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Active K^+ secretion through multiple K_{Ca}-type channels and regulation by IK_{Ca} channels in rat proximal colon

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Joiner, William J., Srisaila Basavappa, Sadasivan Vidyasagar, Keith Nehrke, Selvi Krishnan, Henry J. Binder, Emile L. Boulpaep, and Vazhaikkurichi M. Rajendran. Active K^+ secretion through multiple K_{Ca}-type channels and regulation by IK_{Ca} channels in rat proximal colon. *Am J Physiol Gastrointest Liver Physiol* 285: G185–G196, 2003. First published February 26, 2003; 10.1152/ajpgi.00337.2002.—Colonic K^+ secretion stimulated by cholinergic agents requires activation of muscarinic receptors and the release of intracellular Ca^{2+}. However, the precise mechanisms by which this rise in Ca^{2+} leads to K^+ efflux across the apical membrane are poorly understood. In the present study, Northern blot analysis of rat proximal colon revealed the presence of transcripts encoding rSK2 [small conductance (SK)], rSK4 [intermediate conductance (IK)], and rSlo [large conductance (BK)] Ca^{2+}-activated K^+ channels. In dietary K^+-depleted animals, only rSK4 mRNA was reduced in the colon. On the basis of this observation, a cDNA encoding the K^+ channel rSK4 was cloned from a rat colonic cDNA library. Transfection of this cDNA into Chinese hamster ovary (CHO) cells led to the expression of Ca^{2+}-activated K^+ channels that were blocked by the IK channel inhibitor clotrimazole (CLT). Confocal immunofluorescence confirmed the presence of IK channels in proximal colonic crypts, and Western blotting demonstrated that IK protein sorted to both the apical and basolateral surfaces of colonic epithelia. In addition, transcellular active K^+ secretion was studied on epithelial strips of rat proximal colon using unidirectional $^{86}Rb^+$ fluxes. The addition of thapsigargin or carbachol to the serosal surface enhanced net $^{86}Rb^+$ secretion. The mucosal addition of CLT completely inhibited carbachol-induced net $^{86}Rb^+$ secretion. In contrast, only partial inhibition was observed with the BK and SK channel inhibitors, iberiotoxin and apamin, respectively. Finally, in parallel with the reduction in SK4 message observed in animals deprived of dietary K^+, carbachol-induced $^{86}Rb^+$ secretion was abolished in dietary K^+-depleted animals. These results suggest that the rSK4 channel mediates K^+ secretion induced by muscarinic agonists in the rat proximal colon and that transcription of the rSK4 channel is downregulated to prevent K^+ loss during dietary K^+ depletion.

intermediate conductance Ca^{2+}-activated K^+ channel; intermediate-conductance channel; carbachol; clotrimazole; $^{86}RB^+$ flux; K^+ depletion

THE MAMMALIAN LARGE INTESTINE participates in overall potassium (K^+) homeostasis. After the kidney, the colon is the primary route of K^+ excretion. At least three different K^+ transport processes have been identified in the colon including active K^+ absorption, active K^+ secretion, and passive paracellular K^+ secretion (11, 24). In the basal state, active K^+ absorption is present in the distal colon, whereas active K^+ secretion has been identified in the proximal colon. Active K^+ absorption requires an uptake mechanism at the apical membrane, e.g., a H-K-ATPase, and an exit mechanism at the opposite membrane, e.g., a K^+ channel or a K-Cl cotransporter (KCC). Active K^+ secretion requires an uptake mechanism at the basolateral membrane, e.g., an Na-K-ATPase or an Na-K-2Cl cotransporter, and an exit step at the apical membrane, e.g., a K^+ channel.

Aldosterone or dietary Na^+ depletion stimulates active K^+ secretion, possibly by activation or insertion of apical membrane K^+ channels, whereas blocking these channels inhibits active K^+ secretion (39). Dietary K^+ depletion either inhibits active K^+ secretion or stimulates active K^+ absorption. For example, dietary K^+ depletion is associated with an increase both in the abundance of an apical membrane β-subunit of H-K-ATPase and KCC mRNA abundance and protein expression (32, 33). However, dietary K^+ depletion does not alter colonic H-K-ATPase activity, message or protein levels of its α-subunit (32, 34). In the absence of an effect on absorption, it seems reasonable that K^+ depletion would downregulate active K^+ secretion. In this case, K^+ conservation during dietary K^+ depletion could be affected by downregulating K^+ channels. To test this hypothesis and to study the effect of dietary K^+ depletion on active K^+ secretion independent of K^+ absorptive processes, the present studies were performed in the proximal colon in which active K^+ absorptive processes are absent.

In the colon, Ca^{2+}-activated K^+ (K_{Ca}) channels in particular have been postulated to play a role in K^+ transport (35, 36). Large-conductance (BK_{Ca}) K^+ channels have been identified in the apical (4) and basolateral (3, 22) membranes of colonic surface and crypt cells, respectively, whereas small (SK_{Ca}) and interme-

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diate (IK_{Ca}) conductance K^+ channels have been found in the basolateral membrane of crypt cells from the mammalian colon (2, 3, 7, 22, 27). Apical K_{Ca} channels may facilitate K^+ secretion (4, 15, 23, 36). In contrast, basolateral K_{Ca} channels are thought to facilitate K^+ absorption (35) through recycling of K^+ that enters the cell via the Na-K-ATPase or the Na-K-2Cl cotransporter. The efflux of K^+ through basolateral K_{Ca} channels may also hyperpolarize the basolateral and apical membranes, thereby enhancing the driving force for Ca^{2+}-dependent apical Cl^- secretion (6, 7, 12, 13, 30, 36).

Colonic K^+ secretion by transcellular pathways is regulated by several different means. Agonists that increase intracellular levels of cAMP have been reported to induce K^+ secretion (10, 14, 21). In contrast, activation of muscarinic receptors elevates intracellular Ca^{2+} (8, 9), a messenger that induces K^+ secretion (25), presumably by activating apical K_{Ca} channels. However, the identity of these apical K^+ channels is unknown. In the present study, we used carbachol to stimulate proximal K^+ secretion. Our results demonstrate that *1*) BK_{Ca}, SK_{Ca}, and IK_{Ca} channel transcripts are present in rat proximal colon, but only IK_{Ca} mRNA is downregulated in dietary K^+-depleted animals; *2*) a cDNA encoding the IK_{Ca} channel rSK4 was cloned from rat colon, and its expression in Chinese hamster ovary (CHO) cells reveals IK_{Ca} channel activity that is blocked by IK_{Ca} channel inhibitor clotrimazole (CLT); *3*) K^+ secretion mediated by cholinergic agonists in the rat proximal colon is completely blocked by CLT and partially blocked by the BK_{Ca} and SK_{Ca} channel inhibitors iberiotoxin (IbTX) and apamin (APA), respectively; and *4*) that dietary K^+ depletion abolishes carbachol-induced K^+ secretion. These results suggest that cholinergic K^+ secretion in the rat colon is mediated by $IK_{Ca} > BK_{Ca} \geq SK_{Ca}$ channels and that K^+ depletion causes mRNA encoding the IK channel rSK4 to be downregulated, presumably to prevent loss of K^+.

MATERIALS AND METHODS

Animals. Nonfasting male Sprague-Dawley rats (200–250 g) were divided into two groups: *1*) normal animals were fed Purina rat chow containing 200 μeq Na^+ + 250 μeq K^+/g food ad libitum; *2*) K^+-depleted animals were fed chow containing 200 μeq Na^+ + 0.014 μeq K^+/g food (ICN Pharmaceuticals, Costa Mesa, CA) ad libitum for 21 days, as previously described (12). All animals were given tap water ad libitum.

RNA isolation and Northern blot analysis. Total RNA was isolated by the CsCl cushioning method of Sambrook et al. (31), as described previously (34). Total rat RNA was purified from isolated proximal colonic epithelial cells. Proximal colonic segments from two rats were used for each RNA preparation. mRNA was purified from total RNA using a Qiagen kit (Chatsworth, CA). Five micrograms of mRNA were then electrophoresed on an agarose gel and transferred to a nylon membrane (New England Nuclear, Boston, MA) for use in Northern blots. Hybridizations were performed as described previously (33, 34). In brief, BstXI was used to excise full-length rSK4 from pCDNA1 (see below for cloning). Similarly, *Sac*I was used to isolate an ~0.9-kb fragment from hSlo, and *Evo*RV and *Xho*I were used to isolate an ~1.0-kb fragment from rSK2. All three fragments were similarly labeled by random priming using [^{32}P]dCTP and allowed to hybridize to membranes for 18 h at 42°C. The blots were washed with 2× saline-sodium citrate and 0.1% SDS at 45°C for 30 min. Subsequently, the blots were exposed to hyperfilm at −80°C and developed after 18 h. Individual blots were stripped and hybridized with channel cDNA probes for K_{Ca} channels and GAPDH. The abundance of hybridizing mRNA was quantified with a personal densitometer SI using ImageQuaNT software (Molecular Dynamics, Sunnyvale, CA) and normalized to the level of GAPDH mRNA on the same blot.

rSK4 cDNA isolation and characterization. A rat colonic cDNA library was screened for the rat SK4 cDNA using the human SK4 cDNA [hSK4 (18)] as probe as per the manufacturer's suggestion (Invitrogen, San Diego, CA). The hybridizing plasmid containing the largest insert (~1.9 kb) was sequenced completely in both directions using an automated fluorescence sequencer (W. M. Keck Biotechnology Resource Lab, Yale University). The resulting sequence (accession #AF156554) encodes an open reading frame of 424 amino acids and is 98% identical to the recently cloned rat rSK4 (42) and the rat SMIK (26). In particular, the amino acid sequence encoded by our cDNA differs from that of rSK4 and SMIK at *positions 280, 395*, and *399* and lacks a glutamine at *position 416*. Furthermore, SMIK and the amino acid sequence encoded by our cDNA differ from rSK4 in containing a leucine at *position 213* in place of phenylalanine. These minor differences may reflect sequencing errors. Because our clone so closely resembles rSK4 and SMIK, we will refer to it hereafter as rSK4 out of deference to the nomenclature established for the first rat IK_{Ca} channel that was cloned.

The full-length cDNA was subsequently subcloned into the *Bam*HI site of pCDNA1; this plasmid was then used for transfection studies. Briefly, CHO cells were grown to ~10–20% confluence in 25-cm^2 vented flasks. Growth media for these cells consisted of Iscove's DMEM (GIBCO) supplemented with 10% FBS, hypoxanthine/thymidine (GIBCO), and antibiotic/antimycotic (GIBCO). CHO cells were transfected with 5 μl Lipofectamine (GIBCO) premixed with 0.5 μg rIK1/pCDNA1 + 0.5 μg pGreen Lantern (Invitrogen) in 1 ml Optimem (GIBCO) for 5 h, followed by a 12-h incubation in the same media supplemented with 10% FBS. Subsequently, the transfection media was replaced with normal growth media, and recordings were made from green fluorescent cells within the next 40 h.

Electrophysiology. All recordings were performed at 22°C using an Axopatch 1D amplifier (Axon Instruments, Foster City, CA). Electrodes were fabricated from WPI glass (Sarasota, FL) using a Narishige two-stage puller. Electrodes had a resistance of 3–5 MΩ for whole cell recordings and 10–50 MΩ for inside-out patch recordings when filled with recording solution.

For whole cell recordings, the bath solution consisted of (in mM): 140 NaCl, 1.0 $CaCl_2$, 5 KCl, 29 glucose, and 25 HEPES (pH 7.4). Changes were made to the bath composition during ion-substitution experiments, where K^+ was substituted for Na^+. In pharmacological experiments, the bath solution was either supplemented with various drugs [charybdotoxin (CTX), clotrimazole (CLT), tetraethylammonium (TEA), IbTX, and APA] at concentrations not exceeding 1 mM or, in the case of TEA, 20 mM TEA was substituted for Na^+. In whole cell experiments, pipettes were filled with intracellular solution containing (in mM): 30 KCl, 100 K gluconate, 5 EGTA, 4.27 $CaCl_2$, and 10 HEPES (pH 7.2). The free Ca^{2+} concentration of the intracellular solution was determined to be 1.0 μM using the computer program Cabuffer (kindly

provided by Dr. L. Schlichter). Pulse protocols for whole cell recordings consisted of 500-ms ramps between −120 and +80 mV from a holding potential of −70 mV. Interpulse intervals were 5–10 s.

For inside-out patch recordings of transiently transfected cells, pipettes were filled with (in mM): 30 KCl, 100 K gluconate, 1.0 $CaCl_2$, and 10 HEPES (pH 7.2). The cytoplasmic side of the membrane was perfused with intracellular solution in which the free Ca^{2+} concentration was varied between 0 and 1.0 μM using $CaCl_2$ and readjusted to pH 7.2, as determined by the computer program Cabuffer. For inside-out recordings, patches were held at −80 mV, and data were collected continuously for 30- to 60-s intervals.

All data were acquired online at a sampling rate of 10–20 kHz and filtered at either 2 (whole cell recordings) or 1 kHz (inside-out patch recordings) before analysis with pCLAMP6.0 software. Errors due to series resistance during whole cell recordings were minimized by employing 80–90% series resistance compensation. Results represent means ± SE from five to eight experiments.

$^{86}Rb^+$ flux studies. $^{86}Rb^+$ was used as a K^+ surrogate in in vitro flux studies across proximal colonic segments isolated from both normal and K^+-depleted rats. $^{86}Rb^+$ fluxes were performed at 0 mV under voltage-clamp conditions, as described previously (29). In brief, excised proximal colons were flushed with oxygenated cold Ringer solution, and the muscular layers were stripped. Two mucosal sheets were obtained from each animal, mounted in Lucite chambers (1.13 cm^2), and bathed on both sides with equal volumes of Ringer solution. The bath solutions were maintained at 37°C and continuously gassed with 5% CO_2-95% O_2. The Ringer solution contained (in mM): 115 NaCl, 25 $NaHCO_3$, 5 KCl, 1.2 $CaCl_2$, 1.2 $MgCl_2$, and 10 glucose (pH 7.4). The transepithelial potential difference (V_{te}) of colonic tissues was determined, and, after voltage clamping, the short-circuit current (I_{sc}) was measured. Transepithelial conductance (G_{te}) was calculated from the V_{te} and the I_{sc} (DVC 1000, World Precision Instruments) and normalized to the exposed area of 1.13 cm^2.

$^{86}Rb^+$ (1.0 μCi $^{86}RbCl$; New England Nuclear) was added to either the serosal or mucosal bath solution. After a 50-min equilibration, basal unidirectional fluxes [i.e., mucosal to serosal (m-s) and serosal to mucosal (s-m)] were measured for three 15-min control periods (basal). In other experiments, after basal measurements, $^{86}Rb^+$ fluxes were measured in the presence of 200 μM carbachol in the serosal solution. In the latter experiments, after a 5-min equilibration in carbachol, unidirectional $^{86}Rb^+$ fluxes were measured for two sequential 15-min periods (carbachol I and II). In experiments using K_{Ca}-channel blockers, the blocker was added to either the mucosal or serosal bath solution of both normal and K^+-depleted animals at the end of carbachol I. Thus, after carbachol treatment, 100 nM IbTX, 50 nM APA, or 500 nM CLT were added to the mucosal bath solution, whereas in other experiments, 500 nM CLT was added to the serosal bath solution. In addition, the effect of 100 nM IbTX, 50 nM APA, or 500 nM CLT was tested in the absence of carbachol. In some experiments, $^{86}Rb^+$ fluxes were measured for 15 min after adding 3 μM thapsigargin to both serosal and mucosal solutions. Net $^{86}Rb^+$ transport (J_{net}^{Rb+}) was calculated from the difference between J_{ms}^{Rb+} and J_{sm}^{Rb+} using tissues matched on the basis of the initial G_{te} (with a difference <10%). Positive and negative values represent active absorption and active secretion, respectively. Results are expressed as means ± SE. A nonparametric Friedman test was performed, and a P value <0.05 was considered significant.

$^{36}Cl^-$ flux studies $^{36}Cl^-$ (1.0 μCi; New England Nuclear) was added to either the serosal or mucosal bath solution. After incubation for 15 min, unidirectional fluxes were measured first under control conditions, then following the addition of 200 μM serosal carbachol, and finally in the presence of carbachol plus 500 nM CLT.

Generation of a polyclonal antibody to IK/SK4. At the time of the antibody production, the rat gene for IK_{Ca} had not been identified. Therefore, the anti-IK antibody was directed against a peptide derived from the mouse homolog of rat SK4 (rSK4). This peptide, RQVRLKHRKLTEQVNSMVD, is identical to the sequence encoded by the subsequently cloned rSK4, with the exception that at *position 11*, where T in mouse is substituted for R in rat. No obvious deficiencies in immunoreactivity against native rSK4 have been observed. The anti-IK antibody was custom produced by Research Genetics, a division of Invitrogen (Carlsbad, CA). New Zealand white rabbits were inoculated with a keyhole limpet hemocyanin peptide conjugate mixed with an equal volume of Freund's adjuvant. Animals were injected in three subcutaneous dorsal sites with a total of 0.1 mg per injection. The injection protocol was repeated at *weeks 2*, *6*, and *8*. After 10 wk, terminal bleeds were taken. The serum was collected from clotted blood by centrifugation, and an ELISA was used to assess anti-peptide serum titer. The serum from both rabbits was then combined and affinity purified over an immobilized peptide column.

Western analysis. Mouse brain, kidney, and colon homogenates were used to identify native IK/SK4 protein. Tissues were prepared as previously described (41). After dissection, the tissues were homogenized twice by 10-s strokes at power level 5 with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) using 1 g tissue/5 ml homogenization solution (10 mM HEPES adjusted to pH 7.4 with Tris, 10% sucrose, 1 mM EDTA, 1 mM PMSF) plus 1 tablet of Complete protease inhibitor (Roche Applied Science, Indianapolis, IN) per 50 ml solution. Homogenates were centrifuged at 2,500 *g* for 15 min at 4°C, and the supernatants were saved. The pellets were resuspended in 5 ml homogenization buffer per gram of starting tissue, then homogenized and recentrifuged as above. The supernatants were combined, and crude membrane proteins were precipitated by centrifugation at 22,000 *g* for 20 min at 4°C. The supernatants from this step were discarded, and the pellets were resuspended in PBS containing 1 mM EDTA, 1 mM PMSF, and Complete protease inhibitor (1 tablet/50 ml), then passed once through a 25-gauge needle and once through a 30-gauge needle. Aliquots were quickly frozen in liquid N_2 and stored at −85°C until use. Rat colonic proximal apical membranes were isolated by the modified method of Stieger et al. (38). Basolateral membranes were prepared from scraped mucosa from rat proximal colon by the sucrose density gradient centrifugation described by Biber et al. (1).

Approximately 20 μg of crude membrane proteins were separated by two-phase Tricine polyacrylamide gel electrophoresis (10%T/6%C resolving layer, 4%T/3%C stacking layer) and transferred onto nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech, Piscataway, NJ) in buffer containing 10 mM 3′(cyclohexylamino)-1-propanesulfonic acid (adjusted to pH 11) and 10% methanol. The blot was blocked overnight at room temperature in blocking buffer (PBS containing 0.1% Tween-20, 4% BSA, and 1% normal goat serum). After the blot was blocked, it was incubated for 1 h at room temperature in blocking buffer containing a 1:5,000 dilution of a stock 3.2 mg/ml solution of rabbit anti-mouse intermediate Ca^{2+} activated K channel (anti-mIK_{Ca}) polyclonal affinity-purified antibody, washed three times with PBS containing 0.1% Tween-20 (PBS-T), incubated for 1 h at room temperature in blocking buffer contain-

ing a 1:5,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) and again washed three times with PBS-T. Immune complexes were detected on film using enhanced chemiluminescence (Amersham Pharmacia Biotech; Fig. 1). To assess specificity, the anti-m$IK_{Ca^{2+}}$ (mouse intermediate Ca^{2+} activated K channel) antibody was preincubated for 1 h with a 50-fold molar excess of competitor peptide corresponding to the epitope recognition sequence before use in blotting (Fig. 1).

Confocal Immunofluorescence. Single crypts were isolated as previously described (16). Crypts were adhered with Cell-Tak (Collaborative Biomedical), washed with PBS, and fixed for 10 min at room temperature with 2.5% formaldehyde. After rinsing with PBS, crypts were permeablized with 0.25% Triton X-100 and 0.1% BSA in PBS for 5 min at room temperature. Nonspecific binding was blocked with three washes of PBS containing 1% BSA. Crypts were incubated with a 1:2,000 dilution of anti-m$IK_{Ca^{2+}}$ primary antibody overnight at 4°C. Cells were then washed with PBS containing 0.1% BSA and exposed to goat anti-rabbit immunoglobulin G $(Fab')_2$ fragments conjugated to R-phycoerythrin for 30 min at room temperature. Immunofluorescence was detected by confocal microscopy using excitation and emission wavelengths of 488 and >550 nm, respectively.

RESULTS

Expression and regulation of K_{Ca} channels in the rat proximal colon. The proximal colon participates in Ca^{2+}-dependent K^+ secretion (15, 25). Thus Northern blot analysis was used to identify which K_{Ca}-channel isoforms are expressed in the rat proximal colon. BK_{Ca}, SK_{Ca}, and IK_{Ca} mRNAs were detected as ~4-, 2.1-, and 2.2-kb transcripts, respectively (Fig. 2), whereas SK1 and SK3 mRNAs were not detected (data not shown). Subsequently, the effect of dietary K^+ depletion on K_{Ca}-channel abundance in the proximal colon was investigated on rats maintained on reduced levels of dietary K^+. Of the K_{Ca}-channel isoforms detectable in rats maintained on normal diets, only rSK4 mRNA abundance was significantly decreased in the proximal colons of dietary K^+-depleted animals (Fig. 2). The degree to which rSK4 mRNA abundance was reduced (84%; $n = 3$) suggests that decreased expression of rSK4 in the proximal colon may help conserve K^+ in K^+-depleted animals.

Electrophysiological characterization of rSK4. Because rSK4 message was selectively regulated by dietary K^+ depletion, rSK4 was cloned from rat colonic cDNA library, and its electrophysiological properties were studied in a transiently transfected CHO cell line. As shown in Fig. 3*A*, in transfected cells, several nanoamperes of current were observed in whole cell mode in the presence of 1.0 μM free intracellular Ca^{2+}. In contrast, whole cell currents were not observed in untransfected cells in 1.0 μM free intracellular Ca^{2+} (Fig. 3*A*). In the absence of intracellular Ca^{2+}, no significant current was observed in transfected or untransfected cells (Fig. 3*B*). These results indicate that the currents measured in CHO cells were caused by rSK4 and that intracellular Ca^{2+} activates the channels encoded by this cDNA.

To establish the K^+ selectivity of rSK4, the extracellular concentration of K^+ was varied from 3 to 100 mM by substituting K^+ for Na^+ in the bath solution. As predicted by the Nernst equation, the unclamped membrane potential shifted by 55.0 ± 0.7 mV per 10-fold change in extracellular K^+ concentration (Fig. 3*C*). With 3 mM K^+ and 140 mM Na^+ in the bath, the permeability ratio of K^+ to Na^+ was 72, as calculated by a derivation of the Goldman-Hodgkin-Katz equation. These results indicate that rSK4 is highly selective for K^+ over Na^+.

The current-voltage (*I-V*) relationship of rSK4 currents changed significantly on changing the extracellular K^+ concentration. In 5 mM K^+, the *I-V* relationship was nearly linear over the physiological voltage range (Fig. 3*D*). In contrast, in 100 mM extracellular K^+, the *I-V* relationship rectified significantly in the inward direction. In this example, the slope conductance in 100 mM extracellular K^+ measured between −120 and −80 mV was 100 nS and between 0 and +40 mV it was 50 nS. These values yield a conductance ratio of 2.0 for inward/outward currents, indicating that rSK4 channels rectify strongly in high extracellular K^+, a property observed for some native colonic K_{Ca} channels (5, 26).

To establish that the pharmacological characteristics of rSK4 are similar to those of other mammalian IK_{Ca} channels, the effects of various K_{Ca}-channel inhibitors were examined on rSK4 whole cell currents in the presence of 1.0 μM free intracellular Ca^{2+} (Figs. 3, *E* and *F*). After perfusion with 1.0 μM CLT, a specific blocker of IK_{Ca} channels, whole cell currents were inhibited by 76 ± 4% ($n = 5$) at 0 mV (Figs. 3, *E* and *F*). Similarly, currents from cells perfused with 20 nM CTX, a blocker of some maxi-K_{Ca} and IK_{Ca} channels, were inhibited by 88 ± 2% ($n = 5$) at 0 mV (Fig. 3*F*). In contrast, 20 mM TEA or 100 nM IbTX, blockers of maxi-K_{Ca} channels, inhibited whole cell currents by only 36 ± 2 ($n = 5$) and 22 ± 2% ($n = 5$), respectively (Fig. 3*F*). Similarly, 10 nM APA, a toxin that blocks some SK_{Ca}-channel isoforms but not members of the IK_{Ca} subfamily, did not significantly inhibit rSK4 currents (Fig. 3*F*). The efficacy of the specific IK_{Ca}-channel blocker CLT contrasted with the weak or negligible effects of the inhibitors of other K^+ channels, supporting the notion that the colonic rSK4 is the rat isoform of an IK_{Ca} channel found in colonic epithelial cells (7, 30).

Single-channel characteristics. To establish the single-channel conductance of rSK4, inside-out patch-clamp recordings were performed with 130 mM K^+ in both the pipette and bath solutions. As illustrated in Fig. 4*A*, an increase in channel activity was observed when free Ca^{2+} was raised from 0 to 300 to 600 nM, and this effect was reversible. An all-points histogram of the recording yielded two distinct peaks (Fig. 4*B*). Gaussian fits to these and other patch recording data yielded a chord conductance of 36 ± 2 pS ($n = 4$) between 0 and −80 mV. These results confirm that rSK4 encodes an IK_{Ca} channel similar to that found in colonic epithelia (5). Due to the increase in conductance and rectification observed for rSK4 when extracellular

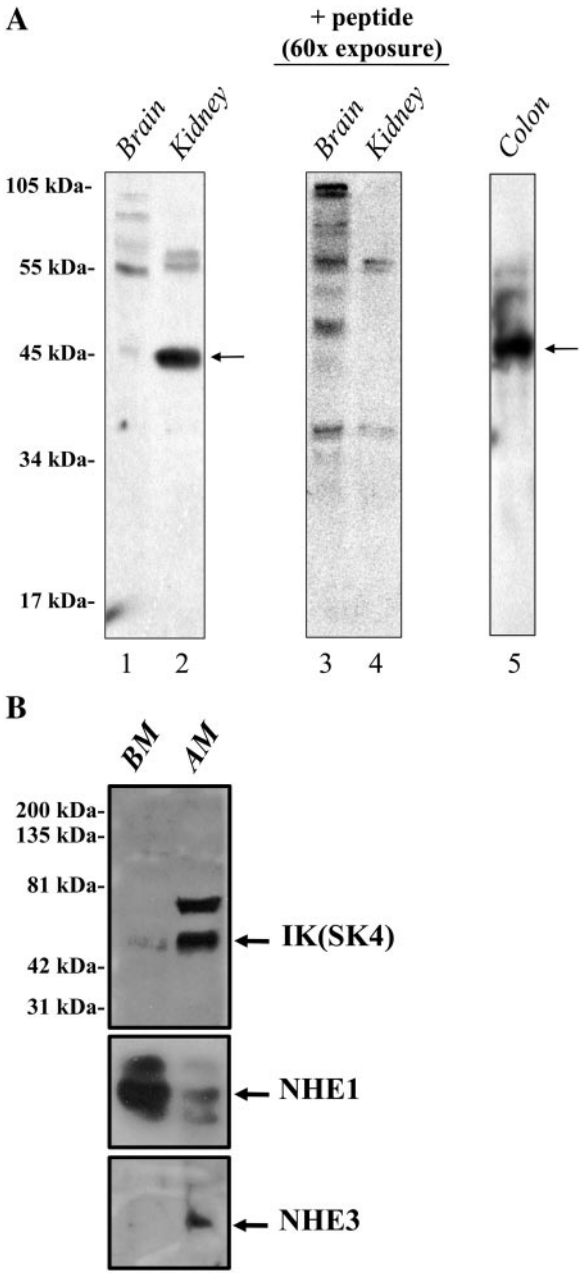

Fig. 1. Characterization of the anti-mIK_{Ca} antibody. *A*: mouse brain (*lanes 1* and *3*), kidney (*lanes 2* and *4*), and colon (*lane 5*) membrane preparations were assayed via Western analysis for intermediate-conductance K^+ channels (IK_{Ca}) protein expression. A strong 45-kDa band in *lanes 2* and *5* (arrow) corresponds to the apparent molecular mass expected for IK_{Ca}; the lack of a band of that size in *lane 1* is consistent with a much lower expression level of IK_{Ca} in the brain. Several minor larger molecular weight bands are also present in both brain and kidney. However, we did not observe formation of a homotetrameric complex in this gel system. Preincubation of the anti-mIK_{Ca} antibody with purified peptide containing the recognition epitope results in a dramatic loss of reactivity, particularly of the 45-kDa band (*lanes 3* and *4*). Note that *lanes 3* and *4* display a 60 times the amount of exposure relative to *lanes 1* and *2*; similar exposure times demonstrate a nearly complete loss of apparent reactivity following peptide preincubation (data not shown). *B*: in the rat proximal colon, IK(SK)4 protein was detected in both apical (AM) and basolateral membrane (BM) preparations. However, expression of IK was greater in the AM. Na^+/H^+ exchanger (NHE)1 and NHE3, which are expressed in the BM and AM, respectively, were used as markers to confirm the relative purity of these membranes.

K^+ is elevated (Fig. 3*D*), it is likely that some of the smaller conductances recorded for K_{Ca} channels in colonocytes under conditions of low K^+ or at depolarized potentials also reflect rSK4 channels (2, 3, 22, 27).

$^{86}Rb^+$ flux studies. After pharmacological properties of rSK4 were established, various K_{Ca}-channel blockers were used to study $^{86}Rb^+$ fluxes across isolated intact proximal colonic mucosa. $^{86}Rb^+$ fluxes were first performed under basal conditions. Over three consecutive 15-min time periods, unstimulated basal net ^{86}Rb fluxes did not change significantly ($n = 4$; Table 1*a*). Fluxes were also measured following exposure of tissues to carbachol, an agonist known to elevate intracellular Ca^{2+} (8, 9). Carbachol increased net $^{86}Rb^+$ secretion from -0.07 ± 0.01 to -0.28 ± 0.01 $\mu eq \cdot h^{-1} \cdot cm^{-2}$ ($n = 4$; Table 1*b* and Fig. 5), primarily as a result of an increase of J_{sm}^{Rb+} (Table 1*b*). Carbachol-induced secretion was sustained during a subsequent 15-min period.

IbTX (100 nM) partially but significantly reduced carbachol-induced net $^{86}Rb^+$ secretion when applied to the apical membranes of proximal colonic mucosa

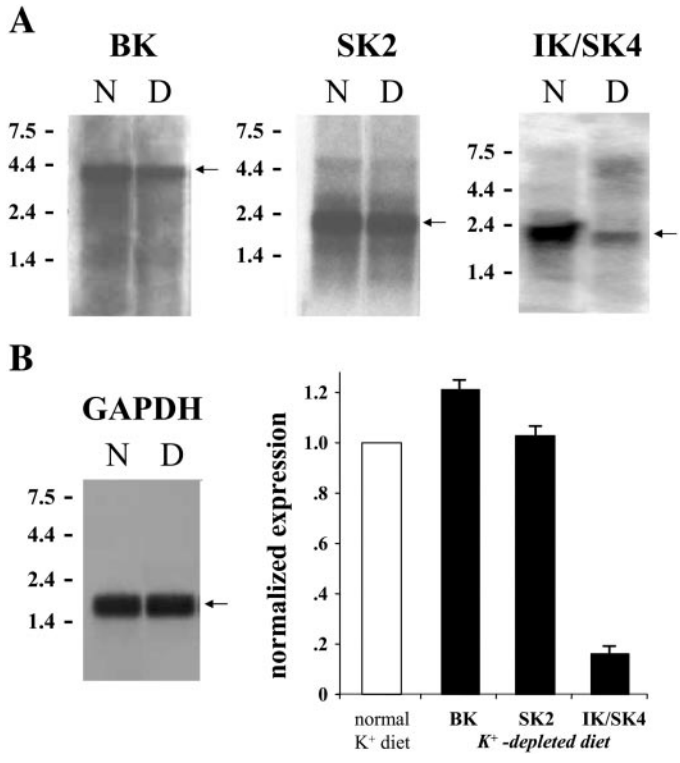

Fig. 2. Regulation of K_{Ca} mRNA abundance in the rat colon by dietary K^+ depletion. mRNA purified from the proximal colon of rats grown on diets with normal (N) or depleted (D) amounts of K^+ were analyzed by Northern blot hybridization using rSK1, rSK2, rSK3, rSK4, large conductance (BK), and GAPDH cDNAs as probes. The expected size of each transcript is denoted at the *left* (*A*). rSK1 and rSK3 were not detected and thus are not shown here. K_{Ca} mRNA abundance in normal or K^+-depleted rats was quantitated by densitometry from 3 different mRNA preparations per dietary condition (*B*). For each condition, K_{Ca} abundance in the proximal colon is normalized to GAPDH mRNA abundance and then to K_{Ca} abundance in rats raised on normal diets.

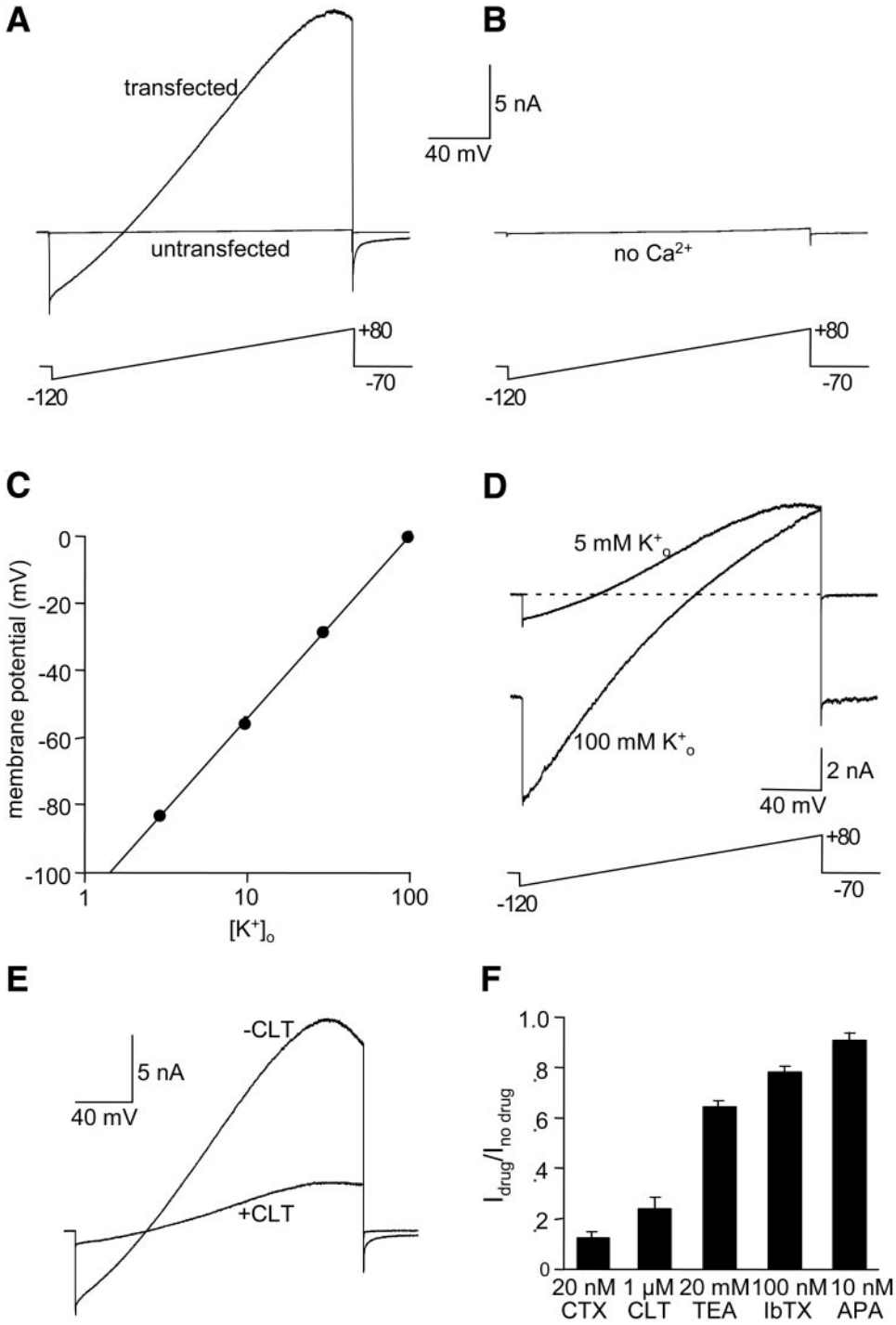

Fig. 3. rSK4 generates Ca^{2+}-dependent, clotrimazole (CLT)-sensitive K^+ currents when expressed in chinese hamster ovary (CHO) cells. *A*: typical whole cell recording of a CHO cell transfected with rSK4 shows large inward and outward currents that are absent in untransfected cells. The recording pipette contained 1.0 μM free Ca^{2+}, and currents were elicited during 500-ms ramps between −120 and +80 mV from a holding potential of −70 mV. *B*: whole cell recording of a typical cell transfected with rSK4 shows no significant inward or outward currents in the absence of free Ca^{2+} in the recording pipette. *C*: unclamped (zero current) membrane potentials of CHO cells transiently transfected with rSK4 were measured with K^+ substituting for Na^+ in the bath solution. The points were fit with a line with a slope of 55 mV per 10-fold change in K^+ concentration (n = 5; error bars are not evident due to their small size relative to the size of the symbols). *D*: representative whole cell recording of a CHO cell transfected with rSK4 shows nearly linear currents with 130 mM K^+ in the pipette and 5 mM K^+ in the bath, whereas currents from the same cell rectify inwardly when extracellular K^+ is raised to 100 mM. *E*: typical whole cell recording of a CHO cell transfected with rSK4 before and after bath application of 1.0 μM CLT. *F*: sensitivity of rSK4 to various blockers of K_{Ca} channels. Membrane current measurements were made at 0 mV before and after bath application of 20 nM charybdotoxin (CTX), 1.0 μM CLT, tetraethylammonium (TEA), 100 nM iberiotoxin (IbTX), or 20 mM or 10 nM apamin (APA) and are expressed as the fractional current remaining after application of the blocker.

(n = 4; Table 1*c* and Fig. 5). A similar partial but significant inhibition of net $^{86}Rb^+$ secretion was observed with 50 nM APA (n = 7; Table 1*d* and Fig. 5). Simultaneous exposure to IbTX and APA resulted in an additional but statistically insignificant decrease in the carbachol-induced net $^{86}Rb^+$ compared with IbTX or APA alone (Table 1*e*). In contrast, 500 nM CLT applied to the mucosal or serosal solution significantly reduced J_{net}^{Rb+} to basal values (Table 1, *f* and *g*, and Fig. 5). Neither the partial inhibition of the carbachol-stimulated J_{net}^{Rb+} by mucosal IbTX and APA nor the complete inhibition by CLT can be attributed to a reduction in the unstimulated flux, because these drugs did not significantly affect the basal J_{net}^{Rb+} (Fig. 5, *inset*). Together, these results suggest that apical BK_{Ca}, SK_{Ca}, and IK_{Ca} channels and basolateral IK_{Ca} channels contribute to Ca^{2+}-activated $^{86}Rb^+$ secretion across the normal rat proximal colon but that IK_{Ca} channels may play a more prominent role in this phenomenon.

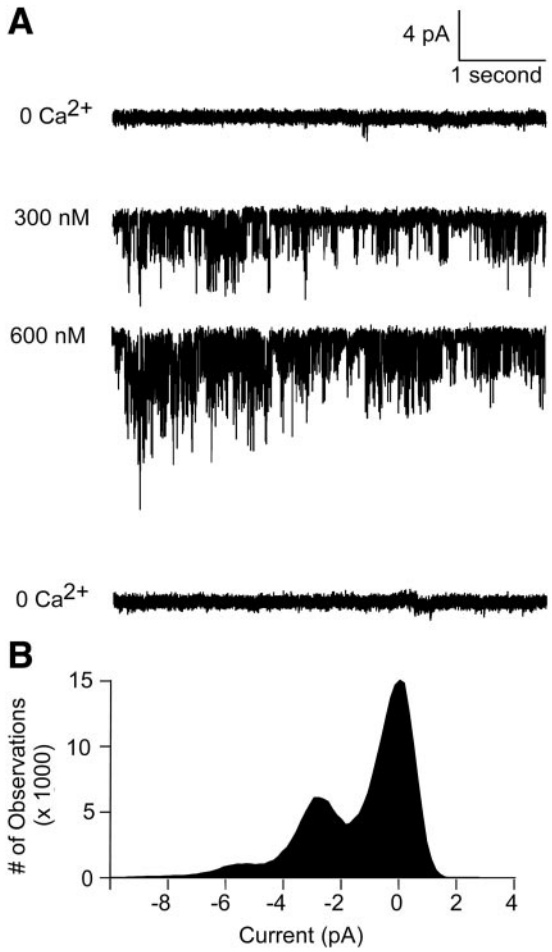

Fig. 4. rSK4 generates IK Ca^{2+}-activated K^+ channels. *A*: an inside-out patch excised from a CHO cell transfected with rSK4 shows increasing channel activity as free Ca^{2+} on the intracellular surface of the patch is elevated. Activity is reversible with washout of Ca^{2+}. This patch was held at −80 mV (inside negative) with 130 mM K^+ in both the pipette and bath solution. *B*: an all-points histogram of the recording in *A*. Between 0 and −80 mV, rSK4 has a chord conductance of 36 pS in this patch.

Regulation of $^{86}Rb^+$ secretion by dietary K^+ depletion. Northern blot analyses demonstrated that under conditions of dietary K^+ depletion, the abundance of rSK4 transcript in the proximal colon decreased (Fig. 2). To determine whether this downregulation of rSK4 transcript might be accompanied by a functional change in K^+ flux, $^{86}Rb^+$ flux studies were performed on proximal colonic segments of rats deprived of dietary K^+. Basal $^{86}Rb^+$ secretion was still present in K^+-depleted rats. However, in K^+-depleted animals, carbachol did not elevate J_{sm}^{Rb+} or J_{net}^{Rb+} significantly above basal values and CLT did not inhibit secretion (Table 2*a*). Diminished active secretion appeared to be due to downregulation of IK_{Ca} channels rather than downregulation of muscarinic receptors or their transduction. This was illustrated by the addition of thapsigargin, an inhibitor of the endoplasmic reticulum Ca-ATPase and thus an activator of intracellular Ca^{2+} release. Thapsigargin failed to elevate J_{net}^{Rb+} significantly above basal values in K^+-depleted animals (Table 2*b*). In contrast, in animals raised on normal levels of dietary K^+, J_{net}^{Rb+} rose significantly above basal values in response to thapsigargin (Table 2*c*). Thus the insensitivity of K^+ secretion to elevated intracellular Ca^{2+} and to CLT in K^+-depleted animals may be caused by the downregulation of IK_{Ca} channel expression, which would thereby facilitate retention of K^+ during periods of diminished K^+ intake.

$^{36}Cl^-$ flux studies. As with $^{86}Rb^+$ studies, proximal colonic epithelia were studied under basal conditions and following exposure to serosal carbachol. Exposure to carbachol induced a large, sustained, net $^{36}Cl^-$ secretion above basal values. The subsequent addition of CLT to either the apical or basolateral membranes of proximal colonic mucosa abolished carbachol-induced net $^{36}Cl^-$ secretion ($n = 7$). The abolition of carbachol-induced secretion by CLT was so profound that proximal colons switched from secreting to absorbing Cl^- (Table 3, *a* and *b*). The inhibition of both $^{36}Cl^-$ and $^{86}Rb^+$ fluxes by CLT suggests that both Cl^- and K^+ transport mechanisms are coupled to IK_{Ca} channels.

Membrane localization of IK_{Ca} channels. To study the polarization of IK_{Ca} channel distribution in the proximal colon, both confocal immunohistochemistry and Western bolts were performed. Intact rat colonic crypts were isolated as previously described (16) and imaged by confocal microscopy. Figure 6*A* depicts a typical crypt preparation, as shown by phase-contrast microscopy. With the use of immunofluorescence, IK_{Ca} channels were observed predominantly in the basolateral membranes of crypt cells (Fig. 6*B*). In addition, IK_{Ca} channels were also observed on surface cells but with more intense staining compared with crypt cells. In the absence of IK_{Ca} antibody and in the presence of the secondary antibody, no significant fluorescence was detected. The absence of IK_{Ca} channel expression on the apical membrane of crypt epithelial cells may result from limited access of the antibody to the lumen of colonic crypts. This issue was resolved with Western blots using apical and basolateral membranes from rat proximal colonic crypt cells. As illustrated in Fig. 1*B*, IK_{Ca} was expressed in both the apical and basolateral membrane preparations as a protein of ~45 kDa, a molecular mass similar to that predicted by the open reading frame of SK4. However, a greater level of expression of IK_{Ca} was detected in the apical membrane. An additional band at ~70 kDa of unknown origin was also observed in the apical membrane preparation. The purity of the membrane preparations was confirmed using antibodies that specifically recognize the apical membrane Na^+/H^+ exchanger (NHE)3 transporter or the basolateral membrane NHE1 transporter.

DISCUSSION

In the proximal colon, cholinergic agonists increase intracellular Ca^+ concentration ($[Ca^{2+}]_i$) and stimulate K^+ secretion (15). The rise in $[Ca^{2+}]_i$ acts on the Na-K-2Cl cotransporter to enhance uptake of K^+ across the basolateral membrane (15). Elevated $[Ca^{2+}]_i$ also

Table 1. *Effect of K_{Ca} channel blockers on carbachol-induced K^+ ($^{86}Rb^+$) secretion in rat proximal colon*

Condition	I_{sc}, μEq·h^{-1}·cm^{-2}	G_{te}, mSiemens/cm^2	J_{ms}, μEq·h^{-1}·cm^{-2}	J_{sm}, μEq·h^{-1}·cm^{-2}	J_{net}, μEq·h^{-1}·cm^{-2}
a					
Basal	1.52 ± 0.07	11.5 ± 0.50	0.08 ± 0.01	0.15 ± 0.02	−0.07 ± 0.01
Basal I	1.25 ± 0.06	13.3 ± 0.42	0.15 ± 0.01	0.24 ± 0.03	−0.10 ± 0.02
Basal II	0.96 ± 0.06	14.5 ± 0.54	0.21 ± 0.02	0.30 ± 0.03	−0.10 ± 0.01
b					
Basal	1.63 ± 0.06	11.7 ± 0.30	0.06 ± 0.01	0.13 ± 0.01	−0.07 ± 0.01
Carbachol I (S)	1.23 ± 0.09	13.5 ± 0.48	0.15 ± 0.01*	0.43 ± 0.01*	−0.28 ± 0.01*
Carbachol II (S)	1.04 ± 0.08	14.5 ± 0.51	0.24 ± 0.01*	0.50 ± 0.02*	−0.26 ± 0.01*
c					
Basal	1.51 ± 0.09	11.8 ± 0.60	0.07 ± 0.02	0.13 ± 0.01	−0.06 ± 0.01
Carbachol I (S)	1.23 ± 0.08	13.1 ± 0.44	0.13 ± 0.03*	0.34 ± 0.03*	−0.21 ± 0.02*
Carbachol (S) + IbTX (M)	1.04 ± 0.09	14.5 ± 0.50	0.23 ± 0.02	0.34 ± 0.02	−0.11 ± 0.02†
d					
Basal	1.57 ± 0.07	10.6 ± 0.40	0.05 ± 0.01	0.14 ± 0.02	−0.08 ± 0.02
Carbachol I (S)	1.25 ± 0.07	12.4 ± 0.50	0.08 ± 0.01	0.40 ± 0.03*	−0.31 ± 0.04*
Carbachol (S) + APA (M)	1.04 ± 0.09	13.2 ± 0.47	0.17 ± 0.02	0.33 ± 0.02	−0.16 ± 0.02†
e					
Basal	1.17 ± 0.07	11.65 ± 0.88	0.02 ± 0.01	0.07 ± 0.01	−0.05 ± 0.01
Carbachol I (S)	0.87 ± 0.08	12.72 ± 0.98	0.13 ± 0.01*	0.39 ± 0.02*	−0.27 ± 0.01*
+IbTX + APA (M)	0.75 ± 0.06	13.13 ± 0.93	0.31 ± 0.01	0.42 ± 0.02†	−0.12 ± 0.01†
f					
Basal	1.05 ± 0.11	9.6 ± 0.40	0.07 ± 0.01	0.15 ± 0.01	−0.08 ± 0.01
Carbachol I (S)	0.87 ± 0.11	13.3 ± 0.42	0.15 ± 0.01*	0.39 ± 0.04*	−0.24 ± 0.04*
Carbachol (S) + CLT (M)	0.79 ± 0.10	14.1 ± 0.43	0.21 ± 0.01	0.25 ± 0.02	−0.04 ± 0.01†
g					
Basal	1.14 ± 0.06	10.2 ± 0.70	0.10 ± 0.03	0.14 ± 0.03	−0.05 ± 0.01
Carbachol I (S)	0.92 ± 0.07	11.0 ± 0.52	0.21 ± 0.05*	0.38 ± 0.05*	−0.17 ± 0.01*
Carbachol (S) + CLT (S)	0.78 ± 0.06	11.9 ± 0.56	0.36 ± 0.05	0.40 ± 0.05	−0.04 ± 0.02†

Values are means ± SE. Unidirectional fluxes of $^{86}Rb^+$ were measured across stripped rat proximal colonic mucosa under voltage clamp conditions as described in MATERIALS AND METHODS. Carbachol I and carbachol II represent fluxes measured over 2 sequential 15-min periods in the presence of 200 μM serosal carbachol. Carbachol + drug [where drug is 100 nM iberiotoxin (IbTX) 50 nM aparnin (APA) or 500 nM clotrimazole (CLT)] represents fluxes measured over 15 min following carbachol I and the addition of drug to the mucosal (M) solution. Four tissue pairs were studied in each group except *d* when 7 tissue pairs were studied. J_{ms}, J_{sm}, and J_{net} indicate mucosa to serosa, serosa to mucosa, and net fluxes ($J_{ms} - J_{sm}$), respectively. G_{te} indicates transepithelial conductance. In this set of experiments, IbTX was added first and equilibrated for 5 min before APA addition. *$P < 0.05$ when carbachol is compared with basal time periods and †$P < 0.05$ when carbachol plus blocker(s) is compared with carbachol alone. I_{sc}, short-circuit current; S, serosal.

opens as yet unidentified apical K_{Ca} channels in the proximal colon, providing intracellular K^+ with a means of exit to the lumen (Fig. 7) (15).

Both paracellular and transcellular pathways contribute to ion transport in polarized epithelia. In the case of the former, ions do not cross a membrane barrier, and they therefore passively distribute between the lumenal and serosal compartments according to their individual electrochemical gradients. In the case of the latter, the segmental localization, polarized distribution, and differential regulation of the various molecular components of the absorptive and secretory machinery are critical for determining how the proximal colon performs net K^+ secretion and the distal colon performs net K^+ absorption. Both paracellular and transcellular transport mechanisms contribute to K^+ secretion in the proximal colon. Although the cotransporters and pumps have been extensively studied, the molecular identities of the channels participating in colonic K^+ absorption and secretion have been less well defined. However, it is apparent that K_{Ca} channels are important conduits for transcellular K^+ transport and that different isoforms may have distinct roles on different membranes. For example, in patch-clamp studies, BK_{Ca} channels have been identified in both apical and basolateral membranes of rat enterocytes (3, 4, 19, 20, 22), whereas SK_{Ca}/IK_{Ca} channels have been identified only in basolateral membranes (2, 3, 22, 27). Although the specific roles of these K_{Ca} channels are not known, some insight into which channels participate in K^+ absorption and secretion has been gleaned from flux studies. In the distal colon, for example, carbachol stimulates an m-s $^{86}Rb^+$ flux that is inhibited by quinine at the basolateral membrane, whereas carbachol stimulates a mucosal $^{86}Rb^+$ efflux that is inhibited by TEA at the apical membrane (15). In contrast, in the proximal colon, mucosal and serosal effluxes are blocked by the inhibitor of SK_{Ca}/IK_{Ca} channels, Ba^{2+} (15, 17), but not by the BK_{Ca}-channel inhibitor TEA (15). This suggests that SK_{Ca} or IK_{Ca} channels may play significant roles in active K^+ secretion in the proximal colon.

In the present study, the potential roles of K_{Ca} channels in mediating K^+ secretion in the proximal colon were investigated. There are five known K_{Ca} channels in mammals: one BK_{Ca} isoform [Slo (28, 40)], three SK_{Ca} isoforms [SK1–3 (20)], and one IK_{Ca} [SK4 (18)]. With the use of Northern analysis, the present study demonstrates that under normal dietary conditions, only the BK_{Ca}, $SK2_{Ca}$, and IK_{Ca} isoforms are expressed in rat proximal colon.

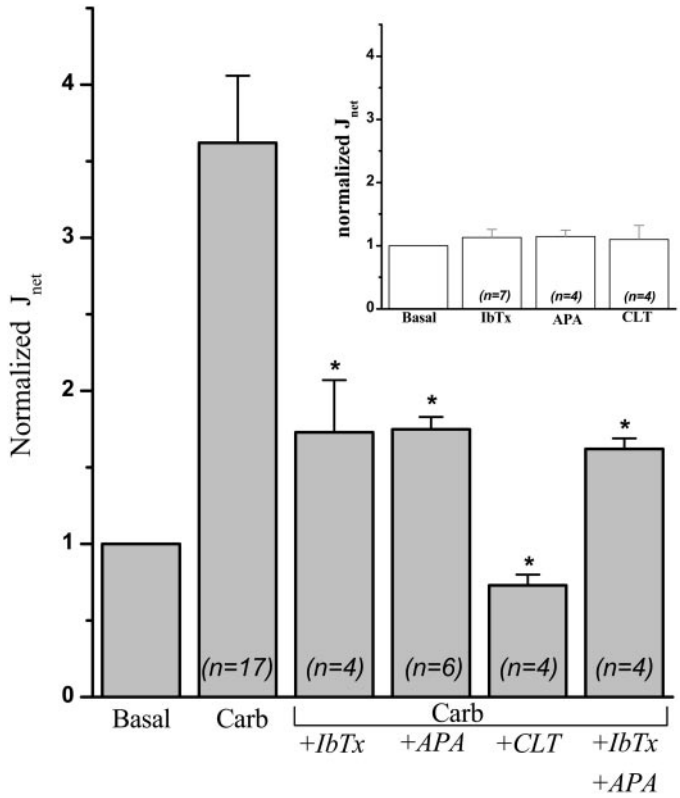

Fig. 5. Effect of CLT on carbachol-induced $^{86}Rb^+$ secretion. Basal net fluxes across rat proximal colonic epithelia were determined in Ringer solution (basal). Subsequently, carbachol was added to the serosal side (Carb) and net fluxes were determined. After exposure to carbachol, IbTX, APA, or CLT was added to the mucosal side and net fluxes were again determined. In the absence of carbachol pretreatment, IbTX, APA, or CLT did not reduce net fluxes significantly (*inset*). All values shown are normalized to the net fluxes determined under basal conditions. Number of measurements is shown in parentheses. *$P < 0.05$ compared with carbachol.

The roles of BK_{Ca}, $SK2_{Ca}$, and IK_{Ca} in regulating proximal colonic K^+ secretion were confirmed in flux studies under zero voltage-clamp conditions using pharmacological activators and inhibitors of the three classes of K_{Ca} channels. Because of the absence of any overall transepithelial gradient for K^+, the observed net secretion only represents active transcellular transport. In the absence of elevated intracellular Ca^{2+}, none of the K_{Ca}-channel antagonists have any effect on $^{86}Rb^+$ fluxes, indicating that K_{Ca} channels do not participate in basal secretion. In contrast, carbachol-induced $^{86}Rb^+$ secretion is partially inhibited by the mucosal application of IbTX or APA, indicating that apical BK_{Ca} and $SK2_{Ca}$ channels probably contribute to K^+ secretion. The greater degree of inhibition of carbachol-induced secretion by CLT compared with the inhibition measured during simultaneous exposure to IbTX and APA suggests that IK_{Ca} channels may play a prominent role in K^+ secretion in the proximal colon. The complete abolition of carbachol-induced $^{86}Rb^+$ secretion by mucosal CLT also suggests that in addition to its basolateral distribution, which has been confirmed by patch-clamp studies (2, 22, 27), IK_{Ca} channels may also be expressed at the apical membrane, from which few recordings have been made. The present data suggest that all three types of K_{Ca} channels are involved in K^+ secretion (Fig. 7).

It is possible that CLT is sufficiently permeant that, when added to the mucosal surface, it enters the cell. Indeed, heterologous expression studies with SK4 indicate that CLT probably crosses the plasma membrane to inhibit IK_{Ca} channels (44). As a consequence, CLT applied to the mucosal surface may also have access to and subsequently block IK_{Ca} channels at the basolateral membrane.

CLT applied to the serosal surface also abolishes carbachol-induced K^+ secretion as an indirect consequence of blocking basolateral IK_{Ca} channels. Indeed, closing basolateral IK_{Ca} channels should initiate the following sequence of events in proximal colonic epithelial cells. Depolarization of the basolateral membrane would abolish the driving force for apical Cl^- efflux, an effect that has been well described by others (7, see also Ref. 36 for review). The cessation of intracellular Cl^- loss would lead to a decrease in basolateral Na-K-2Cl cotransporter activity, which would diminish not only Cl^- uptake but also K^+ uptake across the basolateral membrane. Consequently, the intracellular K^+ concentration would fall, and apical efflux through BK_{Ca} and SK_{Ca} channels would decrease. This model links Cl^- and K^+ at their point of uptake at the baso-

Table 2. *Effect of diet on Ca^{2+}-activated K^+ ($^{86}Rb^+$) secretion in rat proximal colon*

Condition	I_{sc}, $\mu Eq \cdot h^{-1} \cdot cm^{-2}$	G_{te}, mSiemens/cm^2	J_{ms}, $\mu Eq \cdot h^{-1} \cdot cm^{-2}$	J_{sm}, $\mu Eq \cdot h^{-1} \cdot cm^{-2}$	J_{net}, $\mu Eq \cdot h^{-1} \cdot cm^{-2}$
a K^+-depleted diet					
Basal	1.51 ± 0.34	22.0 ± 1.4	0.40 ± 0.80	0.83 ± 0.16	−0.43 ± 0.08
Carbachol I (S)	1.20 ± 0.41	26.3 ± 2.3	0.44 ± 0.07	0.84 ± 0.13	−0.40 ± 0.10
Carbachol (S) + CLT (M)	0.74 ± 0.10	22.6 ± 2.1	0.46 ± 0.04	0.78 ± 0.11	−0.32 ± 0.08
b K^+-depleted diet					
Basal	1.60 ± 0.20	18.9 ± 2.3	0.33 ± 0.07	0.62 ± 0.10	−0.30 ± 0.13
Thapsigargin (S + M)	1.50 ± 0.10	19.3 ± 2.2	0.43 ± 0.08	0.68 ± 0.07	−0.25 ± 0.11
c Normal diet					
Basal	1.60 ± 0.30	13.3 ± 1.2	0.13 ± 0.03	0.58 ± 0.13	−0.45 ± 0.13
Thapsigargin (S + M)	2.20 ± 0.30	13.9 ± 2.4	0.15 ± 0.03	0.85 ± 0.16*	−0.70 ± 0.15*

Values are means ± SE. Unidirectional fluxes of $^{86}Rb^+$ were measured across stripped rat proximal colonic mucosa under voltage clamp conditions as described in MATERIALS AND METHODS and Table 1, except in some experiments thapsigargin was substituted for carbachol. Five, six, and four tissue pairs were studied in groups a, b, and c, respectively. The animals used for the experiments described in this table were obtained from a different lot than those used for the experiments described in Table 1. *$P < 0.05$ compared with basal time periods.

Table 3. *Effect of clotrimazole on Cl^- ($^{36}Cl^-$) secretion in rat proximal colon*

Condition	I_{sc}, μEq·h^{-1}·cm^{-2}	G_{te}, mSiemens/cm^2	J_{ms}, μEq·h^{-1}·cm^{-2}	J_{sm}, μEq·h^{-1}·cm^{-2}	J_{net}, μEq·h^{-1}·cm^{-2}
a					
Basal	1.43 ± 0.05	11.17 ± 1.06	6.24 ± 0.47	6.34 ± 0.55	−0.10 ± 0.32
Carbachol I (S)	1.20 ± 0.06	12.20 ± 1.02	5.63 ± 0.56	6.82 ± 0.52	−1.20 ± 0.27*
Carbachol (S) + CLT (M)	0.95 ± 0.06	12.83 ± 1.03	7.95 ± 0.52	6.89 ± 0.25	1.06 ± 0.55†
b					
Basal	1.38 ± 0.08	10.14 ± 1.08	8.52 ± 1.38	8.09 ± 1.36	0.42 ± 0.14
Carbachol I (S)	1.26 ± 0.08	11.43 ± 1.20	7.29 ± 0.71	10.92 ± 1.28*	−3.63 ± 0.83*
Carbachol (S) + CLT (S)	0.88 ± 0.07	12.56 ± 1.31	9.97 ± 1.13	8.37 ± 1.02†	1.60 ± 0.65†

Values are means ± SE. Unidirectional fluxes of $^{36}Cl^-$ were measured across stripped rat proximal colonic mucosa under voltage clamp conditions as described in MATERIALS AND METHODS. Seven tissue pairs were studied in each group. The animals used for the experiments described in this table were obtained from a different lot than those used for the experiments described in Table 1. *$P < 0.05$ when carbachol is compared to basal time periods and †$P < 0.05$ when carbachol plus blocker is compared with carbachol alone.

lateral membrane and at the level of secretion across the apical membrane: inhibiting the transport of either ion at either membrane would shut down transport of both ions at both membranes (Fig. 7). Consistent with this model, the present study shows that mucosal or serosal application of CLT inhibits both K^+ and Cl^- secretion in the proximal colon.

The model predicts that blocking of IK_{Ca} channels present at either membrane would inhibit net $^{86}Rb^+$ secretion. The present immunofluorescence data suggest that IK_{Ca} channels are expressed predominantly at the basolateral membrane. In contrast, Western blots demonstrate that IK_{Ca} channels are present in both apical and basolateral membranes, with a higher expression in the apical membrane. This discrepancy between immunohistochemistry and Western blot data suggests that the anti-IK_{Ca} antibody had limited accessibility to the crypt lumen in immunofluorescence experiments. Regardless, the present data indicate that IK_{Ca} channels are present on both the apical and basolateral membranes in rat proximal colonic epithelia.

In addition to possible modes of action on apical or basolateral IK_{Ca} channels, there have been reports in other systems of CLT acting on BK_{Ca} channels and on *P*-450 enzymes. However, other evidence bolsters the notion that the effects of CLT described in this study are caused by CLT acting exclusively on IK_{Ca}. First, the concentration of CLT used in this study is less than 10% of the measured K_d of CLT for 12 K^+ channels, including BK_{Ca} and SK_{Ca} channels (43). Second, inhibition of *P*-450 enzymes with the IK_{Ca}-insensitive CLT analog econazole does not disrupt inducible transepi-

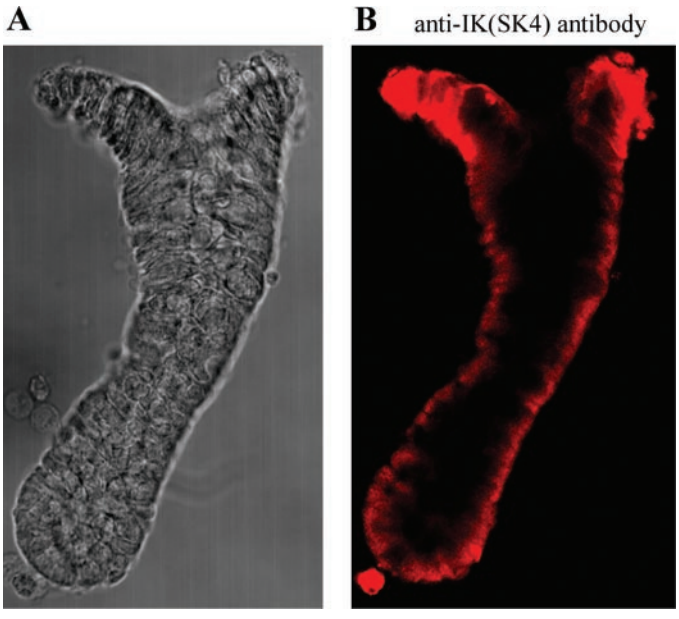

Fig. 6. IK_{Ca} expression in proximal colonic crypts by confocal immunofluorescence. Colonic crypts were isolated from the rat proximal colon as described in MATERIALS AND METHODS. *A*: phase contrast image of a typical colonic crypt. After exposure to the anti-mIK_{Ca} antibody, fluorescence was mainly localized to the basolateral membrane (*B*). In the absence of anti-mIK_{Ca} antibody, minimal fluorescence was observed.

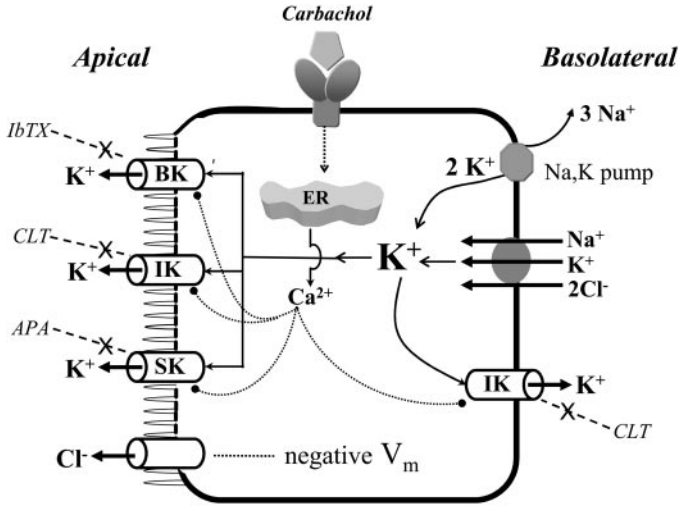

Fig. 7. Model of K^+ secretion in the rat proximal colonic epithelium. Transcellular K^+ secretion requires a basolateral uptake mechanism and an apical exit step. An increase in cytosolic Ca^{2+} by cholinergic agonists, such as carbachol, leads to opening of apical IbTX-sensitive BK_{Ca}, CLT-sensitive IK_{Ca}, and APA-sensitive SK_{Ca} channels, which allow K^+ to be secreted into the lumen. In addition, the elevation in intracellular Ca^{2+} also opens basolateral CLT-sensitive IK_{Ca} channels, allowing K^+ recycling. Intracellular K^+ is replenished by the combined basolateral activities of the Na-K-2Cl cotransporter and the Na-K-ATPase. The activation of apical and basolateral K_{Ca} channels also leads to hyperpolarization of the cell membrane, which favors Cl^- secretion.

thelial transport properties in the rat colon (37). Thus, in the present study, the inhibition of proximal colonic $^{86}Rb^+$ secretion by CLT cannot be ascribed to CLT acting on *P*-450 enzymes.

Because the present study suggests that several K_{Ca} channels are involved in active proximal colonic K^+ secretion, the regulation of K_{Ca}-channel expression by changes in dietary K^+ was examined. Under normal dietary conditions, activation of K_{Ca} channels by thapsigargin or carbachol stimulates a large increase in J_{net} in the proximal colon. In contrast, in K^+-depleted rats, neither thapsigargin nor carbachol significantly elevates J_{net}. Because basal $^{86}Rb^+$ secretion is similar in K^+-depleted rats and animals raised on normal K^+ diets (see Table 2), dietary K^+ depletion must specifically abolish the Ca^{2+}-activated component of proximal colonic K^+ secretion without affecting basal secretion.

In the present study, depletion of dietary K^+ in rats results in the downregulation of mRNA encoding the IK_{Ca} isoform rSK4 but not of the mRNAs of the other two colonic K_{Ca} channels BK_{Ca} and rSK2. Thus downregulation of rSK4 mRNA probably underlies the inability of the proximal colon to secrete K^+ in response to carbachol in the K^+-depleted state. Selective transcriptional downregulation of rSK4 is expected to help conserve K^+ in the K^+-depleted condition if this channel normally either underlies or regulates a major conductance pathway for K^+ secretion in the proximal colon. Because CLT, IbTX, and APA each are capable of reducing net secretion, IK_{Ca}, BK_{Ca}, and SK_{Ca} channels probably each contribute to secretion in the normal rat proximal colon. However, the inability of IbTx and APA together to reduce J_{net} to baseline values plus the strong correlation between dietary K^+, the abundance of rSK4 transcript, and carbachol-induced CLT-sensitive J_{net} suggests that IK_{Ca} channels are the major regulators of Ca^{2+}-activated K^+ secretion by the proximal colon in response to changes in dietary K^+.

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