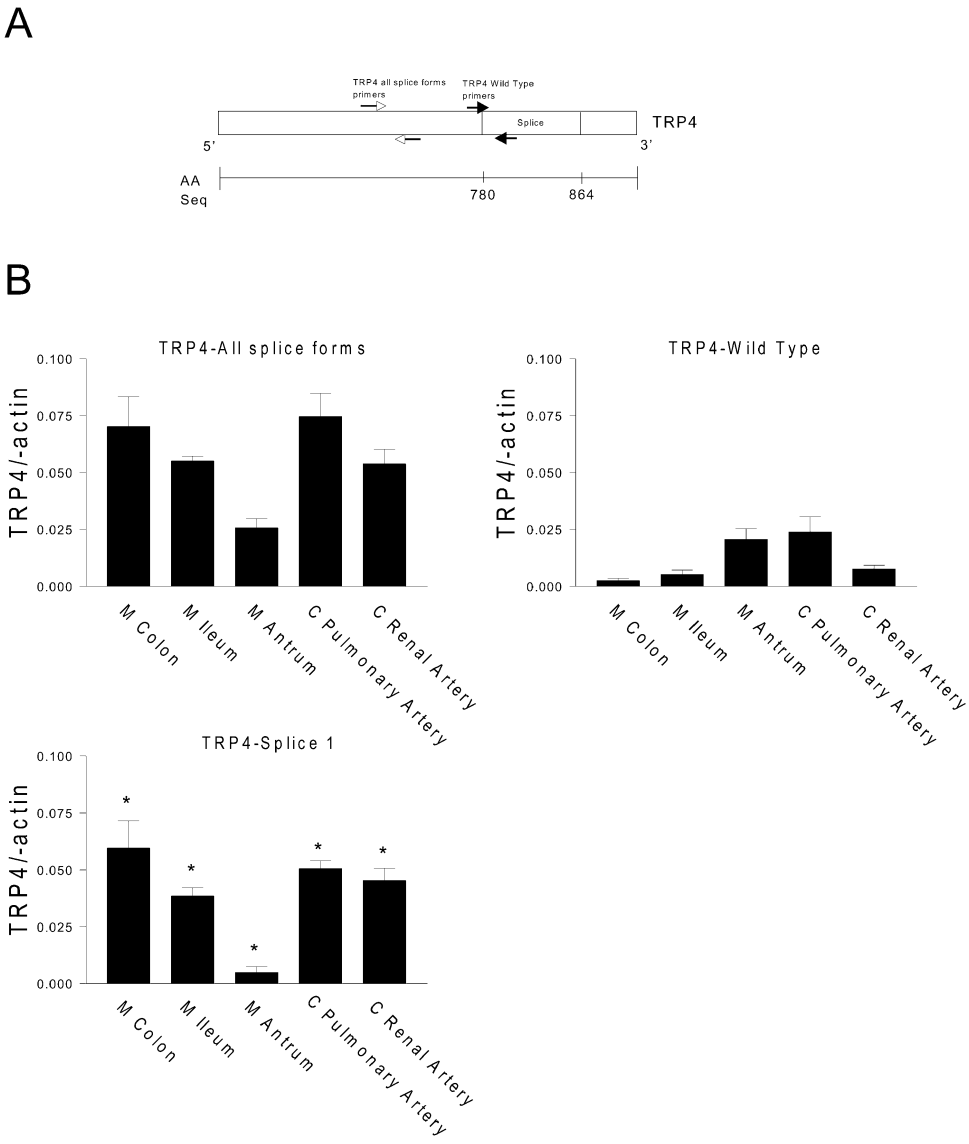

Fig. 5. Quantitative RT-PCR for TRP4 and splice variant expression relative to β-actin. *A*: primer design for amplification of TRP4 and an alternatively spliced transcript deleted for a 252-bp exon. *B*: values for TRP4 steady-state transcripts relative to β-actin in the same preparation. See text for strategy of primer design and estimation for quantitative steady-state transcript values. Results are expressed as means ± SE. *Significant difference between TRP4-splice 1 and TRP4-wild type transcripts ($n = 3$, $P < 0.05$).

(arbitrary units) was 0.0060 ± 0.0015 for murine colon, 0.0049 ± 0.0017 for murine jejunum, 0.0065 ± 0.0032 for murine antrum, 0.001 ± 0.0001 for canine pulmonary artery, and 0.0077 ± 0.0042 for canine renal artery. There was quantitatively more TRP7-splice 1 transcript expressed in murine colon and jejunum than TRP7-wild type ($n = 3$, $P < 0.05$).

DISCUSSION

TRP channels have been receiving increasing attention as molecular correlates for capacitative Ca^{2+} calcium entry (CCE) or store-operated cation channels (1, 7). These channels are activated in response to emptying of intracellular Ca^{2+} stores and act to replenish those stores (23). This Ca^{2+} entry mechanism is present ubiquitously in mammalian cells, although the mechanism of channel activation differs depending on cell type (reviewed in Ref. 7). A more specialized form of Ca^{2+} entry in response to receptor activation occurs in smooth muscles (I_{cat}) and involves the coupling of G protein-linked receptors to isoforms of PLC. This mechanism is independent of store depletion and may involve the direct activation of channels by G proteins (17). TRP channels have been implicated in both store-dependent and store-independent Ca^{2+} entry mechanisms and may be responsible for the receptor-operated channels (I_{cat}) reported in smooth muscles (2, 27). Given the importance of these channels to receptor-operated mechanisms in smooth muscles, it is important to identify which of these channels are expressed in different types of smooth muscles and their molecular characteristics. In this study we have detected TRP3, TRP4, TRP6, and TRP7 in several canine and murine GI and vascular smooth muscles and have analyzed the molecular pattern of their expression, including a detailed analysis of alternatively spliced transcripts expressed in these tissues.

TRP channels can be functionally organized into two groups, those that are dependent on store depletion for activation and those that are independent. While the

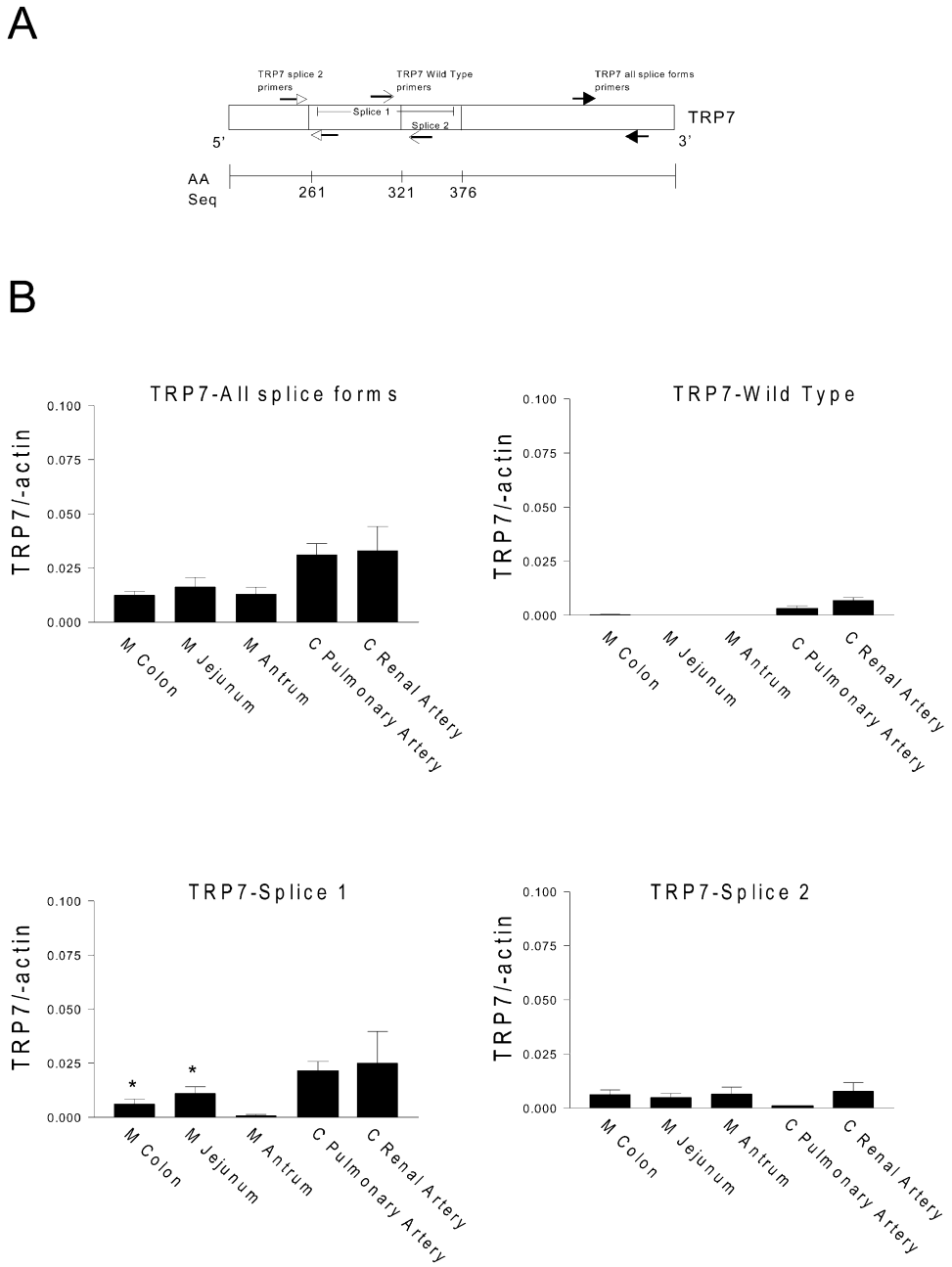

Fig. 6. Quantitative RT-PCR for TRP7 and splice variant expression relative to β-actin. *A*: primer design for amplification of TRP7 and alternatively spliced transcripts deleted for either 2 exons and 345 bp or for the upstream exon alone, deleting 165 bp. *B*: values for TRP4 steady-state transcripts relative to β-actin in the same preparation. See text for strategy of primer design and estimation for quantitative steady-state transcript values. Results are expressed as means ± SE. *Significant difference between TRP7-splice 1 and TRP7-wild type transcripts ($n = 3$, $P < 0.05$).

mechanism for channel activation is probably multifaceted for both groups, those that are store-depletion dependent are more likely to be CCE channels, and the store-independent TRP channels are more likely to encode receptor-operated nonselective cation channels, responsible for I_{cat} in many types of smooth muscle. Current research suggests that TRP1, TRP3, TRP6, and TRP7 encode channels of the latter type and that TRP2, TRP4, and TRP5 are CCE-type channels (reviewed in Ref. 1). It is striking that, for both canine and murine smooth muscles, the predominant TRP forms expressed are TRP4, TRP6, and TRP7. This suggests that a gene regulatory protein(s) expressed in smooth muscles directs this expression in concert with promoter specificity of these TRPs for smooth muscles. In a study performed on rat tissues including liver, lung, heart, kidney, brain, testis, ovary, and nodose ganglia, TRP1 and TRP3 were detected in all these tissues except liver (5). This finding suggests that these TRP channels might be ubiquitous CCE-type channels. In fact, this study also detected high levels of TRP1 expression in five different mammalian cell lines. However, our results indicate that this is not the case for smooth muscle cells, and TRP4 may carry out this function. Because TRP proteins can form heteromeric channels (16, 28), it is possible that a combination of TRP4, TRP6, and TRP7 may encode CCE in smooth muscles. In bovine adrenal cells, TRP4 was the only TRP channel detected at significant levels (21). A direct connection was made between TRP4 and CCE in these

cells by blocking CCE with antisense cDNA directed specifically against TRP4.

In this study we detected specific splice variants of TRP4 and TRP7 expressed in smooth muscles in a species- and tissue-specific expression pattern. Whether these differences are manifest in channel proteins is not known; however, it is striking that in all the murine smooth muscle cells examined, no TRP7 full-length transcripts could be detected. This result was confirmed at the quantitative level by using RNA derived from intact smooth muscles. In addition, the exon-deleted form of TRP4 predominated in all the canine and murine smooth muscles except antrum. It will be important to determine if any differences in channel kinetics or regulation exist between the alternatively spliced forms of these channels.

In conclusion, with the identification of TRP channel expression in several smooth muscles, it now becomes important to critically examine their biophysical and electrophysiological properties. Functional expression of the TRP isoforms will allow investigators to compare channel properties with native receptor-operated and CCE-type channels in smooth muscles and provide information necessary for transgenic studies, delineating their functions in smooth muscle cellular physiology.

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