Molecular properties of small-conductance Ca$^{2+}$-activated K$^+$ channels expressed in murine colonic smooth muscle

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Ro, Seungil, William J. Hatton, Sang Don Koh, and Burton Horowitz. Molecular properties of small-conductance Ca$^{2+}$-activated K$^+$ channels expressed in murine colonic smooth muscle. Am J Physiol Gastrointest Liver Physiol 281: G964–G973, 2001.—Small-conductance Ca$^{2+}$-activated K$^+$ (SK) channels are important participants in inhibitory neurotransmission in gastrointestinal smooth muscles. Three isoforms of an SK channel family were cloned from murine proximal colon smooth muscle. The transcripts encoding these subunits (SK1, SK2, and SK3) were detected in murine proximal colon and other peripheral tissues. The mRNA quan-
titative ratio of SK transcriptional expression in murine and canine colonic smooth muscles. The mRNA quan-
titative ratio of SK transcriptional expression in murine proximal colon is SK2 > SK3 > SK1; transcriptional expres-
sion of SK isoforms in canine proximal colon is minimal. SK3 immunohistochemical localization in murine small intestine (jejunum) and proximal colon showed immunoreactivity in circular and longitudinal muscularis. In transversely sec-
tioned muscularis, staining was localized at the cell mem-
brane in smooth muscle cells. Immunoreactivity was more intense in myenteric ganglia between longitudinal and cir-
cular muscularis and neuronal processes in circular and longi-
tudinal muscularis. Transient expression of mSK1, mSK2, and mSK3 in COS cells resulted in Ca$^{2+}$-activated voltage-
dependent channels. mSK1 is less sensitive to apamin compared with SK2 and showed intracellular Ca$^{2+}$ sensitiv-
ity (10$^{-8}$ to 10$^{-6}$ M) in asymmetrical K$^+$ (5/140 mM K$^+$) gradients. Our results suggest that SK channel expression var-
es in colonic myocytes from different species and may con-
tribute differentially to inhibitory junction potentials.

gastroenteric motility; apamin; small-conductance calcium-
activated potassium channel isoform

ACTIVATION OF SMALL-CONDUCTANCE Ca$^{2+}$-activated K$^+$ (SK) channels is voltage independent and depends on increases in the intracellular Ca$^{2+}$ concentration (7), as occurs from the action of purinergic neurotransmit-
ters on smooth muscle cells (7). In gastrointestinal (GI) tissues, ATP released from enteric inhibitory motoneu-
rons mediates a significant portion of inhibitory neuro-
transmission. The patterns of inhibitory junction po-
tentials (IJPs) evoked by neuronal stimulation are dif-
ferent in murine (11, 17) and canine colon (16). Many studies (1–3, 5, 18) of GI muscles have described at least two components of hyperpolarization in postjunc-
tional cells in response to inhibitory nerve stimulation. The initial fast component of hyperpolarization is attrib-
uted to the release of ATP and the slow component is due to nitric oxide (NO) release from inhibitory nerves. Both distinct components are present in murine colon, but only the slow component can be detected in canine colon (16). Apamin inhibits the fast component by ~30% in murine colon, whereas it has no effect on canine colon (16). The fast component of the IJP is at least partially due to activation of SK channels. However, the differences in apamin sensitivity between colonic muscles from differ-
ent species suggest the possibility of differential expres-
sion of SK channel genes and/or different properties for those channels.

A family of at least three closely related genes, SK1, SK2, and SK3, encode SK channels (4). They have been shown to share high overall structural homology with little similarity to other known K$^+$ channel families. Pharmacologically, each of the SK channel subunits has been shown to have a different sensitivity to the bee venom peptide toxin apamin, which blocks certain classes of SK channels. SK2 channels are highly sen-
tive to apamin, being half blocked by 60 pM apamin, and SK3 channels are half blocked by 1 nM apamin, whereas SK1 channels are not affected by 100 nM apamin (4, 10).

The aim of the present study was to determine whether the differences in the properties of inhibitory neurotransmission in murine and canine colonic smooth muscles could be partially due to differential expression of SK channel isoforms. Therefore, we have compared the molecular levels of SK channel expres-
sion from these two tissues. We have cloned and char-
acterized the cDNAs encoding SK channels in murine colon. We have also expressed the murine isoforms of SK in a mammalian cell line (COS) and determined several properties of mSK1 relevant to its role in inhibitory neurotransmission.

MATERIALS AND METHODS

RT-PCR. A homology search was performed using DNASIS version 2.5 (Hitachi Software Engineering) on the amino acid

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sequences of rat SK1 (rSK1), rSK2, rSK3, human SK1 (hSK1), hSK2, hSK3, and Gallus SK2 (gSK2) retrieved from the database of GenBank (accession nos. AP1000973, U68892, U68884, NM002248, NP067627, CAB61331, and AAP21783). The degenerate oligonucleotide DFSK (AGCATGGVGGCCTBAA) and DRSK (TCCATCATRAAGTTGTCG) (Y = CT, V = G/A/C, H = A/T/C, B = G/T/C, and R = A/G) corresponding to the pore region (see Fig. 2) were synthesized for use in RT-PCR (Bio-source, California).

Total RNA was extracted from mouse or canine proximal colon muscles stripped free of mucosa or from 20 freshly dispersed colonic smooth muscle cells as described previously (8). Poly(A)^+ RNA was isolated from the total RNA using Oligotex mRNA minikit (Qiagen). First-strand cDNA was synthesized using 200 U SuperScript II RNase H^− RT (GIBCO BRL) at 42°C for 50 min in the presence of 1 µg total RNA or 100 ng poly(A)^+ RNA in a 20 µl reaction volume. PCR reactions were performed using GeneAmp PCR system 2400 (Perkin-Elmer) by adding 1 U Taq DNA polymerase (recombinant, GIBCO BRL), 2 µl of the synthesized cDNA, 50 µM of the degenerate primers, 2.5 mM MgCl_2. The amplification procedure was as follows: 1 cycle at 95°C for 1 min, 35 cycles at 95°C for 15 s, 1 min at 50°C, and 2 min at 72°C. After PCR, 10 µl of the RT-PCR product was analyzed on a 2% agarose gel. The fragments amplified by RT-PCR were gel eluted and sequenced.

Rapid amplification of cDNA ends PCR. The DNA sequences obtained from RT-PCR allowed us to design SK gene-specific primers for rapid amplification of cDNA ends (RACE). The forward and reverse primers were as follows: for murine SK1, mSK1–1/mSK1–2rev; for mSK2, mSK2–1/mSK2–2rev; and for mSK3, mSK3–1 and mSK3–2rev (see Fig. 1). The cDNAs for 5′-RACE and 3′-RACE were prepared using a SMART RACE cDNA amplification kit (Clontech). RACE-PCR was performed in 25 µl of reaction mixture containing 2.5 µl of the cDNA, 40 pmol of each primer, 200 µM of each dNTP, 2.5 µl of 10× Advantage 2 PCR buffer, and 0.5 µl of 50× Advantage 2 polymerase mix (Clontech). PCR conditions were as follows: 1) 5 cycles at 94°C for 10 s and at 74°C for 3 min; 2) 5 cycles at 94°C for 10 s and at 72°C for 3 min; and 3) 25 cycles at 94°C for 10 s, at 68°C for 20 s and at 72°C for 2 min, with 25 cycles. PCR products were electrophoresed on a 2% agarose gel. RACE-PCR products were cloned for DNA sequencing into pCR2.1-TOPO (Invitrogen).

DNA sequencing and analysis. DNA sequencing was performed with an ABI 310 automated sequencer (Perkin Elmer). The DNA and the deduced amino acid sequences were analyzed by DNASYN version 2.5 (Hitachi Software Engineering) and by BLAST (National Center for Biotechnology Information).

Northern Blot analysis. DNA probes of 331, 340, and 386 bp were amplified by PCR from the cloned mSK1, mSK2, and mSK3, respectively, with the SK gene-specific primers between the regions sharing the least DNA sequence homology among the mouse SK genes. The DNA fragments were labeled with [α^32P]dCTP (ICN Biomedicals, Irvine, CA) by using the Prime-a-Gene labeling kit (Promega) and purified through a QiAquick gel extraction column (Qiagen). Northern blot was performed using OriGene (Rockville, MD) contained 2 µg of poly(A)^+ mRNA of each of the isolated mouse tissues. Northern blots containing colon tissue RNA were prepared by using a downward transfer system (Tuboblotter) and a Nytran SuperCharge nylon membrane (Schleicher & Schuell) with 1 µg of poly(A)^+ mRNA isolated from mouse or canine proximal colon muscles. The blots were then hybridized in UltraHyb hybridization buffer (Ambion) at 42°C for 24 h with 2 × 10^6 cpm/ml of the α^32P-labeled DNA probes. The blots were washed in 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) and 0.1% SDS twice at 42°C for 5 min and then washed twice in 0.1× SSC and 0.1% SDS at 56°C for 15 min. After washing, the blots were exposed for 3 days, and hybridization signals were visualized by using a membrane blot imager (GS-366 Sample Loading Dock, Bio-Rad, Hercules, CA) for detection of the hybridization signals. After hybridization was completed with the mSK1 probe, the probe was stripped by boiling in 1× SDS and 1× TE buffer (Tris-HCl and EDTA) for 5 min and reprobed successively with the mouse SK2, SK3, or β-actin genes.

Quantitative RT-PCR. cDNAs synthesized from RNAs of colon were amplified by RT-PCR. Real-time quantitative PCR was performed using Syber Green chemistry on an ABI Prism 5700 sequence detector (PE Biosystems, Foster City, CA). 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 set of SK primers were calculated yielding the transcriptional quantitation of SK relative to the endogenous β-actin standard. The data were plotted using Excel Chart Wizard and graphed using the GraphPad Prism. The PCR samples were analyzed on a 2% agarose gel and sequenced.

Tissue dissection and immunohistochemistry. BALB/c mice (9–15 days old) of either sex were killed by asphyxia (CO_2) followed by cervical dislocation. The use and care of the animals was approved by the Institutional Animal Use and Care Committee at the University of Nevada.

The small (jejunum) and large intestine (proximal colon) animals were removed and placed in cold Krebs-Ringer buffer (KRB) containing (in mM): 120.4 NaCl, 5.9 KCl, 15.5 NaHCO_3, 11.5 glucose, 1.2 MgCl_2, 1.2 Na_2HPO_4, and 2.5 CaCl_2 (pH 7.3–7.4). The luminal contents were washed with cold KRB. The intact tissues were pinned to the base of a Sylgard dish and fixed with paraformaldehyde (4%) in PBS for 20 min, washed in PBS for 10 min twice, and then cut into ~0.5-cm sections (tubes). The fixed sections of colon and jejunum were cryoprotected in increasing gradients of sucrose in PBS (5%, 10%, and 15% wt/vol) for 30 min each and in 20% sucrose in PBS overnight. Tissues were then embedded in Tissue Tek embedding medium (Miles, Lake Zurich, IL) and 20% sucrose in PBS (1:2, vol/vol) and rapidly frozen in isopentane precooled in liquid nitrogen. Cryosections were cut at 8 µm on a cryotome (Leica CM 3050).

After cryosectioning, sections were washed for 10 min twice with PBS. The sections were then blocked in 1% BSA containing 0.1% Triton X-100 for 1 h at room temperature. Excess blocking serum was removed, and sections were incubated with polyclonal anti-SK-3 primary antibodies (Alomone Labs, Jerusalem, Israel) at a dilution of 1:100 for 24 h at 4°C (both antibodies were raised in rabbit). For negative controls, primary antibody was omitted and PBS added in its place. Immunoreactivity was detected using Alexa 488 (green fluorescence) conjugated secondary antibody (Alexa 488 anti-rabbit IgG, Molecular Probes, Eugene, OR) at 5 µM/ml in PBS for 1 h at room temperature. Sections were then washed for 15 min three times in PBS and mounted with an aqueous mounting medium (Aqua-Mount, Southern Biotech, Birmingham, AL). Sections were then examined with a Zeiss LSM 510 confocal microscope. The confocal images were assembled and analyzed using MetaVue software (Universal Imaging Corporation, West Chester, PA). The immunoreactive area was calculated using the Image-Pro Plus software (MediaCybernetics, Silver Spring, MD).
SK channels in colonic myocytes.

Sections were examined with a Bio-Rad MRC 600 confocal microscope with excitation wavelengths appropriate for Alexa 488 (488 nm). Confocal micrographs were obtained from digital composites of serial sections (Z sections) taken through the section thickness, typically 14 sections at 0.48-μm steps. Z series were constructed with Bio-Rad Comos software, and final images were prepared using Adobe Photoshop software.

**SK channel expression in COS cells.** All the SK channel cDNAs were subcloned into the expression vector pCNA3.1/V5/His-TOPO (Invitrogen, Carlsbad, CA) for expression. COS cells were transfected using Lipofectamine 2000 reagent (Life Technologies, Grand Island, NY). pcDNA3.1/CT-GFP-TOPO (200 ng) was cotransfected in 24-well plates with 500 ng of pcDNA3.1-mSK1, pcDNA3.1-mSK2, or pcDNA3.1-mSK3. A green fluorescent protein-expressing plasmid was used as a reporter, and transfected cells were detected with fluorescence microscopy.

**Voltage-clamp methods.** The whole cell patch-clamp technique was used to record membrane currents from COS cells expressing SK channel cDNAs. Currents were amplified with an Axopatch 200B amplifier (CV-4 headstage; Axon Instruments, Foster City, CA) and digitized with a 12-bit analog-to-digital converter (Axon Instruments). Data were filtered at 1 kHz with pClamp software (Axon Instruments). For the single-channel recordings, data were sampled at 5 kHz and filtered at 1 kHz. For the whole cell configuration of the patch-clamp technique, COS-7 cells were bathed in a solution containing (in mM): 5 KCl, 135 NaCl, 2 CaCl2, 1.2 MgCl2, 10 glucose, and 10 HEPES adjusted to pH 7.4 with Tris. The pipette solution contained (in mM): 140 KCl, 5 MgCl2, 2.7 K₂ATP, 0.1 Na₂GTP, 2.5 creatine phosphate disodium, 0.1 EGTA, and 5 HEPES adjusted to pH 7.2 with Tris.

For recordings of K⁺ channels in excised patches, the bath solution contained (in mM): 140 KCl, 1 EGTA, and 10 HEPES adjusted to pH 7.4 with Tris. To test Ca²⁺ sensitivity of channels in the patch, we added Ca²⁺ to bath solution buffered by 1 mM EGTA to create Ca²⁺ activities from 10⁻⁸ to 10⁻⁶ M. Activities were calculated with a program developed by C.-M. Hai (University of Virginia, Charlottesville, VA). The pipette solution was identical to the whole cell bath solution and included 200 nM charybdotoxin to inhibit large-conductance Ca²⁺-activated K⁺ channels.

**RESULTS**

Cloning of SK channel cDNAs from mouse proximal colon. Alignment of the sequences of the reported SK genes (rSK1, rSK2, rSK3, hSK1, and hSK3) displayed highly conserved DNA sequences in regions corresponding to pore domain. The highest identity falls into a region of 125 amino acids of the S5, pore, and S6 regions of the three SK isoforms. Based on these conserved regions, the degenerate primers DFSK and DRSK were synthesized and used to amplify all SK subunit homologous sequences by RT-PCR, from total RNA isolated from murine proximal colon. Amplicons corresponding to 377 bp were amplified with degenerate primers by RT-PCR. The amplicons could be organized into four groups based on a detailed restriction enzyme analysis. All four groups were sequenced on both strands. The sequences encoding the conserved domains present in SK genes showed 93–99% homology to rSKs and mSK4 (GenBank accession no. NM008433): group 1, 93.3% to rSK1; group 2, 97.8% to rSK2; group 3, 97.8% to rSK3; and group 4, 99.1% to mSK4. The DNA sequences of groups 1, 2, and 3 were used to design gene-specific primers for isolation of the 5‘ and 3‘ ends of mouse SK1, SK2, and SK3 from mouse colon cDNA templates based on the RACE-PCR method. At least five clones from each 5‘- and 3‘-RACE product were sequenced. The 5‘ and 3‘ sequences were assembled to generate the full-length cDNAs. To prevent the possible joining of 5‘ and 3‘ cDNA fragments derived from two different transcripts, the full-length cDNAs were generated by PCR using primers designed from the extreme 5‘ and 3‘ ends of the assembled cDNAs and sequenced. The assembled cDNAs matched 99–100% with the generated full-length cDNA. Figure 1 summarizes all PCR products (mSK1–377, mSK2–377, and mSK3–377) amplified with degenerated primers (DFSK and DRSK; mSK15, mSK33, mSK25, mSK23, mSK35, and mSK33). Full-length mouse cDNAs of SK1, SK2, and SK3 are 2,136, 2,065, and 2,287 bp long, respectively (GenBank accession nos. AF357239, AF357240, and AF357241; Fig. 2). In the cDNA sequences, mSK2 shares 64% homology with mSK1 and 50.6% with mSK2 (Fig. 2). All primers underlined in Fig. 2 were selected within less homologous sequences, preventing possible cross contamination among mSKs. The SK1 open reading frame covers 1,602 bp, encoding a protein of 534 amino acids.
Fig. 2. cDNA sequence alignment of mSK1, mSK2, and mSK3 genes. Sequences were aligned using DNASIS version 2.5. Gaps are represented by dashes. The putative translational start codon ATG and the stop codons TGA, TAG, and TAA are in bold. Shading denotes perfect matches in aligned sequences. The A sequence of the translational start site ATG is numbered 1. The 5'-untranslated sequences are reverse numbered with a minus denoting upstream from the ATG, whereas the 3'-untranslated sequences are indicated with a plus from the stop codon. Underlined sequences represent those used as primers to analyze quantitative and qualitative mRNA expression (mSK15/mSK1–4r, mSK2–6/mSK2–6r, and mSK3–5/mSK3–5r), to generate probes for Northern bolt (mSK1–5/mSK1–5r, mSK2–6/mSK2–8r, and mSK35/mSK3–6r), and to generate full-length cDNAs for expression vector constructs (mSK15/mSK13, mSK25/mSK23, and mSK35/mSK33). Two degenerate primers (DFSK and DRSK) and gene-specific primers (mSK1–1/mSK1–2r, mSK2–1/mSK2–2r, and mSK3–1/mSK3–2r) for RACE are also underlined.
Fig. 3. Amino acid sequence alignment of mSK1, mSK2, and mSK3 with hSK1, rSK1, mbSK1, gSK2, rSK2, hSK3, and rSK3. Sequences were aligned with the computer program DNASIS version 2.5. Gaps are represented by dashes. The 6 predicted transmembrane domains (S1–S6) and the pore region (P) are overlined. K⁺ selective filter GYG is boxed in the pore. The 2 amino acid residues surrounding the pore that are important for apamin sensitivity are indicated by *. Two glutamine repeats found at the expanded NH₂ terminus of SK3 are boxed. ERS, endoplasmic reticulum retention signal at the NH₂ terminus; CaM BS, putative calmodulin binding site at COOH terminus. The 20 amino acid residues of hSK1 (21) and the 15 amino acid residues of rSK2 (20) boxed at the site are critical for calmodulin binding. Residues conserved among all the SK sequences are shaded. Amino acid numbers for the full-length coding sequences are given at left.
acids with a predicted molecular mass of 58.98 kDa. The SK2 open reading frame covers 1,722 bp, encoding a protein of 574 amino acids with a predicted molecular mass of 63.48 kDa. The SK3 open reading frame covers 2,193 bp, encoding a protein of 731 amino acids with a predicted molecular mass of 81.25 kDa (Fig. 3). The SK1 protein is 99.3% identical (4 amino acids are different) to mouse brain SK1 (Genbank accession no. AF116525), 92.5% identical to rat SK1, and 77.3% identical to human SK1. However, unlike SK1 from other species, three amino acids (AQK) are deleted in the putative calmodulin binding site at the COOH terminus. The 20-amino-acid residues of hSK1 at the calmodulin binding site have been shown to bind to both apocalmodulin and Ca\(^{2+}\) calmodulin (21). The three amino acids deleted in the splice variant were also reported in hSK1 and have the same affinity to calmodulin (21). The SK2 protein is 98.7% identical to rSK2, 96.1% identical to hSK2, and 90.2% identical to gSK2; the SK3 protein is 97.4% identical to rSK3 and 94.6% identical to hSK3. The most diversity in sequence and length between the SK isoforms is found in the NH\(_2\) terminus within which several amino acid repeats are present. SK3 contains extended 175 amino acids at the NH\(_2\) terminus. Two glutamine (Q) repeats are found within the region. The extended glutamine repeats are involved in many human hereditary neurodegenerative diseases and have been identified in the α\(_3\)-P/Q-type Ca\(^{2+}\) channel with 4–17 repeats (21–27 repeats in spinocerebellar ataxia patients) and hSK1 with 12–28 repeats (6, 22). The second repeat of mSK3 ranges from 16–17 repeats (n = 4), which are also polymorphic in length and position in individual mice (data not shown). Two apamin response residues residing on each side of the pore are responsible for the difference in sensitivity to the bee venom peptide apamin (10). These residues in mSKs are matched to other cloned SK subunits aligned. Aspartic acid and asparagine in SK2 are the most sensitive to apamin whereas glutamic acid and histidine in SK1 are almost insensitive. Aspartic acid and histidine in SK3 show medium sensitivity (12).

Expression of SK transcripts in murine and canine tissues. Northern blots containing RNA prepared from various tissues were hybridized with SK subunit probes. Transcripts specific for SK channels were expressed in murine tissues and canine colon (Fig. 4). SK1 transcripts of 3.2 and 2.4 kb were expressed in mouse brain and liver and relatively lower expression was detected in the heart, kidney, testes, and colon. SK2 was highly expressed in mouse brain, liver, and colon and least expressed in the kidney and testes. A major transcript of 3.2 kb was detected in those tissues expressing SK2. Three transcripts (3.2, 2.7, and 1.8 kb) in liver and two (3.2 and 1.8 kb) in colon were detected. SK3 was ubiquitously expressed in all tissues. A major transcript of 4.8 kb was detected in mouse brain, liver, and colon and least expressed in the kidney and testes. A major transcript of 3.2 kb was detected in those tissues expressing SK2. Three transcripts (3.2, 2.7, and 1.8 kb) in liver and two (3.2 and 1.8 kb) in colon were detected. SK3 was ubiquitously expressed in all tissues. A major transcript of 4.8 kb was detected in brain, kidney, liver, small intestine, testes, and thymus, and an additional transcript of 10 kb was detected in liver and colon. Only a 10-kb transcript of SK3 was detected at a low level in canine colon. Transcripts specific for SK1–2 could not be detected from canine colon. However, the
probes did hybridize to SK transcripts from canine brain RNA (see Fig. 4).

To more specifically examine SK channel expression in smooth muscle cells from mouse and canine colon, we performed RT-PCR using the following SK isoform-specific primers: mSK15' and mSK1–4r for the mSK1 gene, mSK2–6 and mSK2–6r for the mSK2 gene, and mSK3–5 and mSK3–5r for the mSK3 gene (see Fig. 2 for primer positions). Products of 81, 140, and 114 bp expected from cDNA sequences of mSK1, mSK2, and mSK3, respectively, were amplified from all smooth muscle cells (Fig. 5A). Although Northern blot detected only SK3 in canine, all SKs were amplified at a low level by RT-PCR. The same results were obtained with two pairs of other primers. All products were sequenced. The sequences of the mouse SK products were 100% identical to the cloned mouse SK cDNAs, whereas the sequences of the canine SK products showed 95% homologies to the mouse cDNAs. There were no amino acid differences in the short regions analyzed.

Quantitative RT-PCR revealed that SK2 is dominantly expressed in mouse colon, whereas SK1 and SK3 are expressed at relatively low levels. The expression levels were normalized relative to β-actin as an internal control. The relative transcriptional expressions of SK in murine colon are shown in Fig. 5B. SK expressions relative to β-actin (in arbitrary units, means ± SE) were 0.00098 ± 0.00063 for SK1, 0.03619 ± 0.01057 for SK2, and 0.00294 ± 0.00183 (n = 3).

**Immunohistochemistry of SK3 channels in murine colon and jejunum.** Immunohistochemical staining for SK2- and SK3-like immunoreactivity in cryostat sections demonstrated that the antibody raised against SK2 was inappropriate for immunohistochemical applications in the tissues and species tested here. SK3-like immunoreactivity was detected in both murine jejunum and proximal colon. Qualitative differences in expression of SK3 were observed between species and regions of the GI tract. In murine jejunum and colon, SK3-like immunoreactivity was observed and found to be discretely located at the cell membrane in smooth muscle cells (Fig. 6, B and D). Intense SK3-like immunoreactivity was observed in other cell types located within the longitudinal and circular muscularis externa and at the level of the myenteric plexus between the longitudinal and circular muscularis externa in both the proximal colon (Fig. 6A) and jejunum (Fig. 6C). The location and morphology of these cell types is characteristic of neuronal processes and myenteric ganglia, respectively.

**Expression of mSK1 channels on COS cells.** The evoked IJP in murine colon is not blocked completely by apamin (300 nM) (11). We found that all three...
isoforms of SK channels are expressed in murine colonic myocytes. SK2 has been reported (10) to be apamin sensitive with an IC50 of ~80 pM, whereas SK3 is relatively apamin insensitive (IC50 51 nM). The properties of mSK1 expressed in mammalian cells have not been reported, and we therefore examined mSK1 in more detail. Cells were held at −80 mV and depolarized to 70 mV by 10-mV increment in dialyzed whole cell configuration. The peak currents at 0, 30, and 60 mV were 77 ± 13, 485 ± 84, and 1,553 ± 178 pA, respectively (n = 4, Fig. 7, A and D). In contrast, mock transfected cells showed small endogenous current, but those currents were smaller than 160 pA at 60 mV (data not shown). Apamin treatment inhibited outward currents in a dose-dependent manner, and IC50 was 28 nM (n = 5 cells, Fig. 7, B, C, and E). Figure 7E demonstrated the summarized data of normalized currents at 50 mV of test potential. To confirm Ca2+ sensitivity of the expressed mSK1 channel, we performed excised single channel recordings. The open probability (Po) decreased when the cytosolic surface was exposed to low Ca2+ (10−6 M, Po = 0.55 ± 0.12) in an asymmetrical K+ condition (5/140 mM, n = 6, Fig. 7F). The holding potential was 0 mV to prevent activation of a nonselective cation channel.

DISCUSSION

The major findings of this report are that 1) SK1, SK2, and SK3 are differentially expressed in murine and canine colonic smooth muscles, 2) the absence of a fast IJP component in canine colon may be due to the extremely low level of SK channel expression in these muscles, and 3) the level of apamin sensitivity for IJP in murine colon may not be solely dependent on the expression of SK channels because the predominant channel is the highly apamin-sensitive SK2, whereas the fast IJP is only blocked 30% by apamin application. Different tissues and species manifest different degrees of the slow and fast IJPs, suggesting considerable heterogeneity in the postjunctional receptors and mechanisms linked to neuron-evoked inhibition. In canine colon, enteric inhibitory inputs are mediated pri-
sensitivity has not been reported for the SK1 form with 28 nM for mSK1 expressed in COS cells (15). Ca\(^{2+}\) channels are voltage independent and Ca\(^{2+}\)-mediated via apamin-insensitive SK channels. SK channels have been identified, and the apamin-insensitive and -insensitive isoforms of SK (19). taenia coli, apamin inhibits the fast component of IJPs in the GI tract, these channels transduce fluctuations in intracellular Ca\(^{2+}\) concentration into changes in membrane potential and can regulate membrane excitability via activity of spontaneous transient outward currents (13). Therefore, we determined the SK channel gene expression in murine and canine colonic myocytes.

In the GI tract, there are many types of cells, including smooth muscle cells, neuronal cells, and interstitial cells of Cajal. To distinguish between smooth muscle and specialized cell expression in the muscle syncytium, we examined both muscle and myocytes. In murine colon, RT-PCR detected significant expression of SK1, SK2, and SK3 from RNA derived from both smooth muscle cells and tissue. Canine colonic smooth muscle cells and tissue also yielded detectable amplification, we expected these discrepancies to account for the lower level of SK channel contribution to IJPs in canine colon.

Two apamin-response residues residing on each side of the pore domain are responsible for the difference in sensitivity to apamin (10). Aspartic acid and asparagine in SK2 impart sensitivity to apamin whereas glutamic acid and histidine in analogous positions in SK1 cause the channel to be relatively insensitive to apamin block. It is not clear whether combinations of SK channel subunits in a heterotetramer arrangement would affect apamin sensitivity of the resulting channel, but expression of all three forms in murine colonic myocytes causes us to speculate about this possibility. Such an arrangement might explain the discrepancy between SK2 predominant expression coinciding with the majority of the IJP being relatively apamin insensitive in murine colon. There have been no reports concerning heterotetramer formation of SK channel isoforms, and detecting heteromeric assembly based on apamin binding would be difficult. Canine colon displays weak expression of SK channels from tissue-derived RNA. This is consistent with the lack of a fast IJP in this preparation (16).

hSK1 is more sensitive to apamin than the murine form reported here, having an IC\(_{50}\) of 2.6 nM compared with 28 nM for mSK1 expressed in COS cells (15). Ca\(^{2+}\) sensitivity has not been reported for the SK1 form expressed in mammalian cells. However, we find that mSK1 is sensitive to micromolar Ca\(^{2+}\) concentrations, which is similar to hSK1 expressed in Xenopus oocytes (12).

The concentration of apamin typically used during intracellular recording of IJPs is in the 100–200 nM range (14). From our data, we would expect these concentrations to block all SK channels, yet the fast hyperpolarization is not completely blocked. One possible way to explain this discrepancy is that apamin-insensitive intermediate-conductance Ca\(^{2+}\)-activated K\(^{+}\) channels are involved in the IJP. Another possibility is that the effective concentration of a peptide blocker such as apamin is much lower in tissue strip intracellular recording experiments than in dispersed cell electrophysiological studies.

In conclusion, we have carefully examined the expression of SK channel isoforms in murine and canine colonic myocytes. We find that although discrepancies exist in apamin sensitivity for IJP and SK isoform transcriptional expression in murine colon, SK2 and SK3 are primary components in inhibitory neurotransmission.

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