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Cloning and expression of a $K_v1.2$ class delayed rectifier K^+ channel from canine colonic smooth muscle

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ABSTRACT **A cDNA (CSMK1) encoding a delayed rectifier K^+ channel of the $K_v1.2$ class was cloned from canine colonic circular smooth muscle and expressed in *Xenopus* oocytes. These channels appear to be uniquely expressed in gastrointestinal muscles and may participate in the electrical slow wave activity. Functional expression of CSMK1 in *Xenopus* oocytes demonstrated a K^+ current that activated in a voltage-dependent manner upon depolarization. This current was highly sensitive to 4-aminopyridine (IC_{50}, 74 μM). A low-conductance K^+ channel was identified in inside-out patches from oocytes injected with CSMK1. This channel displayed a linear current–voltage relation with a slope conductance of 14 pS. The channels were blocked in a concentration-dependent manner by 4-aminopyridine. Northern blot analysis demonstrated that CSMK1 is expressed in a wide variety of gastrointestinal smooth muscles. Portal vein, renal artery, and uterus do not express CSMK1, suggesting that, among smooth muscles, expression of this K^+ channel may be restricted to gastrointestinal smooth muscles. CSMK1 is 91% homologous to RAK, a delayed rectifier K^+ channel cloned from rat heart, but displays unique pharmacological properties and tissue distribution.**

The contractile behavior of smooth muscles depends to a considerable extent upon the electrical activities of these muscles. Electrical activity can vary from a tonic membrane potential that changes slowly in response to regulatory substances to fast Ca^{2+} action potentials that occur in response to excitatory agonists. Gastrointestinal (GI) smooth muscles exhibit the complete range of electrical events, and the cause of this diversity has been a central question of investigation for at least 50 years.

Prevalent in the electrical repertoire of GI muscles is the activity known as electrical slow waves (1–4). The major ionic currents that appear to be responsible for these events have been reviewed (5). K^+ channels found in GI smooth muscles include Ca^{2+}-activated K^+ channels (e.g., ref. 6), delayed rectifiers (e.g., refs. 7 and 8), inward rectifiers (e.g., ref. 9), and a lemakalim-sensitive conductance that may be an ATP-sensitive K^+ channel (10). Among these currents, delayed rectifiers, which are voltage-dependent and non-Ca^{2+}-dependent, appear to play a critical role. For example, in circular muscle cells at physiological temperatures, these currents activate rapidly and tend to limit the amplitude of upstroke depolarization phase of slow waves (7). They may also balance inward Ca^{2+} currents at certain potentials allowing development of the plateau phase of slow waves. This phase is critical for excitation–contraction coupling in some regions of the GI tract (3, 7, 11). Voltage-dependent K^+ currents with different properties appear to be expressed in muscles that exhibit fast Ca^{2+} action potentials, such as the longitudinal muscles of the colon (8). These studies have suggested that the diversity in electrical activity in GI muscles may be related to the expression of different species of voltage-dependent K^+ channels, but definitive descriptions of the properties of these currents have been difficult to obtain because of inadequate means of dissecting specific components of K^+ currents from whole-cell currents.

Molecular biological studies have been extremely useful in identifying and characterizing several classes of voltage-dependent K^+ channels, such as the A-type and delayed rectifier gene family (12), Ca^{2+}-activated K^+ channels encoded by the *slo* gene in *Drosophila* (13), *eag* (14) whose function has yet to be established, and recently, an inwardly rectifying ATP-sensitive K^+ channel (15). Studies of smooth muscle K^+ channels have lagged far behind other excitable cells, and to our knowledge, no K^+ channel from any type of smooth muscle has been cloned, functionally expressed, and studied in isolation of other contaminating currents. In the present study we have sought to better understand the properties of K^+ currents of GI smooth muscles by the cDNA cloning and functional expression of a delayed rectifier K^+ channel from canine colonic circular smooth muscle.† These channels are of the $K_v1.2$ class of voltage-dependent K^+ channels (16), and their pharmacology and expression may be unique to GI smooth muscle.

MATERIALS AND METHODS

Tissue Dissection and mRNA Preparation. GI and other smooth muscle tissues were dissected as described (17, 18). Poly(A)$^+$ RNA was prepared from dissected tissue using the FastTrack kit (Invitrogen) by following the manufacturers instructions. Muscles from four or five animals (0.5–1.0 g) were pooled for each RNA preparation. Each sample of RNA was adjusted to the same concentration. Poly(A)$^+$ RNA was also isolated from canine brain and heart using this method.

cDNA Library Construction and Screening. λZap cDNA libraries were constructed from colonic smooth muscle mRNA by the method of Gubler and Hoffman (19) and assisted by a construction system (Invitrogen). PCR-generated cDNA fragments and subsequent partial cDNA clones were used as radiolabeled probes labeled by the random-primer methods (20). Approximately 1×10^6 recombinants were screened at high stringency (21). Recombinant DNA was excised and analyzed in the Bluescript vector and host system (Stratagene). Several overlapping clones were analyzed by nucleotide sequencing. One was picked for further study and subcloned into a *Xenopus* oocyte expression vector (pSP64T) (22).

Oocyte Injection and Electrophysiological Methods. The plasmid containing the CSMK1 insert was linearized with

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Abbreviations: 4-AP, 4-aminopyridine; *I–V*, current–voltage; GI, gastrointestinal.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. L19740).

*Bam*HI, and capped transcripts were synthesized *in vitro* with SP6 RNA polymerase as described (22). Transcripts were resuspended in 10 mM Tris·HCl, pH 7.4/1 mM EDTA at 1 μg/μl.

Ovarian lobes were removed from anesthetized adult female *Xenopus laevis* frogs (Xenopus 1, Ann Arbor, MI) under sterile conditions. The lobes were then mechanically opened and the oocyte follicular layer was removed by incubation with collagenase (1 mg/ml) in Ca^{2+}-free ND96 (see below) solution at room temperature for 2–3 h. The oocytes were then collected, rinsed, and stored in ND96 solution (2.5 mM pyruvate/96 mM NaCl/1.5 mM $CaCl_2$/2 mM KCl/1 mM $MgCl_2$/5 mM Hepes plus antibiotics [penicillin (100 units/ml)/streptomycin (100 μg/ml)] at 19°C for up to 24 h prior to injection. Only stage V and VI oocytes were selected for injection. mRNA (1 μg/μl) was injected in a total volume of 50 nl and the oocytes were stored until assay for between 2 and 4 days.

K^+ currents were recorded using the double-electrode voltage clamp technique with a Dagan 8500 (Dagan Instruments, Minneapolis) amplifier. Microelectrodes were pulled to have a resistance of 1–3 MΩ and filled with 3 M KCl. Recordings were made at room temperature in Ca^{2+}-free ND96 solution to inhibit the endogenous Ca^{2+}-activated Cl^- current in oocytes. Single-channel K^+ currents were low-pass-filtered at 500 Hz and recorded using an Axopatch-1D amplifier (Axon Instruments, Burlingame, CA). Inside-out patches were obtained from oocytes after mechanical removal of the vitelline membrane as described by Methfessel *et al.* (23). Borosilicate glass pipettes (1–2 MΩ) filled with (*i*) 140 mM KCl/0.1 mM $GdCl_3$/10 mM Hepes or (*ii*) 5 mM KCl/135 mM NaCl/0.1 mM $GdCl_3$/10 mM Hepes, pH 7.2, were used. Bath solution was 140 mM KCl/0.1 mM $GdCl_3$/10 mM Hepes/1 mM EGTA, pH 7.2. All voltage clamp protocols, data acquisition, and analysis were performed by using PCLAMP software (Axon Instruments). $GdCl_3$ was included to inhibit stretch-activated channels. Data are expressed as mean ± SEM.

Northern Blot Analysis. RNA was size-fractionated on 1.0% agarose/formaldehyde gels and transferred to Immobilon filters (24). Filters were baked and prehybridized in 50% (vol/vol) formamide/5× standard saline citrate/50 mM sodium phosphate/5× Denhardt's solution/sonicated salmon sperm DNA (50 μg/ml)/0.1% SDS/10% (wt/vol) dextran sulfate at 42°C overnight. ^{32}P-labeled probes were generated using random hexanucleotide priming (20). Hybridization was performed under the same conditions overnight. The filters were washed at high stringency to assure specificity of labeling. A cDNA of CHO-B, a Chinese hamster ovary cell mRNA expressed at equivalent levels in all tissue examined (25), was used as an internal standard to verify that equal amounts of poly(A)$^+$ RNA was applied. Autoradiography was performed at −80°C with intensifying screens.

RESULTS

cDNA Cloning and DNA Sequence Analysis. Two distinct amplification products were recovered from reverse transcription–PCR that, when used as probes in Northern blot analysis, demonstrated expression in colonic circular muscle (26). These were employed as probes for screening a colonic smooth muscle cDNA library. One 2.1-kb cDNA clone, designated CSMK1, had one open reading frame encoding 499 aa. The amino acid sequence of CSMK1 was compared to other K^+ channels of the K_v1.2 class (Fig. 1).

CSMK1 displayed high homology (91%) to RAK, a rat atrial K^+ channel that, when expressed in oocytes, displayed delayed rectifier properties (27). CSMK1 also displayed high homology to RBK2 (91%) and RCK5 (90%), rat brain K^+

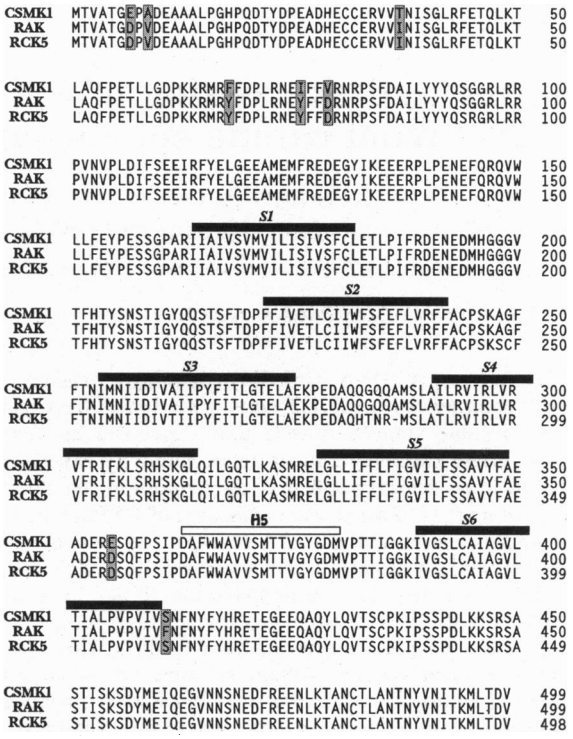

FIG. 1. Amino acid sequence of CSMK1 aligned with other K^+ channels of the K_v1.2 class. RAK was isolated from rat heart atrium (27); RCK5 was isolated from rat brain (12). Divergent amino acids are indicated by shading. Transmembrane-spanning segments S1–S6 and the putative pore region H5 are indicated.

channels (12, 28). All are members of the K_v1.2 class of K^+ channel cDNAs (16).

Electophysiological and Pharmacological Properties of CSMK1 Expressed in *Xenopus* Oocytes. Two days after mRNA injection, large outward currents could be elicited upon depolarization from a holding potential of −60 mV in >90% of injected oocytes examined. Fig. 2*A* shows typical outward currents evoked from an injected oocyte by 500-ms steps from a holding potential of −60 mV to a range of test potentials from −40 mV to +40 mV applied in 10-mV increments. Measurable current could be detected at all test potentials positive to −40 mV and these increased in a voltage-dependent manner. The peak at +40 mV was 2.53 ± 0.22 μA (n = 19). Fig. 2*B* shows the current–voltage (*I–V*) relationship over the range of test potentials examined using a holding potential of −60 mV.

The K^+ selectivity of this current was examined by an investigation of the reversal potential of the tail current in the presence of various concentrations of external K^+ (Fig. 2*C*) from 2 mM to 70 mM. Briefly, the reversal potential of the tail current was monitored in bath solutions containing 2 mM, 10 mM, 20 mM, 50 mM, and 70 mM K^+. Cells were stepped from a holding potential of −60 mV to a potential of +40 mV to fully activate the current and then back to a series of test potentials ranging from +40 mV to −120 mV to determine tail current reversal potentials. The line plotted has a slope of 58 mV per decade change in extracellular K^+ concentration when an internal K^+ concentration of 130 mM is assumed. This is the expected slope for a perfectly selective K^+ electrode.

The activation and inactivation characteristics of the K^+ current were investigated. A clamp step applied from −60 mV to +20 mV rapidly activated the CSMK1 K^+ current with a half-activation time of 7.6 ± 0.2 ms (n = 6). There was a noticeable inactivation of the current over the length of depolarizing step potentials and the rate of inactivation

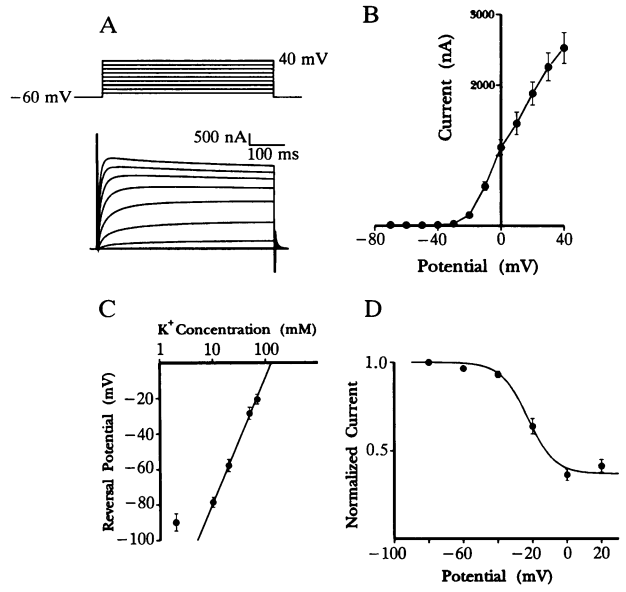

FIG. 2. K^+ currents elicited from oocytes injected with CSMK1 mRNA. (*A*) Representative K^+ currents evoked from an oocyte by a step from a holding potential of −60 mV to test potentials from −40 mV to +40 mV. A measurable current was seen with voltage steps to potentials positive of −40 mV, which increased in a voltage-dependent manner. (*B*) The peak *I–V* relationship for 19 oocytes is shown. (*C*) The K^+ selectivity of CSMK1 is shown. Tail current reversal potentials were measured by stepping from −60 mV to +40 mV for 200 ms, to fully activate the CSMK1 current, and then returning to potentials from +20 mV to −120 mV. This protocol was repeated in five external K^+ concentrations and the reversal potential was plotted against the logarithm of the external K^+ concentration. The line plotted has a slope of 58 mV per decade change in extracellular K^+ concentration when an internal K^+ concentration of 130 mM is assumed. (*D*) Inactivation characteristics from eight oocytes. The oocytes were held at prepulse potentials from −80 mV to +20 mV for 20 s and then stepped to a test potential of +20 mV for 5 s. The results are expressed as current normalized to that elicited by the test pulse after the −80-mV prepulse.

increased with increasing amplitudes of depolarization. The voltage dependence of steady-state inactivation is shown in Fig. 2*D*. Oocytes were held at membrane potentials of −80 mV and stepped to prepulse potentials from −80 mV to +20 mV for 20 s. At the end of these depolarizations, the oocytes were stepped briefly back to −80 mV (5 ms) and then to a test potential of +20 mV for 5 s. The inactivation curve (n = 8) was plotted (Fig. 2*D*) as the current elicited by the test potential after each of the prepulse potentials normalized to the current elicited upon stepping to the test potential in the absence of a prepulse potential. The curve was fitted with a Boltzmann function of the form:

$$I = (C1 - C2)/[1 + \exp(V - Vh/Vs)] + C2,$$

where $C1$ and $C2$ are the maximum and minimum in the normalized current, Vh = −15 mV is the prepulse potential for half inactivation, and Vs = 7.7 is the slope factor. By using 20-s prepulses, inactivation was incomplete; ≈35% of the current failed to inactivate.

We examined block of CSMK1 K^+ current by 4-aminopyridine (4-AP). Currents elicited by steps from a holding potential of −60 mV to test potentials of −20 mV, 0 mV, and +20 mV for 100 ms are shown under control conditions (Fig. 3*A*) and in the presence of 100 μM 4-AP (Fig. 3*B*). The current was reduced at all test potentials. Fig. 3*C* shows the dose–response relationship for 4-AP using a range of doses in nine oocytes. The steady-state response was measured during a step to a test potential of +20 mV at t = 90 ms, a time at which

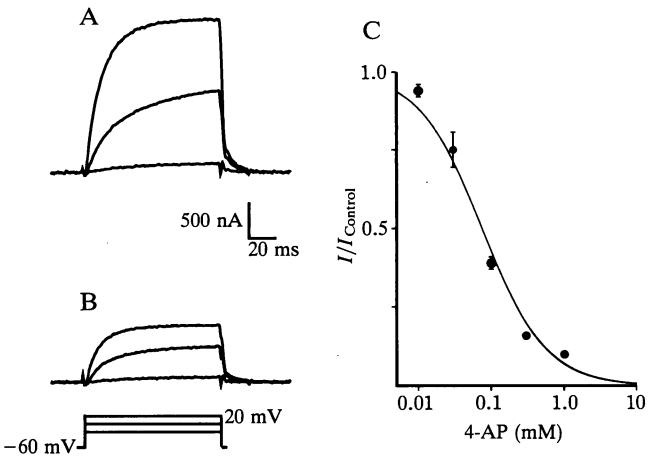

FIG. 3. Pharmacology of CSMK1 K^+ current. Representative current traces in the absence (*A*) and presence (*B*) of 100 μM 4-AP. The oocyte was held at −60 mV and stepped to test potentials of −20 mV, 0 mV, and +20 mV for 100 ms. 4-AP inhibited the current at all test potentials. (*C*) Dose–response relationships for the 4-AP (nine oocytes) steady-state block. The current was measured during a step to a test potential of +20 mV at t = 90 ms. The IC_{50} value for 4-AP was 74.7 μM.

the current had reached a peak, during steps from −60 mV to +20 mV. 4-AP blocked the CSMK1 current with an IC_{50} value of 74.7 μM. The CSMK1 K^+ current was found to be relatively insensitive to external tetraethylammonium with 10 mM reducing the current by only 11 ± 4% (n = 3).

Single-channel currents were recorded in inside-out patches obtained from oocytes after mechanical removal of the vitelline membrane. In patches from oocytes that were kept for at least 4 days after a 50-ng injection of CSMK1 mRNA, K^+-selective channels were found. These channels were activated upon depolarization to potentials positive to −40 mV (Fig. 4 *A* and *B*). The *I–V* curve (Fig. 4*D*) was linear between −40 and +80 mV and had a slope of 14 pS, equivalent to a permeability $P_K = 2.6 \times 10^{-14}$ cm/s (n = 3). With 5 mM KCl/135 mM NaCl in the pipette solution and 140 mM KCl in the bath, the *I–V* curve showed outward rectification and was best-fit by a GHK equation (29) with a permeability value of $P_K = 3.3 \times 10^{-14}$ cm/s (n = 6).

Application of 4-AP to the bath solution (cytoplasmic face of inside-out patches) reduced open probability in a dose-dependent manner by shortening the mean open time (Fig. 4*C*). In one experiment, mean burst durations were reduced from 490 ± 60 ms (n = 30 bursts; control) to 83 ± 13 ms (n = 58 bursts; 0.1 mM 4-AP) and 14 ± 2 ms (n = 118 bursts; 1 mM 4-AP) at a holding potential of +40 mV. The inhibition of open probability by 4-AP was completely and rapidly reversible. Single-channel amplitudes were not affected by 4-AP. Similar results were observed in seven of seven patches from six oocytes.

Tissue Distribution of CSMK1 mRNA. We were interested in the distribution of CSMK1 in muscles throughout the GI tract due to the well-known variations in electrical activity in these muscles (3, 11, 30, 31). Poly(A)$^+$ RNA was prepared from dissected regions of the canine GI tract. Inner and outer regions of circular muscle refer to muscle closest to the submucosal border (inner) and muscle closest to the myenteric border (outer).

Smooth muscle tissue from other sources were also analyzed to determine the smooth muscle distribution of this smooth-muscle-derived K^+ channel in portal vein, renal artery, and uterus. Canine heart and brain tissue were also included due to the numerous K^+ channel cDNAs that have been isolated from these tissues. Full-length CSMK1 was

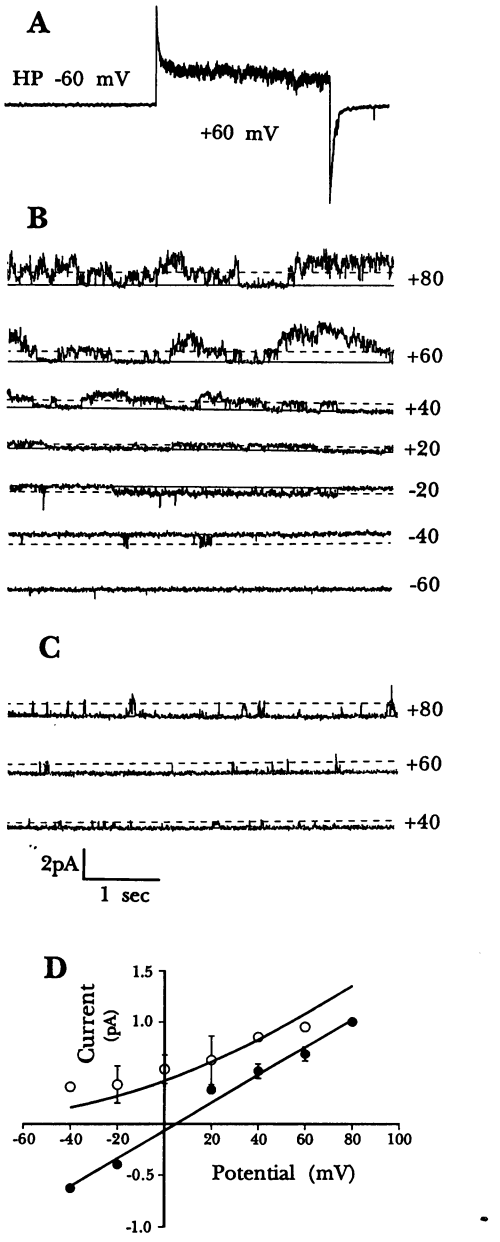

FIG. 4. Single-channel characteristics of CSMK1 in inside-out oocyte patches. (*A*) Current response to a step depolarization from a holding potential (HP) of −60 mV to +60 mV. (*B*) Single-channel currents recorded at various holding potentials. Solid lines indicate baseline (channels closed); broken lines indicate a channel-open state. (*C*) After application of 1 mM 4-AP to the bath solution, channel open times were shortened. Same patch as in *A* and *B*. (*D*) *I–V* relations in symmetrical (pipet solution/bath solution) 140/140 mM KCl (solid circles) and 5/140 mM KCl (open circles). Solid lines indicate best fit to GHK equation (29). Data are pooled from a total of nine patches from seven oocytes.

used as a probe to hybridize to the Northern blots. Fig. 5 displays the autoradiograms resulting from the hybridizations.

CSMK1 hybridized strongly to a transcript of ≈9.5 kb. An additional band with a size of ≈3.3 kb was also detected. CSMK1 transcripts were expressed in all the GI tissues examined with only small differences in expression levels demonstrated. Colonic longitudinal muscle demonstrated the lowest expression levels. Hybridization was undetectable from mRNA prepared from renal artery, portal vein, and heart ventricle even upon long exposure, whereas CSMK1 was expressed in brain.

DISCUSSION

Electrical activity in GI muscles appears to be due to the sequential activation of several voltage- and/or Ca^{2+}-dependent ion channels (5, 32). Although voltage clamp studies of dispersed smooth muscle cells have generated a considerable body of information concerning the properties of these currents, it is difficult to dissect and characterize all of the currents expressed from mixed whole-cell current records. Therefore, we have used molecular techniques to isolate specific K^+ channel proteins expressed in colonic smooth muscles with the goal of assigning these K^+ channel cDNAs to particular K^+ conductances in native cells.

CSMK1 represents one of three distinct K^+ channel cDNAs obtained from colonic smooth muscle mRNA using the reverse transcriptase–PCR and primers designed to hybridize to the conserved S5 and S6 transmembrane domains (26). The electrophysiological and pharmacological characteristics of CSMK1 expressed in oocytes are similar to the 4-AP-sensitive component of delayed rectifier current detected in native colonic circular muscle cells (7).

The single-channel conductance was similar to other channels in the $K_v1.2$ class [CSMK1, 14 pS; NGK1, 18 pS (33)]. The 4-AP sensitivity of CSMK1 was markedly greater than that of other K^+ channels of the $K_v1.2$ subclass [IC_{50}: CSMK1, 74.7 μM; RAK, 600 μM (27); RCK5, 800 μM (12)].

On the basis of electrophysiological studies of native smooth muscle cells, it is not possible to conclude that CSMK1 is different from the delayed rectifier currents in other smooth muscles (e.g., refs. 34 and 35). However, the Northern blot analysis performed in the present study suggests that the delayed rectifiers in portal vein (34) and renal artery (36) are different and distinct from the CSMK1 K^+ delayed rectifier channel found in canine GI muscles.

The electrical characterization of CSMK1 allows some prediction of the role of this current in colonic slow waves. Slow waves in the canine proximal colon circular muscle originate from a resting potential of −80 mV and depolarize to a maximum level of −30 mV to −20 mV (17). The upstroke phase of slow waves develops over ≈50 ms (i.e., 1*V*/sec dV/dt; ref. 17). Even at room temperature a significant amount of CSMK1 current is activated within 50 ms with a depolarization to −20 mV. Furthermore, CSMK1 is highly sensitive to a 4-AP block, whereas in intact muscle 4-AP has been shown to increase the rate of rise and amplitude of the upstroke potential and prolong the plateau phase of the slow wave (7). Thus the CSMK1 current may play a role in regulating the maximal amplitude of the upstroke component, as suggested (7). In addition, CSMK1 does not fully inactivate during prolonged depolarizations, suggesting that this current could balance sustained Ca^{2+} current and contribute to the plateau phase of the slow wave.

In Northern blot analysis employing full-length CSMK1 as a radiolabeled probe, expression of CSMK1 was demonstrated in all GI tissues examined. Although quantitative measurements were not performed, it was apparent that the abundance of CSMK1 mRNA was similar in most regions. The exception to this observation occurred in colonic longitudinal muscles where specific hybridization was low. The electrical activities of circular and longitudinal muscles are quite different (17, 37), and previous studies comparing native circular and longitudinal colonic muscle cells have suggested that different delayed rectifier currents are expressed in the two muscle layers (7, 8). The diversity in expression of delayed rectifiers is a possible explanation for the wide diversity in electrical activity observed in smooth muscles.

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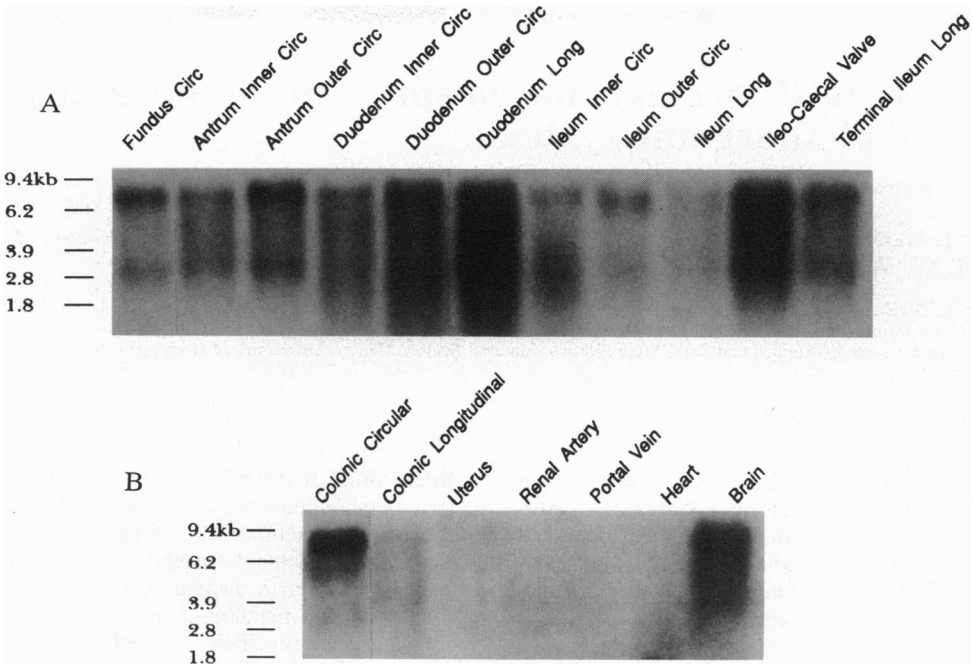

FIG. 5. Northern blot hybridization analysis employing CSMK1 as a probe. (*A*) mRNA isolated from various muscles throughout the GI tract. (*B*) mRNA isolated from colonic circular and longitudinal muscles as well as other smooth muscles, heart, and brain. Note the ubiquitous expression of CSMK1 in GI tissues and lack of expression in vascular and uterine smooth muscles.

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