Cyclic nucleotide-gated cation channels mediate sodium and calcium influx in rat colon

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Cyclic nucleotide-gated cation channels mediate sodium and calcium influx in rat colon. Am. J. Physiol. Cell Physiol. 278: C336–C343, 2000.—We found mRNA for the three isoforms of the cyclic nucleotide-gated nonselective cation channel expressed in the mucosal layer of the rat intestine from the duodenum to the colon and in intestinal epithelial cell lines in culture. Because these channels are permeable to sodium and calcium and are stimulated by cGMP or cAMP, we measured 8-bromo-cGMP-stimulated sodium-mediated short-circuit current (I(sc)) in proximal and distal colon and unidirectional 45Ca2+ fluxes in proximal colon to determine whether these channels could mediate transepithelial sodium and calcium absorption across the colon. Sodium-mediated I(sc) stimulated by 8-bromo-cGMP, were inhibited by dichlorobenzamil and I-cis-diltiazem, blockers of cyclic nucleotide-gated cation channels, suggesting that these ion channels can mediate transepithelial sodium absorption. Sodium-mediated I(sc) and net transepithelial 45Ca2+ absorption were stimulated by heat-stable toxin from Escherichia coli that increases cGMP. Addition of I-cis-diltiazem inhibited the enhanced transepithelial absorption of both ions. These results suggest that cyclic nucleotide-gated cation channels simultaneously increase net sodium and calcium absorption in the colon of the rat.

sodium absorption; calcium absorption; cation channels

UNDER CONDITIONS OF SALT restriction or when aldosterone is elevated, sodium-mediated short-circuit current (I(sc)) across the distal colon is blocked by apical addition of amiloride (20, 31, 32). The channel mediating this sodium transport is the amiloride-sensitive sodium channel (ENaC) (3, 4). In other portions of the intestine, for example, the proximal colon and the ileum, sodium absorption is mediated via the electroneutral sodium-proton exchanger (NHE) (19, 26). ENaC (27) and NHE (15) have been cloned, and the mRNA and protein have been identified in the epithelia of the intestinal mucosa. However, the molecular mechanisms underlying calcium entry and transepithelial calcium absorption are less clear. Although there is evidence for vesicle entry mechanisms (22), calcium entry via ion channels has also been implicated (25, 30). To determine whether cyclic nucleotide-gated (CNG) cation channels might mediate sodium and calcium entry in the intestine, we evaluated the distribution of mRNA for the three isoforms of the cyclic nucleotide-gated cation channel in the intestine and in cultures of intestinal cell lines. We investigated the effect of 8-bromo-cGMP (8-Br-cGMP)-mediated stimulation and transport inhibition by blockers of cyclic nucleotide-gated cation channels on sodium-mediated I(sc) and 45Ca2+ fluxes in the rat colon, because the secondary subunit that increases calcium permeability (5) is expressed in this segment.

Cyclic nucleotide-gated cation channels are not voltage dependent but are gated directly by micromolar amounts of cyclic nucleotides (cAMP and/or cGMP). These channels are equally selective for sodium and potassium and exhibit a large conductance (25 pS) in monovalent cation solutions and a smaller conductance in the presence of calcium (13, 14, 17, 24). The channels are blocked by I-cis-diltiazem and dichlorobenzamil (23) but are rather insensitive to amiloride (13). With use of RNase protection assay and in situ hybridization (8), the presence of a cyclic nucleotide-gated cation channel was documented in rat lung airway epithelia. In primary cultures of rat tracheal airway epithelial cells, a sodium-mediated 8-Br-cGMP-stimulated I(sc) was inhibited by I-cis-diltiazem and dichlorobenzamil, suggesting that this channel contributes to transepithelial sodium transport in this epithelium (28). Thus cyclic nucleotide-gated cation channels carry sodium currents in airway cells that are not blocked by amiloride. This suggested that other epithelia such as the kidney (2) and intestine may also possess these channels. This is the first demonstration that cyclic nucleotide-gated cation channel mRNAs are expressed in all segments of the intestine and that these channels mediate apical calcium and sodium entry and transepithelial transport of these ions across the colon of the rat.

METHODS

Animal Treatments

Male Sprague-Dawley rats (200–250 g; Charles River, Wilmington, MA) were fed a standard rat chow (0.8 g NaCl/kg) or a low-salt (0.02 g NaCl/kg) diet (Laboratory Animal Diet, PMI Feeds, St. Louis, MO) for 2 wk. Evaluation of serum aldosterone and corticosterone levels in control animals showed that the aldosterone level was 14.5 ± 8.5 ng/dl (n = 4). Animals fed the low-salt diet showed an increase in aldosterone to 1,624 ± 51 ng/dl (n = 5). Animals were killed and used for transport studies described below, or...
intestinal segments, including duodenum, jejunum, ileum, and ascending and descending colon, were isolated and perfused with saline solution, then the mucosal layer of each segment was gently scraped from the underlying muscle layer with a clean glass slide. The mucosal scrapes were placed in TRIzol (GIBCO BRL, Gaithersburg, MD), homogenized (Polytron), and frozen at −80°C until RNA was made.

1_{sc}

Male Sprague-Dawley rats (200 g) were killed with an overdose of pentobarbital sodium, and the colon was isolated in a solution of (in mM) 114 NaCl, 5 KCl, 1.65 NaH₂PO₄, 1.25 CaCl₂, 1.1 MgCl₂, 5 HEPES, and 10 glucose (pH 7.4). The proximal or distal colon was quickly stripped of its serosal layer and mounted in Ussing chambers. 1_{sc} was measured using a modified Ussing chamber (model CHM2) with a 9-mm-diameter opening (63.6-mm² area; World Precision Instruments, Sarasota, FL); the chamber was warmed to 37°C with a heated circulating water bath. For 1_{sc} measurements, the apical and basolateral solutions were nominally chloride and bicarbonate free. This 1_{sc} solution was composed of (in mM) 76 Na₂SO₄, 3.3 K₂SO₄, 1.65 NaH₂PO₄, 1.25 CaSO₄, 1.1 MgSO₄, 5 HEPES, and 10 glucose (pH 7.4). The chamber was connected to a voltage-current clamp (model DVC-1000, World Precision Instruments), and 1_{sc} was measured while voltage was clamped at 0 mV. The initial 1_{sc}, along with the initial potential difference, was measured as soon as the epithelium was mounted. From this measurement, the initial resistance was calculated. The resistance was also measured at the end of the experiment, and if it changed >15% from the initial value, the experiment was discarded. For the data from normal rats in Table 1 or 3, the current just before addition of agents that increased 8-Br-cGMP (basal 1_{sc}) was recorded again, and this current was set at 100 as the normalized current for each epithelium. The changes in 1_{sc} for each current were expressed as an increase or decrease compared with the same normalized current, i.e., 100, before the treatments. The averaged normalized currents are expressed as means ± SE.

### 45Ca²⁺ Fluxes

The proximal colon was isolated, and two portions from the same animal were mounted in Ussing chambers, as described above for 1_{sc}. The resistance of the epithelium for the unidirectional mucosal-to-serosal flux (J_{ms}) was matched for resistance within 15% to the epithelium from the same animal that was used for the unidirectional serosal-to-mucosal flux (J_{sm}). About 2 µCi/ml of 45Ca²⁺ were added to the mucosal side to measure J_{ms}, or the same amount of 45Ca²⁺ was added to the serosal side to measure J_{sm}. After a 15-min isotope equilibration period, 45Ca²⁺ samples were taken from the opposite side every 10 min. Three control 45Ca²⁺ samples were collected, three more samples were collected after addition of heat-stable toxin from Escherichia coli (STa), and a final three samples were collected after addition of l-cis-diltiazem. The difference in the counts per minute (cpm) of the three time periods was averaged. This average value, collected over 10 min, was multiplied by 6 to give the counts per minute per hour. Because the area of the chamber opening was 63.6 mm², this average value (cpm/hr) was multiplied by 1.57 to give the average counts per minute per centimeter squared per hour. To convert net flux to current, the net flux (µM·cm⁻²·h⁻¹) was multiplied by the Faraday constant (96,500 C/mol) and divided by 3,600 to convert the hours to seconds and multiplied by the charge of calcium to give the current in coulombs per centimeter squared per second or amperes per centimeter squared.

### Materials

2',4'-Dichlorobenzamil was synthesized through the National Institute of Mental Health Chemical Synthesis Program (Research Biochemicals, Natick, MA) and has been shown previously to block cyclic nucleotide-gated cation channels (23). l-cis-Diltiazem was obtained from Tanabe (Saitama, Japan) or from Research Biochemicals. Amiloride and phenylmethylsulfonyl fluoride were obtained from Research Biochemicals. 8-Br-cGMP was obtained from two sources: for experiments in Table 1, rows 1 and 2, the supplier was Sigma Chemical (St. Louis, MO); for experiments in Table 1, rows 3–5, 8-Br-cGMP gave a greater response at the same 2 mM dose and was supplied by BioLog Life Science Institute (La Jolla, CA). STa was purchased from Sigma Chemical.

### Cell Lines

All cells were grown to confluence following standard culture procedures according to American Type Culture Collection methods. HT-29C and T84 are human colon carcinoma cells that secrete chloride. HT-29 cells were cultured in DMEM with 10% fetal bovine serum, 10 µg/ml transferrin, 50 U/ml penicillin, and 50 µg/ml streptomycin. T84 cells were cultured in a 50:50 mixture of DMEM·Ham's F-12 with 10% fetal bovine serum, 50 U/ml penicillin, and 50 µg/ml streptomycin. IEC-6 and IEC-18 cells are rat normal small intestine and rat ileum epithelial cell lines, respectively. IEC-6 and IEC-18 cells were cultured in DMEM with 5% fetal bovine serum, 0.1 U/mL insulin, 50 U/ml penicillin, and 50 µg/ml streptomycin. Panc-1 is a permanent epithelial cell line established from a pancreatic carcinoma of duct origin. CFPAC is a pancreatic duct epithelial cell line established from a cystic fibrosis patient with pancreatic carcinoma. These cells were cultured in DMEM with 10% fetal bovine serum, 50 U/ml penicillin, and 50 µg/ml streptomycin. At 2 days–1 wk after confluence, cells were rinsed in Hanks’ buffer, scraped into TRIzol, and homogenized by shaking.

### RT-PCR

Total RNA was isolated following the TRIzol protocol (GIBCO BRL), then dissolved in 0.01% diethylpyrocarbonate-treated water. The optical density at 260/280 nm was measured to determine the concentration and purity (ratio 1.7–1.9). First-strand cDNA was reverse transcribed using the SuperScript preamplification system for first-strand cDNA synthesis kit (GIBCO BRL). Each PCR of 50 µl was composed of 2 or 5–10 µl of first-strand cDNA, 1× PCR buffer (pH 9.0), 2.5 mM Mg²⁺, hot wax beads (Invitrogen, San Diego, CA), 0.2 mM dATP, dCTP, dGTP, and dTTP, 0.25–0.5 µM channel primers, 0.1 µM β-actin primers, and 2.5 units of Taq DNA polymerase (Boehringer Mannheim, Indianapolis, IN). A master mix composed of primers, deoxyribonucleotides, and Taq polymerase was premixed and then added to Eppendorf tubes to which the cDNA or water was added. The master mix ensured that each cDNA was exposed to equal concentrations of reaction mixture components. RT-PCR (denaturation at 94°C for 1 min, annealing at 55–65°C for 1 min, and extension at 72°C for 1 min) was performed with a DNA thermal cycler (model 480, Perkin-Elmer, Norwalk, CT). RT-PCR products were analyzed by electrophoresis in a 2% agarose gel, then extracted for sequencing. For RT-PCR involving degenerate primers, the initial five cycles of anneal-
Primers

Primers for β-actin were TATGCAACACAGTGTGTCC-TGG (bp 2760–2782) and TACTCCGTTGCTGATCCACAT (bp 3089–3067). This set of primers was chosen to span an intron to check for contamination with genomic DNA. The β-actin RT-PCR product derived from RNA is 217 bp; the product from genomic DNA is 329 bp. Although the data are not shown, RT-PCR for actin was performed on all intestinal segments, and no contaminating genomic cDNA was found. Primers chosen for rCNG1 do not amplify rCNG2 from rat olfactory library or rCNG3 from rat kidney, heart, or testis, as verified by sequencing. Choosing the primers in areas where there were deletions in the CNG2 and CNG3 isoforms gave this specificity. In this manner, the RNA for each of the three channel isoforms could be distinguished in each segment. This is more difficult with a Northern blot, because the mRNA for CNG3 and CNG1 are very close in size; therefore, it is difficult to distinguish them in a Northern blot (8). Primers for rCNG1 were TGTTTGTACACTCTGTGGACTAACCACAA (bp 1282–1307) and GCTTCCATGAGGTGATCTCTT (bp 1724–1715). Primers for rCNG2 were AAGGATGAGGAG-TACCTATTTGTCAT (bp 1042–1067) and GCTGAGACAG-GCATACTGAGTCAT (bp 1550–1505). Rat CNG3 was sequenced from kidney with use of degenerate primers based on the sequence for bovine CNG3, then specific primers for αCNG3 were designed on the basis of the partial rat sequence. The primers correspond to the bovine nucleotide sequence GTAAAGGAGGACGGTTGAGAAA (bp 1665–1690) and TCCGTTGACTCGTGAGGTTCTCCAT (bp 2107–2082) (2). The primers for βrCNG were AAG TAC ATG GCC TTC TTC GA (bp 1582–1598) and TGC CAG GTG TAC TCA TAC CA (bp 2057–2038; accession no. AF068572). The primers for αhCNG1 were TGGTTGACCATCTGACTCATTGG (bp 1051–1057) and AATTTCAAGACCAACTCCACACAGAC ACA (bp 1472–1441). A human retinal library used to amplify message for αhCNG1 was provided by Jeremy Nathans (Howard Hughes Institute, The Johns Hopkins University). A rat olfactory library used to amplify message for αrCNG2 was provided by Randall Reed (Howard Hughes Institute, Johns Hopkins University). All PCRs yielded a single band, which was extracted from the gel and sequenced in both directions at the Johns Hopkins Core Facility. All PCR products were of the expected size and sequence compared with published mRNA sequence, except CNG3 from rat, which has not been sequenced, except in bovine kidney.

Fig. 1. A: RT-PCR products of αrCNG1 amplified from rat intestinal epithelium. Lane 1, 100-bp molecular-weight ladder (M). Most intense band in middle of ladder is 600-bp marker. Amplification of product with primers specific for αrCNG1 from rat eye (E) shows an RT-PCR product with an intense band at 442 bp. Lane 2, human retinal library (HRL), i.e., a positive control for primer reaction expressed in retinal rod outer segment (17). Expression of channel in human retinal cell lines correlates with expression in rat colon. B: RT-PCR products of αhCNG1 from HT-29C1 and T84 cells. Lane 1, 8X 174 RF DNA/Hae III molecular-weight ladder showing base-pair markers from top to bottom as follows: 1353, 1018, 872, 603, 310, 234, 194, 118, and 72. PCR product for αhCNG1 amplified using primers for αhCNG1 is 421 bp. Lane 2, human intestinal library (HRL), i.e., a positive control for primer reaction expressed in small intestinal scrapes, suggesting that channel is expressed in epithelial cells. C: RT-PCR products of αhCNG1 from IEC-6 and IEC-18 cells. Lane 1, 100-bp ladder showing base pairs 600, 500, and 400. Lanes 2 and 3, RT-PCR products of 442-bp αrCNG1. Expression in these cell lines correlates with expression in small intestinal scrapes, suggesting that channel is expressed in epithelial cells. D: RT-PCR products of αhCNG1 from Panc-1 and CFPAC-1 cells. Lane 1, 8X 174 RF DNA/Hae III molecular-weight ladder showing base-pair markers from top to bottom as follows: 1353, 1018, 872, 603, 310, 281, 234, 194, 118, and 72. RT-PCR product αhCNG1 amplified using specific primers is 421 bp. Message levels were greater in cystic fibrosis (CF) cell line, although both cell lines originate in pancreatic duct. WB, water blank.

Fig. 2. RT-PCR products from αrCNG2 mRNA in rat intestinal mucosa. Lane 1, 100-bp ladder. Most intense band in middle of ladder is 600-bp marker. Lane 3, high-intensity 508-bp band from RT-PCR products amplified from rat olfactory library (O), as previously shown (7); rat eye (E) shows very minor amplification of this isoform (lane 2). Duodenum (D) and ileum (I) show expression of this isoform. J eumen (J) and colon (C) do not express this isoform. WB did not show a band.
Fig. 3. RT-PCR products of αCNG3 from rat intestinal epithelial segments. Lane 1, molecular-weight marker with most intense band at 600 bp. Lane 2, RT-PCR product from eye (E); retinal cone cells have CNG3 (16). Lane 3, no RT-PCR product in olfactory library (O), which indicates that primer does not amplify CNG2. Lane 4, abundant product from kidney (K), where CNG3 channel message was shown by Northern blot (2); this channel is also expressed in lung (L). Lanes 6 and 7, RT-PCR from intestinal segments, including duodenum (D), jejunum (J), ileum (I), and colon (C). Expected product size for CNG3 is 442 bp. WB did not show any PCR product.

Northern Blot

Generation of probe. The CNG3 316-bp probe was generated by RT-PCR spanning bp 1529–1845 relative to CNG3 (accession no. AB002801). The RT-PCR product was gel extracted, sequenced, ligated into PCR2.1 plasmid (Invitrogen, Carlsbad, CA), transformed into DH5α competent cells (Life Technology, Gaithersburg, MD), and grown in Luria-Bertani medium containing 50 µg/ml ampicillin. The plasmids were extracted by alkaline lysis and purified using a Mini-plasmid preparation kit for sequencing followed by a Maxi-plasmid preparation kit (QIAGEN, Valencia, CA) to generate the probe. EcoRI was used to cut the probe cDNA from the plasmid, and the insert was extracted from a gel with a QIAquick gel extraction kit (QIAGEN) and then sequenced. The RT-PCR product and the insert were identical to the reported sequence.

Hybridization. RNA was denatured at 65°C and fractionated by electrophoresis in a 1% agarose gel containing 2.2 M formaldehyde. RNA was determined to be intact by ethidium bromide stain and transferred overnight to Nytran filters (Hybond, Amersham) that were cross-linked with ultraviolet light and then stained with methylene blue to determine that transfer was complete. The blot was prehybridized in Rapid-hyb (Amersham Pharmacia Biotech, Piscataway, NJ) at 65°C for 1.5 h. The probe was radiolabeled by random primer extension (Multiprime DNA labeling kit, Amersham), purified using Probe Quant G-50 micro columns (Pharmacia Biotech), diluted to 1 × 106 cpm/ml in Rapid-hyb, and then hybridized at 65°C for 2 h following the manufacturer's protocol. The membrane was washed three times with 2× sodium chloride-sodium citrate (SSC) and 0.1% SDS at room temperature for 30 min, 1× SSC and 0.1% SDS at 65°C for 20 min, and 0.1× SSC and 0.1% SDS at 65°C for 20 min. Autoradiography of the blot was performed at –80°C with Hyper-film (Amersham Pharmacia). Sizes of mRNA were determined using the 0.24- to 9.5-kb RNA ladder (Life Technologies GIBCO BRL).

RESULTS

To determine whether cyclic nucleotide-gated cation channels might contribute to transepithelial sodium transport across intestinal epithelia, we investigated the distribution of mRNA for the three isoforms in all intestinal segments of the rat and in pancreatic and intestinal cell culture lines. Figure 1A shows that αCNG1 mRNA is expressed in the mucosa of the duodenum, jejunum, ileum, and colon. The mRNA is shown in comparison to that in the eye, where mRNA for αCNG1 has been measured previously by Northern blot (17). No mRNA for CNG1 was amplified from rat olfactory library, where αCNG1 is not present (7). Message for αCNG1 is also expressed in HT-29 and T84 cells (Fig. 1B), as well as in IEC-6 and IEC-18 cells (Fig. 1C) and the pancreatic cell lines Panc-1 and CF-PAC-1 (Fig. 1D), when RT-PCR is used to show expression of mRNA transcripts. The mRNA found in these cell lines suggests that the cDNA amplified from the mucosal layer represents mRNA from epithelial cells.

Figure 2 shows that mRNA for αCNG2 is mainly restricted to the mucosa of the duodenum and ileum. The small amount of message for CNG2 detected in the eye has not previously been reported in rods and cones, where CNG1 and CNG3 are abundant (16). Sequencing of the RT-PCR product confirmed that it was CNG2. Expression of CNG2 in rat eye may represent message from the retinal pigment epithelium or cells other than those involved in sensing light. The highest expression of CNG2 is in olfactory organ library. This channel was cloned from the olfactory organ, where the expression has been shown previously by Northern blot (7).

Restriction analysis of plasmid preparations after RT-PCR by use of degenerate primers in intestinal segments and cell lines revealed nine CNG1 plasmids and six CNG2 plasmids in the ileum. For the colon, 10 plasmids were shown to be CNG1 and 6 were CNG2. For HT-29 cells, nine plasmid preparations had CNG1
and two contained CNG2. Sequencing of clones from rat ileum also revealed that nine were CNG1 and six were CNG2, and sequencing revealed eight CNG1 and four CNG2 clones in rat colon. Sequencing of 11 of 15 clones from the HT-29 library suggested that 9 were CNG1 and 2 were CNG2. These results suggest that CNG1 and CNG2 are expressed in multiple intestinal segments. We previously reported that CNG1 mRNA is expressed in the lung (8) and airway cells (trachea, bronchus, bronchioles, and alveoli) and mediates electrogenic transepithelial sodium absorption across tracheal epithelial cells (28). The degenerate primers used here did not amplify CNG3, which is also expressed in all the intestinal segments (Fig. 3). The kidney expresses high levels of CNG3, as previously reported (2). Figure 5 shows that the mRNA for the secondary (β) subunit, which alters calcium affinity or enhances drug affinity (5), is mainly in the kidney and lung (Fig. 3). By use of Northern analysis, Fig. 4 shows that CNG3 is expressed in all the intestinal segments in almost equal abundance to that in the kidney or heart, where this channel mRNA was previously reported (2). Figure 5 shows that the mRNA for the secondary (β) subunit, which alters calcium affinity or enhances drug affinity (5), is mainly in the colon. The colon was used for transport experiments, because the presence of the secondary subunit suggested a better drug efficacy (5).

To determine the functional role of cyclic nucleotide-gated channel currents in intestinal epithelia, we measured $I_{sc}$ across the proximal and distal colon and $^{45}$Ca$^{2+}$ fluxes across the proximal colon. Because cyclic nucleotide-gated channel currents carry cGMP-stimulated sodium currents, we used 8-Br-cGMP-stimulated sodium-mediated $I_{sc}$ to investigate the role of cyclic nucleotide-gated channel currents in sodium entry and transepithelial transport in proximal and distal colon, where CNG1 and CNG3 are expressed (Figs. 1A, 3, and 4). Because amiloride-sensitive (ENaC-mediated) electrogenic sodium absorption has been reported in the distal colon of the rats fed a low-salt diet, electrogenic sodium current in this segment was investigated in the presence of amiloride to block these currents; furthermore, only l-cis-diltiazem was used to completely distinguish cyclic nucleotide-gated channels from ENaC, both of which are blocked by dichlorobenzamil. To distinguish electrogenic sodium currents carried by cyclic nucleotide-gated channel currents, we used two classes of blockers of these channels, l-cis-diltiazem and dichlorobenzamil, which caused inhibition of cGMP-stimulated currents (Table 1), whereas amiloride and phenamil did not. The β-subunit for cyclic nucleotide-gated channel currents was also expressed in the colon (Fig. 5). Because this subunit is thought to increase the calcium conductance of cyclic nucleotide-gated channel currents (5), the role of cyclic nucleotide-gated channel currents (5), the role of cyclic nucleotide-gated channel currents in transepithelial calcium transport was also measured by unidirectional transepithelial $^{45}$Ca$^{2+}$ fluxes. The role of Sta, which increases cGMP-mediated chloride secretion in crypt-derived cells, has been well studied, but the role of Sta (or guanylin) on other transport pathways has not been well defined. Therefore, we investigated the role of Sta in mediating transepithelial sodium and calcium transport. Sta increased transepithelial sodium transport, as measured by $I_{sc}$ and unidirectional and net $^{45}$Ca$^{2+}$ transport across the proximal colon that was blocked by l-cis-diltiazem (Table 2).

We also studied electrogenic sodium absorption in the distal colon of the rats fed a normal-salt diet, where sodium currents mediated by ENaC are minimal (Table 3). $I_{sc}$ was also measured in rats fed a low-salt diet for

### Table 1. Effect of 8-Br-cGMP on $I_{sc}$ in normal rat proximal colon

<table>
<thead>
<tr>
<th>n</th>
<th>PD$_i$, mV</th>
<th>$I_{sc}$, µA/cm$^2$</th>
<th>$R_i$, Ω·cm$^2$</th>
<th>Basal</th>
<th>8-Br-cGMP</th>
<th>l-cis-Diltiazem</th>
<th>Dichlorobenzamil</th>
<th>Amiloride</th>
<th>Phenamil</th>
<th>DMSO</th>
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<td>10</td>
<td>15.0 ± 1.36</td>
<td>83.4 ± 6.4</td>
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<td>100</td>
<td>124.6 ± 5.2$^*$</td>
<td>101.7 ± 3.1†</td>
<td>100.4 ± 8.1†</td>
<td>169.3 ± 20.9‡</td>
<td>133.0 ± 16.6‡</td>
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<td>9</td>
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<td>143.4 ± 10.6</td>
<td>138.3 ± 10.8‡</td>
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Values are means ± SE; n, number of animals. Proximal colons were treated with 2 mM 8-bromo-cGMP (8-Br-cGMP), 200 µM l-cis-diltiazem, 30 µM dichlorobenzamil, 100 µM amiloride, 100 µM phenamil, or 30 µl of DMSO. PD$_i$, potential difference measured when colon was first mounted in Ussing chamber; $I_{sc}$, initial short-circuit current measured when colon was first mounted; $R_i$, initial resistance. At beginning of experimental period, just before addition of 8-Br-cGMP, $I_{sc}$ was standardized to 100 and called basal $I_{sc}$. $I_{sc}$ 8-Br-cGMP, stable current measured after addition of 8-Br-cGMP; all other currents, e.g., $I_{sc}$ amiloride, were stable currents measured after addition of drugs. *Significantly different from basal (P < 0.05); †significantly different from 8-Br-cGMP (P < 0.05); ‡not significantly different from 8-Br-cGMP (P > 0.1, by 2-tailed Student’s t-test).
Table 2. Effect of STa and l-cis-diltiazem on 45Ca2+ fluxes in rat proximal colon

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control (0–30 min)</th>
<th>STa (30–60 min)</th>
<th>STa + l-cis-Diltiazem (30–90 min)</th>
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<td>I_sc Na, µA/cm²</td>
<td>65 ± 7.6</td>
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</tbody>
</table>

Values are means ± SE; n = 6. Heat-stable toxin from Escherichia coli (STa, 2 µg/10 ml) and l-cis-diltiazem (200 µM) were added to apical side. J_min and J_net, unidirectional fluxes of 45Ca2+ in mucosal-to-serosal and serosal-to-mucosal directions, respectively; J_net = J_min - J_min; I_sc, Na, I_sc in chloride-free media; I_sc Ca, I_sc of calcium calculated from J_net. *Significantly different from control (P < 0.005); †significantly different from STa (P < 0.05); ‡significantly different from control (P < 0.01); ‡‡significantly different from STa (P < 0.05); ‡‡‡significantly increased (P < 0.05, by paired t-test) compared with control.

Table 3. Effect of 8-Br-cGMP on I_sc in rat distal colon

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>PD, µA/cm²</th>
<th>I_sc,i, µA/cm²</th>
<th>R_sc, TSc/cm²</th>
<th>I_sc</th>
<th>Basal</th>
<th>Amiloride</th>
<th>8-Br-cGMP</th>
<th>l-cis-Diltiazem</th>
<th>Dichlorobenzamil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>11</td>
<td>6 ± 1.6</td>
<td>38 ± 8.7</td>
<td>236 ± 32.9</td>
<td>100</td>
<td>120+2</td>
<td>4.2+1</td>
<td>8-Br-cGMP</td>
<td>96 ± 7.5†</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>7</td>
<td>9 ± 1.5</td>
<td>65 ± 9.0</td>
<td>198 ± 18.8</td>
<td>100</td>
<td>116+2</td>
<td>3.6+1</td>
<td>l-cis-Diltiazem</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low salt diet</td>
<td>9</td>
<td>27 ± 5.6</td>
<td>210 ± 23.7</td>
<td>198 ± 23.3</td>
<td>177+19.8</td>
<td>100</td>
<td>109+3.7</td>
<td>3.7+1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of animals. Animals were fed normal diet containing sodium or low-salt diet (NaCl-deficient diet) and distilled water for ≥14 days. Distal colon was treated with 10 µM amiloride, 2 mM 8-Br-cGMP, 200 µM l-cis-diltiazem, or 30 µM dichlorobenzamil. See Table 1 footnote for definition of abbreviations. * Significantly different from basals (P < 0.05); †significantly different from 8-Br-cGMP (P < 0.05); ‡significantly different from amiloride (P < 0.05, by 2-tailed Student’s t-test).

DISCUSSION

Intestinal epithelial cells absorb sodium via electro-neutral or electroneutral uptake at the apical membrane and extrude sodium at the basolateral membrane via the Na⁺-K⁺-ATPase. The major fraction of sodium delivered to the small intestine and proximal colon is absorbed via the electroneutral NHE (6, 19, 26), whereas the distal colon mediates electrogenic transepithelial sodium absorption via ENaC under conditions of a low-salt diet (20, 31, 32). Sodium entry and electrogenic sodium absorption via ENaC is increased when circulating aldosterone is high (32) as a result of low salt intake (31). The cloned ENaC is the most completely studied epithelial sodium channel (3, 4). We now show that mRNA for the cyclic nucleotide-gated cation channel αCNG1 is expressed in the mucosa of all intestinal segments, in several intestinal epithelial cell lines, and in two pancreatic cell lines. Other isoforms, CNG2 and CNG3, and the secondary subunit βCNG are also present in intestinal mucosa, suggesting that these channels may also mediate electrogenic sodium and calcium currents into intestinal epithelial cells.

In the proximal and distal colon, cyclic nucleotide-gated cation channels carry an 8-Br-cGMP-stimulated, sodium-mediated I_sc that is inhibited by l-cis-diltiazem. The current stimulated by cGMP is not inhibited by amiloride, suggesting that ENaC does not carry this current. Likewise, phenamil, a more potent blocker of ENaC than amiloride, did not inhibit the 8-Br-cGMP-stimulated, sodium-mediated I_sc in the proximal colon, whereas dichlorobenzamil did inhibit the I_sc. These results are consistent with previous data (31) which suggest that ENaC does not carry an amiloride-sensitive sodium current in the proximal colon of the rat. The transepithelial sodium transport carried by cyclic nucleotide-gated cation channels in the colon is less in magnitude than aldosterone-induced, ENaC-mediated sodium absorption in the distal colon (31, 32) or electroneutral sodium absorption mediated by NHE in the proximal colon (6), but these other pathways are specific for sodium. Opening of cyclic nucleotide-gated

...
cation channels by cGMP increases sodium and calcium conductance through this class of channels (14, 17, 24).

In the same manner, because an inward electrochemical gradient for sodium and calcium exists across the apical membrane of intestinal epithelial cells, the opening of cyclic nucleotide-gated cation channels by cGMP would increase calcium and sodium influx across the apical membrane. We have shown that STa increases sodium-mediated \( I_{sc} \) to increase electrogenic transepithelial transport of sodium, and at the same time, STa increases transepithelial calcium transport. The finding that blockers of cyclic nucleotide-gated cation channels, but not amiloride, inhibit the transepithelial movement of sodium and calcium suggests that cyclic nucleotide-gated cation channels mediate an electroconductive sodium and calcium entry at the apical membrane of the proximal colon. Therefore, unlike ENaC and NHE, cyclic nucleotide-gated cation channels mediate transepithelial sodium and calcium transport stimulated by STa or cGMP.

The receptors for STa and guanylin are distributed from the crypt-to-villus axis along the entire intestine (21, 29). The role of these receptors in transepithelial transport or signal transduction in the villus tip is not clearly defined, but the major localization of guanylate cyclase is in the brush border (29). Guanylin and STa act in the small and large intestine to increase cGMP-mediated chloride secretion from intestinal crypts (11). Likewise, the role of guanylin in the surface epithelia of the colon is not well defined, but this segment also has a surface distribution of this receptor (29).

It was previously shown in porcine distal colon that cGMP, in the absence of chloride, increases sodium-mediated \( I_{sc} \) and inhibits electroneutral sodium chloride absorption, as measured by \( 22^{\text{Na}} \) influx (10). Similarly, atrial natriuretic peptide in this preparation inhibits electroneutral sodium chloride absorption via cGMP- and calcium-mediated mechanisms (1). Extracellular calcium, as well as protein kinase C, was previously shown to activate particulate guanylate cyclase activity in rat colon (18). Thus a calcium influx mediated by cyclic nucleotide-gated cation channels would be expected to enhance increases in cGMP caused by the action of STa in the colon.

Although we did not measure transepithelial \( 22^{\text{Na}} \) fluxes in the small intestine, inhibition of NHE by calcium is well documented (9). Because the mRNA for cyclic nucleotide-gated cation channels also exist in the ileum, it is likely that increased influx of calcium stimulated by cGMP could inhibit transepithelial sodium absorption via NHE in the small intestine and/or enhance chloride secretion in this segment. Our experiments predict that STa, by increasing calcium influx via cyclic nucleotide-gated cation channels, would act to alter sodium absorption or chloride secretion along the entire intestine. Increases in intracellular calcium are not usually associated with the action of STa on chloride secretion (11), but it is more difficult to measure small increases in intracellular calcium, such as those mediated by calcium influx, than to measure the large increases caused by release of calcium from intracellular stores related to inositol trisphosphate (12).

In summary, we have documented, for the first time, that mRNA of the three isoforms of cyclic nucleotide-gated cation channels exist in intestinal mucosa from the duodenum to the distal colon. The finding that the mRNA is also present in intestinal and pancreatic epithelial cell lines is evidence that these channels reside in the epithelial cells. In addition, functional transport assays suggest that these channels mediate transepithelial calcium and sodium across the proximal and distal colon; this transepithelial transport also implies that these channels reside in transporting epithelial cells. Because these channels exist along the entire intestinal tract, we speculate that they are poised to enhance cGMP-mediated activation of guanylate cyclase activity that would inhibit sodium absorption or stimulate chloride secretion, if these channels are expressed along the whole crypt-to-villus axis.

We thank Drs. S. Khurana and J. Wright for helpful discussions regarding flux analysis.

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