Functional and molecular identification of a novel chloride conductance in canine colonic smooth muscle

GREGORY M. DICK, KARRI K. BRADLEY, BURTON HOROWITZ, JOSEPH R. HUME, AND KENTON M. SANDERS
Department of Physiology and Cell Biology, University of Nevada
School of Medicine, Reno, Nevada 89557

Dick, Gregory M., Karri K. Bradley, Burton Horowitz, Joseph R. Hume, and Kenton M. Sanders. Functional and molecular identification of a novel chloride conductance in canine colonic smooth muscle. Am. J. Physiol. 275 (Cell Physiol.: 44): C940–C950, 1998.—Swelling-activated or volume-sensitive Cl− currents are found in numerous cell types and play a variety of roles in their function; however, molecular characterization of the channels is generally lacking. Recently, the molecular entity responsible for swelling-activated Cl− current in cardiac myocytes has been identified as ClC-3. The goal of our study was to determine whether such a channel exists in smooth muscle cells of the canine colon using both molecular and electrophysiological techniques and, if present, to characterize its functional and molecular properties. We hypothesized that ClC-3 is present in colonic smooth muscle and is regulated in a manner similar to the molecular entity cloned from heart. Indeed, the ClC-3 gene was expressed in colonic myocytes, as demonstrated by reverse transcriptase polymerase chain reaction performed on isolated cells. The current activated by decreasing extracellular osmolarity from 300 to 250 mosM was outwardly rectifying and dependent on the Cl− gradient. Current magnitude increased and reversed at more negative potentials when Cl− was replaced by I− or Br−. Tamoxifen ([Z]-1-[p-dimethylaminoethoxy-phenyl]-1,2-diphenyl-1-butene; 10 µM) and DIDS (100 µM) inhibited the current, whereas 25 µM niflumic acid, 10 µM nicardipine, and Ca2+ removal had no effect. Current was inhibited by 1 mM extracellular ATP in a voltage-dependent manner. Cl− current was also regulated by protein kinase C, as phorbol 12,13-dibutyrate (300 nM) decreased Cl− current magnitude, while chelerythrine chloride (30 µM) activated it under isotonic conditions. Our findings indicate that a current activated by hypotonic solution is present in colonic myocytes and is likely mediated by ClC-3. Furthermore, we suggest that the ClC-3 may be an important mechanism controlling depolarization and contraction of colonic smooth muscle under conditions that impose physical stress on the cells.

the ability of mechanical stimuli to elicit smooth muscle contraction has been realized for almost 100 years (2), and the first simultaneous recordings of smooth muscle tension and membrane potential were provided almost one-half century ago (3). During the past decade, the cellular mechanisms underlying depolarization and contraction in response to mechanical distension (i.e., the myogenic response) have begun to be elucidated (5, 6, 11). Of particular importance, mechanosensitive ion channels are thought to transduce mechanical forces into cellular signals such as Ca2+ influx (5, 6). Mechanosensitive ion channels are present in visceral smooth muscle and may regulate their electrical activity (18, 34, 36).

Mechanical stimulation, applied in the form of hypotonic cell swelling, potentiates the nonselective cation current activated by muscarinic stimulation in smooth muscle cells isolated from the guinea pig ileum without effect on other voltage-dependent channels such as outwardly rectifying K+ channels or L-type Ca2+ channels (34). Activation of these nonselective cation channels would be expected to cause membrane depolarization and contraction of smooth muscle. Indeed, such a mechanism has been shown to mediate a portion of the myogenic response of small arteries (23). The nonselective cation channels of arterial myocytes can be activated directly by stretching isolated cells with glass microelectrodes attached at each end (5, 6). Along with nonselective cation currents, Cl− currents may also participate in the depolarization response of smooth muscle to mechanical stimuli, as the Nernst equilibrium potential for Cl− (ECI) is positive to the resting membrane potential in visceral smooth muscles (1).

Mammalian cardiac myocytes possess a swelling-activated Cl− conductance (29), which has been shown to affect electrical activity (31). A molecular entity responsible for a Cl− current activated by cell swelling has recently been cloned from guinea pig heart and expressed in 3T3 fibroblasts (8). The ClC-3 gene encodes a swelling-activated Cl− channel that is a member of the gene superfamily that includes ClC-2 (17), another ion channel proposed to play a part in cellular volume regulation (10). Neither ClC-2 nor other previous candidates for the swelling-activated Cl− channel such as P-glycoprotein (32) or pICln (26) possess properties consistent with a role for these channels as the current carrier in native cells. Evidence supporting ClC-3 as the cardiac swelling-activated Cl− channel includes (8) 1) the magnitude of current through cloned ClC-3 increases and decreases as cells are exposed to hypotonic and hypertonic solutions, respectively; 2) the reversal potential of this conductance shifts with EC1; 3) ClC-3 channels are outwardly rectifying and more permeable to I− than Cl−; 4) DIDS, tamoxifen, extracellular ATP, and phorbol 12,13-dibutyrate (PDBu) inhibit ClC-3 current; and 5) single-channel properties of ClC-3 are similar to those in native cardiac myocytes. These properties are similar to the native cardiac swelling-activated Cl− current. In addition, a single
whether the ClC-3 gene is expressed in visceral smooth muscle. Cl- current activated by hypotonic solution was recently described in guinea pig antral smooth muscle, and this conductance was suggested as a mediator of volume regulation (36). Cl- currents may also be involved in the response to mechanical stimulation of canine pulmonary and renal artery smooth muscle cells, which express the CIC-3 gene (37). The present study tests the responses of smooth muscle cells of the gastrointestinal (GI) tract to hypotonic solutions to determine whether a Cl- conductance is activated. We have also investigated whether the properties of the current activated by hypotonic solution are consistent with CIC-3, the swelling-activated Cl- channel in cardiac myocytes (8). Molecular studies were also performed on isolated smooth muscle cells to determine whether the CIC-3 gene is expressed in visceral myocytes.

MATERIALS AND METHODS

Preparation of smooth muscle cells. Mongrel dogs of either sex were killed with an overdose of pentobarbital sodium (100 mg/kg), and the abdomen was opened along a midline incision. The stomach and proximal colon were removed and placed in Krebs solution (see Solutions and reagents). Sheets of the tunica muscularis were dissected from the overlying mucosal elements, and strips of muscle were cut with a double-bladed scalpel and pinned out in a dissection dish filled with Ca2+-free Hanks' solution (see Solutions and reagents). Smooth muscle cells from the circular muscle layer of the fundus and colon were isolated as described previously (19).

Isolated smooth muscle cells from mouse colon were also prepared. Adult BALB/c mice of either gender were anesthetized with chloroform and killed by cervical dislocation. A segment of proximal colon was removed, placed in Ca2+-free Hanks' solution, opened along the mesenteric border, and washed free of fecal contents. Mucosa and submucosa were removed by blunt dissection, and the remaining tunica muscularis was covered with an enzyme solution containing 230 U/ml collagenase (Worthington Biochemical, Freehold, N J) and 100 U/ml elastase (Sigma Chemical, St. Louis, MO) in Ca2+-free Hanks' buffer, opened along the mesenteric border, and washed free of fecal contents. Mucosa and submucosa were removed by blunt dissection, and the remaining tunica muscularis was covered with an enzyme solution containing 230 U/ml collagenase (Worthington Biochemical, Freehold, N J) and 100 U/ml elastase (Sigma Chemical, St. Louis, MO) in Ca2+-free Hanks' buffer. Tissues were incubated 20–30 min at 37°C, washed free of enzymes with Ca2+-free Hanks' solution, and cut into six to eight pieces that were ~10–15 mm2. Tissue pieces were then triturated to create a cell suspension. The cells were stored at 4°C and used within 8 h.

Current measurements. Drops of cell suspensions were placed on a glass coverslip forming the bottom of a 300-µl chamber mounted on an inverted microscope. Cells were allowed to adhere to the coverslip and then solutions were suffused by gravity. The bath solution flowed at a rate of ~3 ml/min, and therefore, the solution in the chamber was exchanged ~10 times per minute. “Giga seals” were made with fire-polished glass pipettes having tip resistances of 3–4 MΩ. The whole cell, dialyzed configuration of the patch-clamp technique was used to record ionic currents. The headstage ground wire was connected to the bath via an agar bridge containing 3 M KCl to prevent junction potential errors when the Cl- concentration was altered. An Axopatch 1-D amplifier and CV-4 headstage (Axon Instruments, Foster City, CA) were used to measure ionic currents. The currents were sampled at 4 kHz and filtered at 1 kHz. A personal computer running pCLAMP software (version 5.5.1, Axon Instruments) was used to collect data. All recordings were performed at room temperature (22–25°C).

Solutions and reagents. Nicardpine, PDBu, 1-(5-isouquinolinesulfonyl)-2-methylpiperazine (H-7), and chelerythrine chloride were purchased from Research Biochemicals (Natick, MA). CaCl2, KCl, KH2PO4, NaCl, NaHCO3, Na2HPO4, sucrose, and glucose were from Fisher Scientific (Fair Lawn, N J), and all other chemicals were from Sigma. Krebs solution contained (in mM) 125 NaCl, 5.9 KCl, 2.5 CaCl2, 1.5 NaHCO3, 1.2 Na2HPO4, and 11.5 glucose, with pH adjusted to 7.3–7.4 by bubbling with 95% O2-5% CO2. Ca2+-free Hanks' solution contained (in mM) 125 NaCl, 5.36 KCl, 15.5 NaHCO3, 0.336 Na2HPO4, 0.44 KH2PO4, 10 glucose, 2.9 sucrose, and 11 HEPEs, pH adjusted to 7.4 with NaOH. Isotonic bath solution contained (in mM) 125 NaCl, 5 CsCl, 1.3 MgCl2, 2 CaCl2, 10 HEPEs, 5 Tris, and 10 glucose, with pH adjusted to 7.4 with NaOH. Osmolarity of all solutions was checked with a freezing point depression osmometer (model 5004, Precision Systems). Isotonic bath osmolarity was adjusted to 300 mosM with mannitol. Hypotonic bath solution had the same composition as isotonic bath solution except that the NaCl concentration was reduced to 100 mM and adjusted to 250 mosM with mannitol. Hypertonic bath solution was prepared by adding 50 mosM mannitol to the normal isotonic bath solution, thus giving a final strength of 350 mosM. Extracellular Cl- was removed by equimolar replacement with aspartate, isethionate, Br-, or I- as indicated in Figs. 1–10 or text. The pipette solution contained (in mM) 100 tetraethylammonium (TEA) chloride, 10 HEPEs, 2 EGTA, 5 Mg-ATP, 1 NaGTP, and 2.5 phosphocreatine and was brought to pH to 7.1 with TEA hydroxide. The osmolarity of the pipette solution was adjusted to 300 mosM by adding mannitol.

Molecular biology techniques. Single smooth muscle cells isolated from the various species and tissues were differentiated from other cell types by their characteristic morphology; that is, smooth muscle cells were taken to be those that were spindle-shaped with a length of 50–100 µm and a width of 5–10 µm. Smooth muscle cells were collected by aspirating them into a wide-bore patch-clamp pipette. Sixty cells from each source were collected and frozen in liquid nitrogen for molecular biological work. Total RNA was prepared from colon and fundus muscle cells and tissue isolated from dogs and mice by use of the SNAP Total RNA Isolation kit (Invitrogen, Carlsbad, CA) as per the manufacturer’s instructions. First-strand RNA (1 µg) was reverse transcribed by use of an oligo(dT)12–18 primer (500 µg/µl). Specific CIC-3 primers were used that anneal to the sequence near the carboxy terminus of the amino acid sequence forward, nucleotides 1891–1911, and reverse, 2130–2150 (gene accession no. U83464). The housekeeping gene control sequence (β-actin) was amplified from the same cDNA by using primers that anneal at nucleotides 2382–2400 or 3071–3090 (gene accession no. V01217). Sense and antisense primers (20 µM) were combined with cDNA and 1 mM dNTP, 40 mM Tris·HCl (pH 8.3), 100 mM KCl, 3 units Taq (Promega, Madison, WI), 1 AmpliWax Gen 100 (Perkin Elmer, Foster City, CA), and RNase-free water to a final volume of 50 µl. PCR was performed in a COY II Thermal Cycler under the following
conditions: 32 cycles at 94°C, 1 min; 57°C, 30 s; 72°C, 1 min; and then, incubated at 72°C, 10 min. PCR products were separated by electrophoresis on a 2% agarose gel. Quantitative PCR (Q-PCR) was performed by use of the PCR MIMIC Construction Kit (Clontech, Palo Alto, CA), which is based on a competitive PCR approach, where nonhomologous engineered DNA standards (referred to as PCR MIMICs) compete with target DNA for the same gene-specific primers. PCR MIMICs were constructed for ClC-3 and the control standard (β-actin, n), and competitive PCR was carried out by titration of sample cDNA with known amounts of the desired nonhomologous PCR MIMIC constructs; serial dilutions of these constructs were added to PCR amplification reactions. After PCR, products were separated by electrophoresis on a 2% agarose gel and quantified by use of Molecular Analyst software (Bio-Rad, Hercules, CA). Experiments utilizing PCR to determine ClC-3 expression in visceral smooth muscle cells were performed on cells isolated from at least three different animals. For Q-PCR, the concentration of the target DNA was normalized to β-actin expression.

Statistical analyses. Data are expressed as means ± SE from n number of cells (or n number of animals for Q-PCR). Current-voltage (I-V) relationships were constructed from currents in isotonic solutions to calculate difference currents. The time at which the difference current reached maximum at the most positive test potential was determined, and all current amplitudes were measured at this time point. All statistical analyses were performed using SigmaStat 1.0 software (Jandel, San Rafael, CA). I-V relationships were compared by two-way repeated-measures ANOVA. In all statistical analyses, values of P < 0.05 were considered statistically significant. When differences between I-V curves were found by ANOVA, Student-Newman-Keuls post hoc test was used to identify voltages at which the I-V curves might differ. One-way ANOVA was used to compare reversal potentials and maximum currents in Cl− replacement experiments, and Bonferroni post hoc test was used to discern differences from control. For Q-PCR experiments, one-way ANOVA was used to compare gene expression among the groups, and Student-Newman-Keuls post hoc test was then used to identify differences between the groups.

RESULTS

Whole cell current activated by hypotonic solution. When myocytes isolated from the circular layer of the canine colon were exposed to hypotonic solution (osmolality decreased from 300 to 250 mosM; pipette osmolality 300 mosM), outwardly rectifying currents developed. Experiments were performed to determine the nature of this current. Cells were dialyzed with 100 mM TEA chloride to block K+ currents and the outward flow of monovalent cations through the nonselective cation channels that are known to be present in colonic myocytes (21). Cells were held at −40 mV and stepped from −100 to +120 mV in 20-mV steps before and after exposure to hypotonic solution. Subtraction of currents in isotonic and hypotonic solution yielded difference currents that reversed at an average of −9.8 ± 0.9 mV (Fig. 1B, inset), close to the ECl (calculated to be −8 mV). A current with the same characteristics and reversal potential was observed in identical experiments conducted on isolated muscle cells from the circular layer of the canine fundus and mouse proximal colon (n = 3 each, data not shown). Hypotonic solution activated an outwardly rectifying current that reversed at −10.2 ± 0.8 mV (n = 3) in the presence of a symmetrical 100 mM TEA (ECl = −8 mV), ruling out the possibility that nonselective cation channels carry the current or that extracellular Na+ influences the current. Also, correcting solution osmolality with sucrose produces the same effects as mannitol; that is, hypotonic and hypertonic solutions made with sucrose increase and decrease current amplitude, respectively (n = 2, data not shown).

The same solutions, but a different voltage-step protocol, were used to examine this current in the physiological range of membrane potentials (Fig. 1C and D). Cells were held at −40 mV and stepped from −80 mV to +10 mV in 6-mV increments. Exposing myocytes to hypotonic solution induced current over
the range of potentials tested. Subtraction of currents in isotonic solution from currents in hypotonic solution yielded difference currents that reversed at \(-8.7 \pm 1.4\) mV (Fig. 1, C and D), a value very similar to those found in the experiments detailed above.

The reversal potential of the current activated by hypotonic solution was near E_{CI}, suggesting the current is carried mainly by Cl\(^-\). This hypothesis was tested by altering the Cl\(^-\) gradient. Replacement of extracellular Cl\(^-\) with aspartate decreased magnitude of the outward current in a concentration-dependent manner and shifted the reversal potential to more positive values (Fig. 2, A–C). The reversal potential of the swelling-activated current shifted largely as predicted by the E_{Cl} (Fig. 2D). There was, however, some deviation from the predicted relationship at low concentrations of extracellular Cl\(^-\), as there may be some finite permeability for aspartate ions (37). Replacement of extracellular Cl\(^-\) with varying amounts of isethionate produced effects on current magnitude and reversal potential similar to those of aspartate \((n = 3;\) data not shown).

In additional experiments, extracellular Cl\(^-\) was replaced by Br\(^-\) \((n = 1)\) or I\(^-\) \((n = 4)\). In contrast to the effects of aspartate and isethionate, I\(^-\) enhanced current magnitude and shifted the reversal potential to a more negative value (Fig. 3, A and B). Relative currents in the presence of Cl\(^-\), Br\(^-\), and I\(^-\) were compared (Fig. 3B). Replacement of extracellular Cl\(^-\) with equimolar Br\(^-\) increased current magnitude and shifted the reversal potential to a more negative value. These changes were even greater when Cl\(^-\) was replaced with I\(^-\). Permeability for I\(^-\) was 1.07 \pm 0.1 times that for Cl\(^-\) \((n = 3)\), when calculated by the Goldman-Hodgkin-Katz equation. These data suggest a permeability relationship such that I\(^-\) \(>\) Br\(^-\) \(>\) Cl\(^-\). Such a permeability sequence has been demonstrated previously for the swelling-activated Cl\(^-\) conductance in canine renal and pulmonary artery myocytes (37). The Cl\(^-\) current activated by hypotonic solution exhibited voltage-dependent inactivation at more positive test potentials, as has been shown in other cell types (16, 22, 33). The inactivation could be fit by a single exponential. I\(^-\) replacement of Cl\(^-\) slowed inactivation of the anion current at positive potentials, as demonstrated by the change in time constant \((\tau)\) in Fig. 3D. This suggests that currents carried by I\(^-\) show less voltage-dependent inactivation, as demonstrated in a previous study (33).

**Time and concentration dependence of the Cl\(^-\) current.** Upon exposure to hypotonic solution, Cl\(^-\) current increased as a function of osmolarity and time (Fig. 4). Three cells were exposed to solutions ranging from 250 to 300 mosM. During exposure to each solution, the cells were stepped from \(-40\) mV to potentials ranging from \(-100\) to \(+120\) mV in 20-mV steps. There was an inverse relationship between the osmolarity of the solution and current magnitude, because lowering the osmolarity increased Cl\(^-\) current in a concentration-dependent manner (Fig. 4B). After exposure to hypotonic solution, Cl\(^-\) current developed with a half time of \(2.1 \pm 0.2\) min and reached an apparent steady-state level within 5-10 min. In four additional experiments, we studied the reversal of the activated Cl\(^-\) current when cells were returned to isotonic solution. After full activation of the Cl\(^-\) current, the time for recovery in isotonic solution was \(10.3 \pm 1.7\) min. Experiments showing the time course of the development of the Cl\(^-\) current are summarized in Fig. 4, C and D.

**Pharmacology of the Cl\(^-\) current activated by hypotonic solution.** We tested the effects of known Cl\(^-\) channel inhibitors at potentials from \(-100\) to \(+120\) mV on the current activated by hypotonic solution. After induction of the Cl\(^-\) current, cells were treated with tamoxifen \((10 \mu M; n = 3)\), DIDS \((100 \mu M; n = 3)\), or niflumic acid \((25 \mu M; n = 3)\). DIDS and tamoxifen reduced the Cl\(^-\) current (Fig. 5). Niflumic acid, a cyclooxygenase inhibitor and potent inhibitor of Ca\(^2+\)-activated Cl\(^-\) channels, did not attenuate Cl\(^-\) current (data not shown). Tamoxifen, an antiestrogen compound, inhibits swelling-activated Cl\(^-\) channels. Tamoxifen decreased significantly the magnitude of the

**Fig. 2.** Current activated by hypotonic solution is Cl\(^-\) dependent. A: currents elicited by steps from \(-40\) mV to voltages from \(-100\) to \(+120\) mV in 20-mV increments in isotonic and hypotonic solutions. Currents were also measured in hypotonic solutions in which Cl\(^-\) was partially replaced with equimolar aspartate (as noted). Group data for these experiments \((n = 3)\) are shown in B. Open squares, solid squares, and open triangles represent I-V relationships in hypotonic solutions containing 114.4 mM Cl\(^-\), 61.4 mM Cl\(^-\)/50 mM aspartate, or 114 Cl\(^-\)/100 mM aspartate, respectively. Asterisks indicate voltages where currents in presence of 50 or 100 mM aspartate differ from currents in control hypotonic solution. B, inset, contains Cl\(^-\)-sensitive current. C: additional data from experiments described in B, but axes are expanded to reveal reversal potentials \((E_{\text{reversal}})\) in presence of varying Cl\(^-\) gradients. Average reversal potentials are shown in D, and straight line shows predicted relationship.
Fig. 3. Effect of I\(^-\) and Br\(^-\) replacement on current activated by hypotonic solution. An example of effect of 100 mM I\(^-\) replacement of Cl\(^-\) on current is shown in A. Hypotonic difference was determined by subtracting isotonic current from current in hypotonic Cl\(^-\) solution. I\(^-\) replacement increased peak and sustained portions of current as a relatively time-independent difference current was activated. I\(^-\) difference current was determined by subtracting hypotonic Cl\(^-\) current from hypotonic I\(^-\) current. An example of relative currents carried by Cl\(^-\), Br\(^-\), and I\(^-\) is contained in B. In these tracings, where cell was held at −40 mV and potential ramped from −100 to +120 mV, current magnitude increased and showed less voltage-dependent inactivation in presence of Br\(^-\) or I\(^-\). C: group data (n = 4) illustrating effect of I\(^-\) on peak I-V relationship, where open and solid symbols represent currents in presence of Cl\(^-\) and I\(^-\), respectively. Change in time constant (τ) due to Cl\(^-\) replacement with I\(^-\) can be seen in D (symbols are same as C).

Cl\(^-\) current activated by hypotonic solution at both positive and negative test potentials, as has been shown in vascular smooth muscle (37). The tamoxifen-sensitive Cl\(^-\) current reversed at −8.8 ± 2.7 mV (Fig. 5B). In contrast, DIDS, a stilbene derivative that antagonizes various anion channels and transporters, produced a striking effect on the outward Cl\(^-\) current but much less inhibitory effect on the inward current, as is shown in the example in Fig. 5C and has been demonstrated previously (8, 36, 37). In the three experiments with DIDS, the DIDS-sensitive Cl\(^-\) current reversed at −12.3 ± 1.5 mV. The pharmacology of Cl\(^-\) current induced by hypotonic solution was also examined within the physiological range of potentials by stepping cells in 6-mV increments from −80 to +10 mV (Fig. 5D). The tamoxifen- and DIDS-sensitive Cl\(^-\) currents reversed at −10.8 ± 0.6 and −8.5 ± 2.7 mV, respectively.

Ca\(^{2+}\) independence of the Cl\(^-\) current activated by hypotonic solution. Ca\(^{2+}\)-activated Cl\(^-\) channels are present in numerous smooth muscle cell types (20), so we performed experiments to test whether the Cl\(^-\) current activated in hypotonic solution was Ca\(^{2+}\) dependent. Resistance of the current activated by hypotonic solution to 25 µM niflumic acid suggests that it is not mediated by Ca\(^{2+}\)-activated Cl\(^-\) channels; however, this does not rule out some other dependence on Ca\(^{2+}\). Replacement of extracellular Ca\(^{2+}\) with Mn\(^{2+}\) or addition of nicardipine has been shown in previous studies to reduce currents through Ca\(^{2+}\)-dependent conductances in canine colonic muscle cells (4). Replacement of extracellular Ca\(^{2+}\) with equimolar Mn\(^{2+}\) had no effect on the Cl\(^-\) current activated by hypotonic solution (Fig. 6A and B). The lack of dependence on Ca\(^{2+}\) entry was confirmed in the physiological range of membrane potentials, and data from these experiments are dis-

Fig. 4. Concentration and time dependence of Cl\(^-\) current. Effect of reduced extracellular osmolarity on current magnitude can be seen in A. This cell was held at −40 mV and stepped to +120 mV repeatedly as extracellular osmolarity was reduced from 300 to 250 mosM. B: concentration dependence of Cl\(^-\) current over potentials ranging from −100 to +120 mV (symbols are omitted for clarity; group data are contained in inset, n = 3). Time and concentration dependence of current is shown in example in C. This cell was held at −40 mV and stepped to −100 and +120 mV every 20 s. When solution osmolarity was reduced to 275 mosM, current increased to a new steady state at both test potentials within a few minutes. Same effect was seen when osmolarity was reduced to 250 mosM and was reversed when cell was returned to isotonic solution. A protocol similar to that in A was used to determine time course of onset of Cl\(^-\) current when osmolarity was reduced to 250 mosM (D). In this example, cell was held at −40 mV and stepped to +120 mV every 30 s after application of hypotonic solution.
played in Fig. 6B, inset. Cl\(^-\) current in the presence of Ca\(^{2+}\) or Mn\(^{2+}\) reversed at \(-11.7 \pm 0.9\) and \(-11.7 \pm 1.9\) mV, respectively. There was no significant difference in the magnitude or the reversal potential of the Cl\(^-\) current in the presence and absence of Ca\(^{2+}\). Addition of nicardipine (10 \(\mu\)M) to inhibit Ca\(^{2+}\) entry through L-type Ca\(^{2+}\) channels in these cells (35) did not affect the ability of hypotonic solution to activate the Cl\(^-\) current (Fig. 6, C and D).

Inhibition of Cl\(^-\) current by extracellular ATP. CIC-3 was recently suggested as a candidate for volume-activated Cl\(^-\) current in mammalian cells (8). Extracellular ATP has been shown to attenuate current carried by CIC-3 (8, 37) and other volume-sensitive Cl\(^-\) channels (14–16). We tested the effects of ATP on the Cl\(^-\) current activated by hypotonic solution in canine colonic myocytes. ATP (1 mM) reduced the amplitude of the Cl\(^-\) current activated by hypotonic solution (Fig. 7A). In another volume-sensitive Cl\(^-\) channel, pCl\(_{in}\), this effect has been proposed to be due to ATP binding to a nucleotide binding site in the area of the predicted pore region (26). Jackson and Strange (15, 16) have suggested that ATP access to such a nucleotide binding site would function as an open channel blocker.

In seven of seven canine colonic myocytes, ATP (1 mM) blocked the outward portion of the Cl\(^-\) current (e.g., at +10 mV current was reduced by 30 ± 4 pA, 53 ± 11%). More variable results were obtained at negative test potentials (e.g., the inward portion of the Cl\(^-\) current was reduced in 4 of 7 cells by 70 ± 19 pA or 57 ± 12% at −80 mV); however, the current was unchanged in the remaining three cells. This variability could be due to other conductances affected by ATP, or ATP may inhibit the Cl\(^-\) current via a purinergic mechanism that is unrelated to the voltage-dependent, open-channel block seen in other cells. Therefore, we performed three additional experiments to test the effects of ATP on the Cl\(^-\) current activated by hypotonic solution in the presence of symmetrical 100 mM TEA. In these experiments, only outward current was blocked (Fig. 7B).

![Diagram](http://apcell.physiology.org/)

**Fig. 5.** Pharmacology of Cl\(^-\) current: inhibition by tamoxifen and DIDS. Representative current tracings illustrate effect of 10 \(\mu\)M tamoxifen on current activated by hypotonic solution (A). Holding potential was −40 mV, and cells were stepped from −100 to +120 mV in 20-mV increments. Group data for these experiments (n = 5) are shown in B along with tamoxifen-sensitive current (Inset). Open and solid symbols represent currents in hypotonic solution and in absence and presence of tamoxifen, respectively. Asterisks indicate differences in I-V relationships. Effect of 100 \(\mu\)M DIDS on swelling-activated current can be seen in example shown in C, where same voltage protocol described for A was also used. D: group data (n = 5) summarizing effect of tamoxifen (■) and DIDS (▲) on activated current (●) in physiological range of membrane potentials. Cells were held at −40 mV and stepped from −80 to +10 mV in 6-mV increments. Asterisks represent specific differences from control for tamoxifen and DIDS (above and below symbols, respectively).

![Diagram](http://apcell.physiology.org/)

**Fig. 6.** Ca\(^{2+}\) independence of Cl\(^-\) current activated by hypotonic solution. A: representative current tracings obtained in hypotonic solution containing either 2 mM Ca\(^{2+}\) or 2 mM Mn\(^{2+}\) as major extracellular divalent cation. This cell was held at −40 mV and stepped in 20-mV increments from −100 to +120 mV. I-V relationship from this experiment can be seen in B. Open and solid symbols represent currents in presence of 2 mM Ca\(^{2+}\) and 2 mM Mn\(^{2+}\), respectively. Group data (n = 3) for currents in physiological range of membrane potentials are shown in inset of B. Open circles, open squares, and solid squares represent currents under isotonic and hypotonic with or without Ca\(^{2+}\), respectively. Effects of L-type Ca\(^{2+}\) channel antagonism were tested in another cell in hypotonic solution in presence or absence of 10 \(\mu\)M nicardipine (C). I-V relationship from this experiment can be seen in D. Open and solid symbols represent currents in absence and presence of nicardipine, respectively.
Regulation of the Cl⁻ current by protein kinase C. ClC-3 has also been shown to be regulated by protein kinase C (PKC; Ref. 8). We tested the effect of PKC activation on the current induced by hypotonic solution in canine colonic myocytes (Fig. 8). PDBu (300 nM) reduced the amplitude of the Cl⁻ current recorded in hypotonic solution (Fig. 8, A and B). PDBu, a diacylglycerol mimetic, activates PKC. Difference currents revealed that the current inhibited by PDBu was an outwardly rectifying, inactivating current that reversed at −10.3 ± 1.2 mV. Under isotonic conditions (bath and pipette both 300 mosM), chelerythrine chloride (30 µM), a specific inhibitor of PKC, activated a current similar to the Cl⁻ current induced by hypotonic solution (Fig. 8, C and D). The reversal potential of the chelerythrine-activated Cl⁻ current was −7.5 ± 2.6 mV. H-7 (100 µM), a broad-spectrum kinase inhibitor, also activated a Cl⁻ current similar to the current elicited by hypotonic solution (n = 2, data not shown).

Evidence for basal activation of the Cl⁻ current. Under the control conditions of these studies (bath and pipette solutions were both 300 mosM and of the same ionic composition as in Fig. 1), we observed a current with the same properties as the Cl⁻ current activated by hypotonic solution (250 mosM). Experiments were performed to determine whether the basal current was due to the same Cl⁻ conductance activated when cells were exposed to hypotonic solution. The current observed under basal conditions was outwardly rectifying and reversed near E_{Cl} (−8.8 ± 1.9 mV, n = 5). The basal current was also dependent on the Cl⁻-gradient, as replacing extracellular Cl⁻ with aspartate reduced current magnitude and shifted the reversal potential to more positive values (15.0 ± 4.6 mV, n = 5). Basal current was inhibited by 10 µM tamoxifen, and only the outward portion of the current was blocked by DIDS (100 µM). The basal current, similar to the current activated by hypotonic solution, was insensitive to niflumic acid (25 µM). ATP blocked the outward portion of the basal current in a voltage-dependent manner. The magnitude of the basal Cl⁻ current was also reduced when cells were exposed to hypertonic solution (increased to 350 mosM with mannitol). Taken together, these data suggest that the same population of
channels responsible for Cl\textsuperscript{−} current elicited by hypotonic solution were active under isotonic conditions. Results of these experiments are shown in Fig. 9.

Molecular studies indicating CIC-3 gene expression. The electrophysiological studies suggest that phasic and tonic regions of the GI tracts of two species possess a current that is activated by exposure to hypotonic solution. This current has properties similar to CIC-3 (8, 37). Molecular studies were therefore performed to determine whether CIC-3 is expressed in colonic and fundus myocytes. RT-PCR was performed on isolated smooth muscle cells. Qualitative RT-PCR indicated that CIC-3 mRNA was present in colonic and fundus smooth muscle cells. Quantitative RT-PCR was performed to determine the relative amount of CIC-3 transcripts in mRNA isolated from murine and canine smooth muscle; a representative gel used for digital analysis is shown in Fig. 10A. These RT-PCR experiments were quantified by comparing CIC-3 gene expression to the amount of \(\beta\)-actin gene expression (37). These quantitative RT-PCR experiments revealed significantly greater amounts of CIC-3 transcripts in colonic smooth muscle than the fundus (n = 3). No difference in CIC-3 gene expression was observed between murine and canine colon. The normalized values for CIC-3 expression are smaller (approximately one-tenth magnitude) compared with those in canine renal and pulmonary arteries (37); however, current density in canine colon is virtually identical to that in canine vascular tissues. For example, current density for the six cells shown in Fig. 1B was \(-5.0 \pm 1.0, -2.1 \pm 0.4,\) and \(5.8 \pm 0.9\) pA/pF at \(-100, -40,\) and \(+40\) mV, respectively. This apparent difference in CIC-3 gene expression between this and our previous study is most likely due to the fact that more specific \(\beta\)-actin primers were used here. However, at this time, differences in \(\beta\)-actin isoforms between visceral and vascular smooth muscle cannot be excluded, nor can an actual difference in gene expression.

**DISCUSSION**

Volume-regulated or swelling-activated Cl\textsuperscript{−} currents are present in numerous cell types and play important roles in the control of cell volume, pH, and membrane potential (9, 12). The molecular identity of these anion channels has been a matter of debate (25). Recently, CIC-3 has been identified as the molecular entity that underlies the swelling-activated Cl\textsuperscript{−} current in guinea pig cardiac myocytes (8) and in canine vascular smooth muscle cells (37). A recent report has demonstrated a volume-sensitive Cl\textsuperscript{−} current in smooth muscle cells of

![Fig. 9. Cl\textsuperscript{−} current under isotonic conditions. Current tracings in A and B demonstrate that basally active current has a strong dependence on Cl\textsuperscript{−} gradient, as equimolar 100 mM aspartate replacement decreased current and shifted reversal potential to a more positive value. Open and solid squares represent current in presence of Cl\textsuperscript{−} or aspartate, respectively. Triangles represent difference current. Tamoxifen inhibited current present under isotonic conditions (C and D). Open and solid squares represent current in absence and presence of tamoxifen, respectively. Triangles represent difference current. Increasing extracellular osmolarity attenuated basally active current (E and F). Open and solid squares represent current in 300 and 350 mosM, respectively. Triangles represent difference current.](image-url)
systematic studies comparing responses of stretch-sensitive ionic channels to stimulation from osmotic perturbation and radial stretch have not been performed in smooth muscles. It is likely that the volume-sensitive Cl\(^-\) current would be activated by changes in cell shape, because this type of current may be controlled by mechanisms involving deformation of cytoskeletal elements (38).

We isolated the Cl\(^-\) current activated by hypotonic solution under conditions that minimized contributions from other ionic currents present in GI muscle cells: K\(^+\) currents (removal of intracellular and extracellular K\(^+\) by replacement with TEA), Ca\(^{2+}\) currents (replacement of extracellular Ca\(^{2+}\) with Mn\(^{2+}\) and addition of nicardipine), and nonselective cation currents (replacement of Na\(^+\) and K\(^+\) with symmetrical TEA). Under these conditions, the reversal potential of the current activated by hypotonic solution shifted with the E\(_{Cl}\) demonstrating that the current is a Cl\(^-\) current. The current was outwardly rectifying and possessed properties similar to the swelling-activated Cl\(^-\) current in cardiac myocytes and in fibroblasts transfected with ClC-3 (7, 8). A channel of \(~40\) pS was responsible for the current in those studies. The single-channel conductance that mediates the Cl\(^-\) current activated by hypotonic solution in smooth muscle cells has not yet been determined. However, on the basis of the presence of ClC-3 gene transcripts in vascular (37) and visceral (this study) smooth muscles and high degree of similarity in characteristics to ClC-3 (7, 8), it is likely that ClC-3 channels mediate a significant portion of the swelling-activated current in smooth muscles.

The current attributed to ClC-3 was sensitive to known Cl\(^-\) channel antagonists such as tamoxifen and DIDS. DIDS was more effective at blocking outward current than inward current as demonstrated by Ca\(^{2+}\) currents (8, 26, 37). At the present time, we do not know whether the inhibition of Cl\(^-\) current activated by hypotonic solution, suggesting this conductance was not a Ca\(^{2+}\)-activated Cl\(^-\) current. In fact, there is no evidence for a Ca\(^{2+}\)-activated Cl\(^-\) current in canine colonic cells. Niflumic acid has been shown to inhibit volume-sensitive Cl\(^-\) channels in some other cell types (33, 36); however, this difference might be explained by cell and species differences.

Purinergic agonists are known to regulate the activity of numerous ion channels. In some cases, extracellular ATP directly activates conductances, such as P\(_{2x}\) receptors (30). In other cases, ATP regulates ion channels via changes in intracellular Ca\(^{2+}\) concentration or through other second messenger systems. ATP inhibits swelling-activated Cl\(^-\) currents (8, 26, 37). At the present time, we do not know whether the inhibition of ClC-3 by ATP is receptor mediated or due to direct access of ATP to an intrinsic nucleotide binding site, as...
has been reported for plClN and the anion channels of rat C6 glioma cells and skate hepatocytes (14–16, 26). The physiological significance of the effects of ATP on the swelling-activated Cl\(^{-}\) conductance is not understood at present. ATP is proposed as inhibitory neurotransmitter in many GI muscles (13), and it is possible that this agent could participate in the regulation of myogenic activation in response to distension.

PKC was shown to regulate cloned CIC-3 channels and the native conductance found in cardiac muscle cells (8). PKC suppressed the Cl\(^{-}\) current-activated hypotonic solution in GI muscles in the present study, and inhibitors of PKC (e.g., cholerythrine chloride and H-7) activated the current. These results are consistent with the recently proposed role of endogenous PKC and phosphatases in the regulation of CIC-3 channels by cell volume (D. Duan, S. Cowley, B. Horowitz, and J. R. Hume, unpublished observations). Thus agonists coupled to activation or inactivation of PKC may have important effects on regulating responses to stretch in the GI tract. For example, CIC-3 may provide negative feedback to excitatory agonists coupled through G\(_{q}\) and phospholipase C, such as the neurotransmitters, acetylcholine, and tachykinins, since the net inward current activated by stretch would be lessened by PKC inhibition of the Cl\(^{-}\) conductance. Swelling-activated Cl\(^{-}\} current in cardiac muscle cells may additionally be regulated by tyrosine kinase (27). The role of tyrosine kinases in regulating CIC-3 in smooth muscle or in cells transfected with cloned CIC-3 has not been evaluated.

It should be noted that myogenic tone and membrane potential of rat cerebral arteries are sensitive to the same pharmacological agents that inhibit CIC-3 current (24). DIDS and indanyloxyacetic acid 94 hyperpolarized and dilated pressurized arteries, whereas niflumic acid was without effect. A similar effect may occur in GI smooth muscles; that is, antagonists of CIC-3 may hyperpolarize and relax visceral smooth muscles. This hypothesis remains to be tested and may require CIC-3 to be activated by an agonist or stretch (analogous to conditions in pressurized cerebral arteries) before an inhibitory effect of Cl\(^{-}\} channel antagonists is evident.

In summary, we have found that the CIC-3 gene is expressed in tonic and phasic smooth muscles of the dog and mouse GI tracts. An outwardly rectifying Cl\(^{-}\} current is induced in visceral myocytes on exposure to hypotonic solution. This current is strikingly similar to the swelling-activated Cl\(^{-}\} conductance in mammalian cardiac myocytes (7, 29) and the current resulting from the expression of CIC-3 in fibroblasts (8). Our data suggest that CIC-3 channels convey at least a portion of the response to changes in extracellular osmolarity and possibly radial stretch in GI smooth muscle cells. In the physiological range of membrane potentials, currents resulting from CIC-3 activation are significant and as great in magnitude as currents carried by L-type Ca\(^{2+}\} channels in this tissue (35). Therefore, depending on the state of distension, CIC-3 could provide a significant source of inward current in GI smooth muscle cells and a depolarizing influence in intact muscles. This conductance may have an important effect on the electrical activity of GI muscles, as has been demonstrated in guinea pig cardiac myocytes and rat cerebral arterioles (24, 31).

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Address reprint requests to K. M. Sanders.

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