Cl⁻ transport in an immortalized human epithelial cell line (NCM460) derived from the normal transverse colon

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Sahi, J., asminder, Selvaraj G. Nataraja, Thomas J. Layden, Jay L. Goldstein, M. P. Moyer, and Mrinalini C. Rao. Cl⁻ transport in an immortalized human epithelial cell line (NCM460) derived from the normal transverse colon. Am. J. Physiol. 275 (Cell Physiol. 44): C1048–C1057, 1998.—Cells of a newly described, immortalized, epithelial, human transverse colonic cell line, NCM460, reach ~90% confluence on plastic and develop transepithelial resistances of 120–250 Ω·cm² on porous substrates. Its utility as a model for the transverse human colon was validated by comparing second messenger-mediated Cl⁻ transport, using the fluorescent probe 6-methoxy-quinolyl acetoethyl ester, in NCM460 cells and colonocytes isolated from human transverse crypts. Basal Cl⁻ influx was increased (P < 0.01) by PGE₁ (1 µM), forskolin (1 µM), 8-bromoadenosine 3'5'-cyclic monophosphate (100 µM), heat-stable Escherichia coli enterotoxin (STa; 1 µM), 8-bromoguanosine 3'5'-cyclic monophosphate (100 µM), histamine (1 µM), and phorbol 12,13-dibutyrate (1 µM) in both cell types. The Cl⁻ channel blocker dihydropyrimidin e2-carboxylic acid (50 µM) and the Na⁺-K⁺-2Cl⁻ cotransport inhibitor furosemide (1 µM), but not the K⁺ channel blocker Ba²⁺ (3 mM), inhibited these Cl⁻ permeabilities. These cells possess transcripts for cystic fibrosis transmembrane conductance regulator, Na⁺-K⁺-2Cl⁻ cotransporter, STa receptor, and intestine-specific cGMP-dependent protein kinase II. Thus cAMP+, cGMP+, and Ca²⁺-dependent secretagogues act on NCM460 and primary colonocytes to stimulate Cl⁻ transport. This validates the utility of NCM460 as a model for transverse colonic crypts and is the first demonstration of a colonic cell line whose origin is known.

Colonic crypts; primary cultures; 6-methoxy-quinolyl acetoethyl ester; second messenger regulation; resistance

CHLORIDE TRANSPORT IN THE HUMAN COLON is regulated by a variety of intracellular messengers, including cAMP, cGMP, Ca²⁺, and protein kinase C (PKC). An understanding of human colonic ion transport has been derived from in vivo perfusion models (6), intact colonic epithelial sheets (21), isolated colonocytes (8), primary colonocyte cultures (16, 19, 8), vesicles derived from colonic tissues (4) and transformed cell lines (10). Each of these has limitations. Thus, although in vivo perfusion studies in the human colon are the most physiological, they at best represent a composite picture of net transport. To delineate the individual ionic components of transport, in vitro studies have been performed using intact colonic epithelial sheets. However, even when stripped of underlying muscle layers, these tissues remain a heterogeneous preparation comprised of surface and crypt epithelial cells and the underlying lamina propria and submucosal layers. Primary cultures of isolated colonocytes, a preparation we have characterized in some detail, have proven to be useful models (16). However, they are limited in their long-term (>72 h) viability in culture and are subject to the vagaries of tissue availability. Apical and basolateral membrane vesicle preparations of tissues obtained at the time of autopsy are good models (4) for delineating events at the membrane level but require larger amounts of tissue and are not amenable to study of the signaling cascades underlying neurohumoral regulation of ion transport. Extensive studies evaluating ion transport have also been performed using animal models with the assumption that they have applicability to humans. However, there are species-specific differences in colonic transport mechanisms. Human colon crypts are good models for study of epithelial ion transport in general, but there are some limitations to the available cell lines. First, all but one of the ~40 commercially available human colon cell lines are transformed, having been derived from carcinomas. Second, some cell lines, such as T84, develop transepithelial resistances (R<sub>t</sub>; ~1,000 Ω·cm²) much greater than does normal human colon (~140–160 Ω·cm²). Third, some cell lines, such as Caco-2 cells, exhibit characteristics more representative of fetal and small intestinal tissues; e.g., they exhibit sucrase activity and display intercellular cysts (7a). Fourth, there are differences between Cl⁻ transport regulation in normal human colonocytes and in colon cells. For example, neither 8-Br-cGMP (4b), a cGMP analog, nor phorbol esters (20), which are PKC activators, stimulate Cl⁻ secretion in T84 cells, but both stimulate Cl⁻ transport in isolated primary human colonocytes (7, 16). Fifth, and of greatest relevance to the present study, the segmental origin of most colonic cell lines is not known, and therefore they cannot be used to study differences along the cephalo-caudal axis.

We have now established a new model to study ion transport in colonocytes, using an immortalized, non-transformed, human colon cell line (NCM460) that negates some of the inadequacies of the earlier models. The NCM460 line is derived from the normal human transverse colonic mucosa (13). These cells do not form tumors in nude mice (13) and have tested negative for the colonic neoplasm markers (MDM2, DCC, K-ras, and CEA; Moyer, unpublished observations). These cells are epithelial in origin, since they stain positively for cytokeratin (17), human secretory component, vilin, and the colon-specific glycoprotein 5E113 (13). Because these cells are not of tumor origin, they better...
represent the normal human colon than the transformed cell lines. In addition, because NCM460 cells are immortalized, their availability is not a limiting factor. NCM460 cells grow in culture as an attached population (attached cells) and a floating population (“floaters”), and we have concentrated on delineating the characteristics of the attached cells. In a recent study, we demonstrated that these cells exhibit Na+/H+ exchange activity with characteristics of the NHE-1 and NHE-2 isoforms but not of the NHE-3 isoform (18). This indicated that NCM460 cells are crypt in origin, as in situ hybridization studies in human transverse colon depict NHE-1 and NHE-2 isoforms in the crypts, whereas surface cells possess all three isoforms (4a). Another ion transport characteristic ascribed to crypts is Cl− transport, and little is known about this process in NCM460 cells.

The current paper therefore characterizes the growth and attachment of NCM460 cells and the Cl− transport characteristics of the attached cell population. To validate whether these cells are indeed representative of colonic crypts, we compared Cl− transport in NCM460 cells with those in primary isolates from the transverse human colon. As in our earlier studies with human and rabbit colonocyte primary cultures (3a, 7, 16, 19), we used the Cl−-sensitive fluorescent probe 6-methoxy-quinolyl acetoethyl ester (MQAE). Our studies show that NCM460 cells form resistive monolayers with R0 akin to those of human colonic epithelial sheets. The NCM460 cells exhibit Cl− permeabilities that are regulated by agents acting via the cAMP, cGMP, Ca2+, and PKC pathways. The basal and stimulated Cl− permeabilities are partially, although significantly, decreased by inhibitors of the Cl− channel and the Na+/K+2Cl− cotransporter pathways but not by inhibitors of the K+ channel. Equally important, these responses are qualitatively similar to those of primary cultures of human transverse crypt colonocytes. Both NCM460 cells and primary transverse colonocytes possess transcripts for the cystic fibrosis transmembrane conductance regulator (CFTR), the secretory form of the Na+/K+2Cl− cotransporter, the heat-stable Escherichia coli enterotoxin (Sta) receptor, guanylate cyclase C (GCC), and the cGMP-dependent protein kinase (PKG) II isoform.

METHODS

Materials. NCM460 cells were obtained from In Cell (San Antonio, TX). M3:10 culture medium (In Cell) for NCM460 colonocytes was provided through the University of Texas Health Science Center (San Antonio, TX) Center for Human Cell Biotechnology, and Ham’s F-12 nutrient mix and Iscove’s modified Dulbecco’s medium for GIBCO Laboratories (Grand Island, NY). Sterile lactated Ringer was from Baxter Health Care (Deerfield, IL). Biocell culture inserts were from Collaborative Research (Bedford, MA), and all other supplies for cell culture were from Costar (Cambridge, MA). MQAE was purchased from Molecular Probes (Junction City, OR). Diphedylamine-2-carboxylate (DPC) was purchased from Aldrich (Milwaukee, WI). All other reagents were of analytical grade and were purchased from Sigma Chemical (St. Louis, MO).

NCM460 cell culture. The NCM460 colonocytes were counted and plated at a density of 2 × 10⁴ cells/ml in Costar 75-cm² flasks at 37°C with 6% CO₂. The tissue culture medium used was M3:10 nutrient mix containing 10% fetal bovine serum and antibiotics. The cells were passaged by using a cell scraper and splitting them 1:2. The epithelial origin of the cells was confirmed by intermediate filament immunofluorescence, using the method of Yang et al. (27).

Human colonocyte isolation and culture. Human transverse colonic tissue was obtained from individuals undergoing colonic resection at the University of Illinois Hospital and Clinics for benign or malignant tumors. Donors had not received preoperative irradiation or chemotherapy, and use of human tissue was approved by the Institutional Review Board (University of Illinois at Chicago, Chicago, IL). The tissue pieces used were taken from transverse colon of normal appearance, at sites at least 2 cm away from the tumor. For the transport studies, human transverse colonic epithelial cells were isolated as described previously (16). The transverse colonic tissues were transported on ice in oxygenated lactated Ringer containing 5 mM dextrose and antibiotics (in µg/ml: 25 ampicillin, 120 penicillin, 270 streptomycin, and 1.25 amphotericin B). The colonic mucosa was stripped off the underlying muscle and digested (0.1% pronase, 0.03% collagenase) for 90 min at 37°C, in the presence of 5 mM dithiothreitol. The cells were filtered to remove residual tissue and centrifuged to enrich for crypt cells as described previously (16). The colonocytes were plated at 2 × 10⁴ cells/ml in tissue culture medium (Ham’s F-12 nutrient mix) supplemented with 20% FCS, 0.5 U/ml insulin, 4 mM L-glutamine, 1 µM hydrocortisone, 10.5 mM selenium, 0.5 mM sodium butyrate, and antibiotics for 24 h. Isolated, nonattached colonocytes were used for the transport studies.

Resistance measurements. NCM460 cells were grown on Millipore filters precoated with different extracellular matrix proteins. Resistance measurements were made using an ohmmeter (World Precision Instruments, Sarasota, Florida). Background resistance (coated and uncoated filters not plated with cells) was ~30 Ω·cm² and was deducted from each value. Resistance was measured over an 8-day period.

Ionton transport. MQAE fluorescence is quenched by all halides in a dose-dependent way (3a, 19, 25). NCM460 cells were grown on plastic Leighton tubes (Costar) until they reached 95% confluence. Primary human colonocytes were used 24 h postplating and used in suspension. The cells were washed free of the tissue culture medium and dye loaded for 90 min on ice in buffer A, which contained (in mM) 5 MQAE, 110 NaCl, 1 MgCl₂, 1 CaCl₂, 5 dextrose, 50 mannitol, and 1 KCl. The cells were then resuspended in a Cl−-free solution (buffer B) containing (in mM) 110 sodium isethionate, 1 MgSO₄, 5 dextrose, 50 mannitol, 1 K₂SO₄, and 1 CaSO₄. Fluorescence was measured at an excitation wavelength of 355 nm and an emission wavelength of 460 nm in a PRTI Alphascan spectrofluorometer (Princeton, NJ). The rate of change of fluorescence was monitored in the NCM460 cells as buffers A and B were alternately perfused in the presence and absence of different agents and inhibitors. For the nonattached human colonocytes, initial fluorescence was observed in buffer B, and the rate of change of fluorescence was monitored as 5 mM Cl− was added to the cells under different conditions. Cl− influx was calculated as previously described (16), using the formula JCl = (Fₐ/F₀)(dF/dt), where JCl is the Cl− influx rate (mM/s), dF/dt is the slope of the initial rate of change of fluorescence upon addition of Cl− (fluorescence units/s), F₀ is the Stern-Volmer constant for quenching of intracellular MQAE by Cl−, and Fₐ and F are absolute fluorescence units in the absence and presence of Cl−, respec-
the presence of various Ca\(^{2+}\) channels using Ba\(^{2+}\) and attached cells. To study the effect of inhibiting K\(^{+}\) channels, we blocked the K\(^{+}\)-mediated Cl\(^{-}\) transport. We used an FK506 modulator. Although short-term exposure to forskolin activates PKC, long-term exposures are considered statistically significant. In all experiments, values of P < 0.05 were considered statistically significant. In all experiments, n values represent the number of experiments, in each of which the measurements were made in triplicate; n = 5 or more for NCM460 cells, and n = 3 for the primary cultures, unless otherwise indicated.

RESULTS

Growth. The growth characteristics of only the NCM460 cells were studied, since we had previously observed that the viability of human colonocytes in primary culture declines after 48–72 h (16). When grown in 12-well plastic culture plates, NCM460 cells (7.5 × 10\(^5\)) approximately doubled in 48 h, yielding 1.80 ± 0.33.2 on October 13, 2017 http://ajpcell.physiology.org/ Downloaded from
observed in this cell population up to day 10. The floaters, in contrast, did not show any significant growth until after day 4. At this stage, these cells grew rapidly, and by day 6 postplating there was a threefold increase (P < 0.001) in the floating cell population. This trend continued through day 8 postplating, when the numbers of floaters increased to $2.15 \times 10^6 \pm 0.3 \times 10^6$. No reasonable quantitation could be done past day 8, as the medium could not support this large quantity of cells and the rapidly growing floating cell population had to be passed.

Measurement of $R_t$. To determine whether the attached NCM460 cells were capable of developing $R_t$, we grew the cells on a variety of extracellular matrices and $R_t$ was measured. Cells were plated either on uncoated Millipore filters or filters coated with one of the following: laminin, Matrigel, collagens I and IV, and fibrillar collagen (collagen type 1, which is treated to form large collagen fibrils with a normal cross-striation pattern). The $R_t$ was measured over an 8-day period and, regardless of the matrix used, was found to plateau by day 6 postplating. An example in which maximal resistance was developed 5 days postplating in cells grown on complex fibrillar collagen is shown in Fig. 2, inset. The differential effects of the extracellular matrices on $R_t$ are shown in Fig. 2. The data are derived from culture wells, 7 days postplating, after subtraction of baseline resistance ($-30 \ \Omega \cdot \text{cm}^2$). The lowest resistance was with cells grown on the uncoated filters ($95 \pm 7.1 \ \Omega \cdot \text{cm}^2$). Resistance was significantly increased, 1.25-fold over the basal, when cells were grown on laminin ($P < 0.05$), Matrigel ($P < 0.05$), and collagen I ($P < 0.01$). The increase was even greater (2-fold) when the cells were grown on collagen IV (201.52 $\pm$ 4.5 $\Omega \cdot \text{cm}^2$; $P < 0.001$) or on complex fibrillar collagen (218.2 $\pm$ 17.8 $\Omega \cdot \text{cm}^2$; $P < 0.001$), respectively.

Ion transporters: activity and transcripts. To study ion transport, we first determined the $K_{\text{Cl}}$ in NCM460 cells and found it to be 20 M$^{-1}$. In earlier studies, the $K_{\text{Cl}}$ for human colonocytes was determined to be 24 M$^{-1}$ (16). To identify the pathways involved in $\text{Cl}^-$ transport, we characterized $\text{Cl}^-$ permeability based on DPC ($\text{Cl}^-$ channel) and furosemide ($\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransport) sensitivity (Fig. 3). We have previously demonstrated that these inhibitors, unlike bumetanide, do not interfere with MQAE fluorescence (16). Basal $\text{Cl}^-$ permeability (0.214 $\pm$ 0.02 mM/s) was inhibited 87% by 50 $\mu$M DPC ($P < 0.001$) and 35% by 10 $\mu$M furosemide ($P < 0.01$). When both inhibitors were added together, $\text{Cl}^-$ permeability was further suppressed, to ~95% of the basal value and significantly ($P < 0.05$) more than with DPC alone.

Similar studies were conducted on primary cultures of colonocytes derived from the transverse human colon. Basal $\text{Cl}^-$ permeability (0.162 $\pm$ 0.03 mM/s) was inhibited 76% by DPC ($P < 0.001$) and 52% by furosemide ($P < 0.001$). The two inhibitors together decreased $\text{Cl}^-$ permeability to a greater extent (89%) than that of either inhibitor alone.

The transport results suggest that the NCM460 cells and the primary colonocytes contain $\text{Cl}^-$ channels and the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter. To demonstrate that they contain the transcripts for such proteins, we designed primers to detect, by RT-PCR amplification, the transcript for $\text{CFTR}$ and the secretory form of the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter. $\text{CFTR}$ is highly conserved in the transmembrane domains across species, and we used primers based on the rabbit sequence to amplify a 530-bp region in the first transmembrane domain. The primers for the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter were based on the known human sequence. As shown in Fig. 4, both NCM460 cells and primary human colonocytes contain transcripts for $\text{CFTR}$ and the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter.

$\text{Cl}^-$ influx induced by the cAMP pathway. To dissect out the effect of cAMP on $\text{Cl}^-$ permeability, PGE$_1$, a
receptor-G protein-mediated activator of adenylate cyclase (1 µM), forskolin, a direct activator of adenylate cyclase (1 µM), and the cAMP analog 8-BrcAMP (100 µM) were tested (Fig. 3). The secretagogues were tested in the presence and absence of the inhibitors DPC and/or furosemide to assess the transport pathways involved.

All three agents caused a significant increase in the influx rate of Cl⁻ in both the NCM460 cells and the primary cultures. In the NCM460 cells, a 2.3-fold increase in Cl⁻ influx was found with PGE₁, a 1.8-fold increase with forskolin, and a 2.5-fold increase with 8-BrcAMP (P < 0.001 for all 3; Fig. 3A). This secretagogue-stimulated Cl⁻ influx was partially and significantly inhibited by DPC (PGE₁, 65%; forskolin, 83%; and 8-BrcAMP, 66.1%; P < 0.001) and furosemide (PGE₁, 37.6%; P < 0.05; forskolin, 55.3%; P < 0.001; and 8-BrcAMP, 47.3%; P < 0.01). When both inhibitors were added together along with the secretagogue, the effect was partially additive. The residual Cl⁻ permeability after addition of both inhibitors in the stimulated cells is higher (P < 0.05) than that in the nonstimulated cells (0.012 mM/s). These studies were conducted on attached NCM460 cells. To determine whether qualitatively similar results are observed when these cells are not attached to a substratum, we examined the Cl⁻ permeabilities in suspended NCM460 cells (note that these cells are not the floaters population). Cells were scraped with a rubber policeman, and Cl⁻ transport was studied with and without PGE₁ (1 µM). PGE₁ caused a 2.44-fold increase (inhibitor-sensitive influx in mM/s: PGE₁, 0.44 ± 0.06; basal, 0.18 ± 0.02; n = 5).

Similar results were obtained with the primary colonocytes (Fig. 3B). PGE₁, forskolin, and 8-BrcAMP significantly (P < 0.001) increased the Cl⁻ influx rates, and these were inhibited by DPC (PGE₁, 72%; forskolin, 83% and 8-BrcAMP, 77%; P < 0.001) and furosemide (forskolin, 73%; P < 0.001; 8-BrcAMP, 48%; P < 0.01). Although furosemide appeared to cause a modest, ~20% decline in PGE₁ stimulation, this was not statistically significant. As with the NCM460 cells, when both inhibitors were added together along with the secretagogue, Cl⁻ permeability was further decreased, although not to basal levels. It therefore appears that, both in NCM460 and in primary colonocytes, the secretagogues stimulate Cl⁻ channels, Na⁺-K⁺-2Cl⁻ cotransporter, and some other Cl⁻ permeabilities.

Cl⁻ influx induced by the cGMP pathway. Studies in the distal human colon, in human colonic cell lines, and...
in colonic membranes indicate that STa acting via its specific membrane guanylate cyclase receptor increases cGMP and stimulates Cl⁻ secretion (3, 5, 7). The effects of cGMP on the transverse colon per se are not known. The effects of both STa (1 µM) and the cGMP analog 8-BrcGMP (100 µM) in the presence and absence of inhibitors were studied. As shown in Fig. 5A, in NCM460 cells, baseline Cl⁻ influx was significantly increased by STa (2.3-fold, \( P < 0.001 \)) and to a lesser extent by cGMP (1.3-fold, \( P < 0.05 \)). The STa-induced Cl⁻ influx was mainly via the DPC-sensitive Cl⁻ channels (67.2%, \( P < 0.001 \)) and to a lesser extent through the cotransporter (34%, \( P < 0.01 \)). However, furosemide inhibited the 8-BrcGMP-stimulated Cl⁻ influx by 86.1% (\( P < 0.001 \)), and DPC blocked it by 53.9% (\( P < 0.01 \)). The combined effect of the two inhibitors was greater than either inhibitor alone.

In contrast to the NCM460 cells, in the primary cultures (Fig. 5B), basal Cl⁻ influx (0.16 ± 0.03 mM/s) was increased equally by STa (0.74 ± 0.12 mM/s) and 8-BrcGMP (0.64 ± 0.11 mM/s). As in NCM460 cells, STa stimulated Cl⁻ permeability largely via the DPC-sensitive Cl⁻ channels (DPC, 66%; furosemide, 50%), whereas 8-BrcGMP did so mainly through the cotransporter (furosemide, 81%; DPC, 43%).

Their responses to STa and to 8-BrcGMP suggest that both NCM460 cells and primary human colonocytes possess receptors for STa as well as a cGMP-specific signaling mechanism. To determine whether they express the transcripts for STa receptors (i.e., GCC) and the intestine-specific isoform PKG II, RNA from NCM460 cells and primary human colonocytes was amplified by RT-PCR using human-specific primers. As shown in Fig. 6, both cell preparations possess transcripts for GCC and PKG II. As a positive control, the “housekeeping” gene GAPDH was amplified in every RT-PCR experiment. A representative amplification is shown in Fig. 6.

Cl⁻ influx induced by the PKC pathway. It is well recognized that short-term phorbol ester treatment stimulates the PKC cascade, whereas long-term treatment downregulates the enzyme. The effects of short-term exposure to phorbol esters on Cl⁻ secretion in human colonocytes have varied with the source of the cells. Thus, although PDB does not increase short-circuit current in naive T84 monolayers (1, 20), it stimulates Cl⁻ secretion in primary isolates of human colonocytes (16) and in the colon carcinoma cell line HT-29.d19a (24). To determine whether similar differences exist between primary isolates and a cell line of the transverse colon, we studied the effects of PDB. As shown in Table 1, short-term treatment with PDB caused an approximately sixfold (\( P < 0.05 \)) increase in Cl⁻ transport in primary colonocytes and an approximately twofold (\( P < 0.05 \)) increase in NCM460 cells. In
both cell types, either DPC or furosemide inhibited Cl\textsuperscript{-} influx to \(\sim 60-67\%\) (\(P < 0.05\), data not shown). We also examined the long-term effects of PDB on Cl\textsuperscript{-} secretion in the NCM460 cells; again due to viability and availability problems, similar studies could not be carried out in the primary colonocytes. Twenty-four-hour treatment with 0.1 \(\mu\)M PDB resulted in Cl\textsuperscript{-} influx rates no different than those of untreated controls. Addition of PDB to a final concentration of 1 \(\mu\)M for 5 min to these cells also did not stimulate Cl\textsuperscript{-} influx rates. Although Cl\textsuperscript{-} transport rates in cells exposed to 0.1 \(\mu\)M PDB for 48 h were also similar to those in untreated cells, exposure to 1 \(\mu\)M PDB for an additional 5 min caused a small 1.3-fold increase (\(P < 0.05\), by paired analysis) in Cl\textsuperscript{-} influx.

Cl\textsuperscript{-} influx induced by the Ca\textsuperscript{2+} pathway. To determine whether the transverse colon was responsive to Ca\textsuperscript{2+}-dependent secretagogues, we determined the effects of histamine, serotonin, and neurotensin (Tables 2 and 3). Two different types of NCM460 preparations were used, the attached cells (Table 2) and attached cells that had been scraped and studied in suspension (Table 3; note that these cells are not the floater population). Due to limited availability of primary colonocytes, the effects of only one secretagogue could be tested in that preparation. Studies in human and rabbit distal colonocytes had indicated interspecies differences in the actions of histamine, and therefore this agent was tested in primary colonocytes (15, 19).

There was no qualitative difference in the responsiveness of the three different preparations used. However, the relative degree of stimulation varied. Histamine caused significant 1.34-fold and 2.3-fold increases in Cl\textsuperscript{-} influx in attached and suspended NCM460 cells, respectively, while causing a 2.66-fold increase in that of primary colonocytes. Serotonin and neurotensin caused 1.6-to 1.7-fold increases in Cl\textsuperscript{-} permeabilities in NCM460 cells. In contrast to the effects of the cyclic nucleotide-dependent secretagogues, those of the Ca\textsuperscript{2+}-dependent secretagogues were not inhibited by furosemide. However, DPC caused a 70% (\(P < 0.05\)) decrease in serotonin-stimulated Cl\textsuperscript{-} influx and a 40–57% (\(P < 0.05\)) decrease in histamine-stimulated transport. The fact that there is no qualitative difference in the responses of NCM460 cells whether they are studied attached to a matrix (Table 2) or in suspension (Table 3) suggests that these two preparations are similar with respect to detection of Cl\textsuperscript{-} permeabilities. In addition, there is no major qualitative difference between the NCM460 cells and the primary cultures.

In T84 cells, the secretagogue actions of Ca\textsuperscript{2+}-dependent agents are Ba\textsuperscript{2+} sensitive, and these agents are known to activate K\textsuperscript{+} channels (1). To determine whether this is also true for the NCM460 cells, we examined the effects of Ba\textsuperscript{2+} on basal and Ca\textsuperscript{2+}-mediated agents in presence and absence of inhibitors on NCM460 colonocytes and primary cultures of human transverse colonocytes.

### Table 1. Effect of PDB on NCM460 cells and primary cultures of human transverse colonocytes

<table>
<thead>
<tr>
<th>Cl\textsuperscript{-} Influx, mM/s</th>
<th>NCM460 cells</th>
<th>Primary colonocytes</th>
</tr>
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<tbody>
<tr>
<td>No long-term treatment</td>
<td>0.24 ± 0.07</td>
<td>0.29 ± 0.04</td>
</tr>
<tr>
<td>100 nM PDB for 24 h</td>
<td>0.27 ± 0.03</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td>100 nM PDB for 48 h</td>
<td>0.27 ± 0.03</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td>Basal</td>
<td>0.24 ± 0.07</td>
<td>0.29 ± 0.04</td>
</tr>
<tr>
<td>PDB (1 (\mu)M, 5 min)</td>
<td>0.47 ± 0.04</td>
<td>0.29 ± 0.03</td>
</tr>
</tbody>
</table>

Values are means ± SE; \(n = 5\) experiments for NCM460 cells, and \(n = 3\) experiments for the primary cultures. NCM460 cells were grown to 95% confluence on Leighton tubes, and isolated colonocytes were grown in tissue culture flasks for 24 h as described in METHODS. For long-term treatment, NCM460 cells were treated with 100 nM phorbol 12,13-dibutyrate (PDB) in tissue culture media for 24 and 48 h. PDB was maintained in 6-methoxy-quinolyl acetoxymethyl ester (MQAE) loading buffer and in Cl\textsuperscript{-}free buffer. Five minutes before start of experiment, cells were placed in a Cl\textsuperscript{-}-free buffer containing 1 \(\mu\)M PDB. For short-term treatment, cells were loaded with MQAE, Cl\textsuperscript{-} depleted, and treated with PDB for 5 min at room temperature. Initial fluorescence was taken before addition of NaCl, rate of change of fluorescence was monitored, and Cl\textsuperscript{-} influx was calculated in mM/s. Significant decreases below corresponding baseline values: *\(P < 0.05\) and †\(P < 0.001\).

### Table 2. Effect of Ca\textsuperscript{2+}-mediated agents in presence and absence of inhibitors on NCM460 colonocytes and primary cultures of human transverse colonocytes

<table>
<thead>
<tr>
<th>Cl\textsuperscript{-} Influx, mM/s</th>
<th>NCM460</th>
<th>Primary cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>No inhibitor</td>
<td>0.24 ± 0.07</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td>DPC</td>
<td>0.32 ± 0.06</td>
<td>0.16 ± 0.04</td>
</tr>
<tr>
<td>Furosemide</td>
<td>0.37 ± 0.10</td>
<td>0.11 ± 0.02</td>
</tr>
</tbody>
</table>

Values are means ± SE; \(n = 5\) experiments for NCM460 cells, and \(n = 3\) experiments for the primary cultures. NCM460 cells were grown to 95% confluence on Leighton tubes, and isolated colonocytes were grown in tissue culture flasks for 24 h as described in METHODS. DPC, diphenylamine-2-carboxylate. Cells were loaded with MQAE, Cl\textsuperscript{-} depleted, and treated with agent with or without inhibitor for 5 min at room temperature. Initial fluorescence was taken before addition of NaCl, rate of change of fluorescence was monitored, and Cl\textsuperscript{-} influx was calculated in mM/s. Significant decreases below corresponding baseline values: *\(P < 0.05\) and †\(P < 0.001\).

### Table 3. Effect of Ba\textsuperscript{2+} on action of Ca\textsuperscript{2+}-dependent secretagogues in NCM460 cells

<table>
<thead>
<tr>
<th>Inhibitor-Sensitive Cl\textsuperscript{-} Transport, mM/s</th>
<th>+NaCl</th>
<th>+BaCl\textsubscript{2}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>0.18 ± 0.02</td>
<td>0.15 ± 0.03</td>
</tr>
<tr>
<td>Histamine</td>
<td>0.44 ± 0.08*</td>
<td>0.43 ± 0.05*</td>
</tr>
<tr>
<td>Serotonin</td>
<td>0.30 ± 0.04*</td>
<td>0.43 ± 0.05*</td>
</tr>
<tr>
<td>Neurotensin</td>
<td>0.48 ± 0.04*</td>
<td>0.51 ± 0.05*</td>
</tr>
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</table>

Values are means ± SE; \(n = 5\) NCM460 cells in suspension were loaded with MQAE, Cl\textsuperscript{-} depleted either in buffer B or in modified buffer B containing 3 mM barium acetate, and treated with secretagogues with or without inhibitors (DPC and furosemide) for 5 min at room temperature. Fluorescence was measured before addition of 5 mM NaCl or BaCl\textsubscript{2}. NaCl was added for cells in buffer B and BaCl\textsubscript{2} for cells in modified buffer B. Rate of change of fluorescence was monitored, and Cl\textsuperscript{-} influx was measured and calculated as described in METHODS. *Secretagogue-stimulated values are different from their respective basal values: P < 0.05 by ANOVA. There is no difference for any group between NaCl and BaCl\textsubscript{2} treatment.
stimulated Cl⁻ permeabilities. The cells were preincubated with 3 mM barium acetate. Influx was measured by the addition of 5 mM NaCl or BaCl₂ as described in METHODS. As shown in Table 3, preincubation with Ba²⁺ affected neither basal nor secretagogue-stimulated Cl⁻ transport. Equally importantly, there was no difference in the actions of Ca²⁺-dependent secretagogues with and without Ba²⁺.

The studies with the Ca²⁺-dependent secretagogues suggest that, in both NCM460 cells and primary colonocytes, Cl⁻ permeability induced by the Ca²⁺-mediated pathway is via DPC-sensitive and DPC-insensitive Cl⁻ channels but does not appear to activate the Na⁺-K⁺-2Cl⁻ cotransporter. In addition, activation of Ba²⁺-sensitive K⁺ channels does not appear to play a major role in NCM460 cells.

**DISCUSSION**

In this study, we characterized a new human colonic epithelial cell line of known segmental origin. We demonstrate that the immortalized NCM460 colonocytes derived from the transverse colon (13) are similar to primary cultures of cells isolated from the same region.

The NCM460 cells grow as two populations, attached cells and floats. Viability is ≥90% in the floating cell population, and these cells appear to require paracrine factors for division, as they grow faster in conditioned medium. We have not characterized the floating cell population further. The attached cells grow actively in fresh medium and reach 90–95% confluence on nonporous substrates like plastic or plastic coated with collagen type IV. On porous membranes, NCM460 cells form monolayers with RT in the presence of extracellular matrix proteins (cells on uncoated filters 85 ± 252 Ω·cm²; cells on coated filters, 120 ± 252 Ω·cm²) set them apart from transformed cell lines like T84, which can grow on plastic and establish high Rₜ of ≥1,000 Ω·cm² (9).

In the human colon, Cl⁻ absorption is via an electroneutral, Na⁺-independent, HCO₃⁻-dependent process, and Cl⁻ secretion is via apical membrane Cl⁻ channels acting in conjunction with the basolateral membrane Na⁺-K⁺-2Cl⁻ cotransporter (21). To determine whether NCM460 cells are representatives of transverse colonic crypts, we compared their Cl⁻ transport and its regulation with those of primary cultures of human transverse crypt colonocytes. Our results with inhibitors suggest that, in both cell types, in the resting state, Cl⁻ channels such as CFTR are the predominant Cl⁻ permeability, although Na⁺-K⁺-2Cl⁻ cotransporter is also present. This is borne out by the presence of transcripts for CFTR and the Na⁺-K⁺-2Cl⁻ cotransporter in both cell types (Fig. 3). The small residual Cl⁻ permeability seen in the presence of both DPC and furosemide is presumed to be due to DPC-insensitive Cl⁻ channels. Although Cl⁻/HCO₃⁻ exchange is prevalent in colonic crypts, its contributions were presumed to be minimal, since the perfusion buffer was HCO₃⁻ free.

Forskolin, PGE₁, and 8-BrcAMP enhanced Cl⁻ permeabilities via the Cl⁻ channels and the Na⁺-K⁺-2Cl⁻ cotransporter in both cell types. This demonstrated the presence of hormone-specific receptors, an activatable adenylate cyclase, and a cAMP-sensitive Cl⁻ transport mechanism. This is consistent with previous findings in primary human distal colonic cultures (16) and in T84 cells (1). As in the case of basal Cl⁻ transport, combined addition of DPC and furosemide inhibited forskolin-stimulated Cl⁻ permeabilities 89–95% in both cell types, suggesting that Cl⁻ channels and the Na⁺-K⁺-2Cl⁻ cotransporter are the major routes. In contrast, combined addition of the inhibitors caused a significantly smaller (72–74%, P < 0.05) decline in PGE₁-stimulated Cl⁻ transport. Thus PGE₁ appears to activate DPC and furosemide-sensitive Cl⁻ transport processes, perhaps via a cAMP-independent pathway.

In the small intestine, cGMP and 8-BrcGMP act via PKG II to stimulate secretion. However, the cGMP signal transduction cascade in the large intestine appears to vary with cell type. In T84 cells, STα stimulatores cGMP and Cl⁻ secretion, but 8-BrcGMP has no effect (4b). This has been demonstrated to be due to the lack of PKG II and the fact that cGMP, but not its analog, can cross-activate protein kinase A and thereby Cl⁻ secretion (4b). In contrast, our studies demonstrate that both STα and 8-BrcGMP can stimulate Cl⁻ transport in distal colonocytes (7) as well as in NCM460 cells and transverse human colonocytes (Fig. 5), suggesting that these cells have an active PKG II. The data in Fig. 6 confirm that these cells possess transcripts both for PKG II and for the STα receptor GCC. There are two noteworthy differences in the responses elicited by STα and 8-BrcGMP. First, STα-stimulated transport, like those of cAMP- and Ca²⁺-dependent secretagogues, appears to be largely via DPC-sensitive channels, whereas 8-BrcGMP acts mainly via the Na⁺-K⁺-2Cl⁻ cotransporter. This is true for both NCM460 cells and the primary colonocytes. It could be speculated that activation of PKG II (action of 8-BrcGMP) affects the cotransporter, whereas activation of PKG II and protein kinase A (PKA) (action of STα) activate both Cl⁻ channels and the cotransporter. We have previously demonstrated that, in the flounder intestine, 8-BrcGMP regulates Na⁺-K⁺-2Cl⁻ cotransporter phosphorylation and activity (22). The second difference between STα and 8-BrcGMP is that, whereas they elicit responses of similar magnitude in the primary colonocytes, STα has a significantly greater effect than 8-BrcGMP in the NCM460 cells. This interesting difference between these cells and the primary cultures implies that STα is acting via multiple (PKA and PKG II) pathways in NCM460 cells.

The Ca²⁺-mediated and PKC pathways are other examples of the similarity between primary cultures and the immortalized colonocytes. The PKC cascade is
utilized by several secretagogues in the intestine. In primary human colonicocytes (16) and HT-29.c19A cells (23, 24), short-term exposure to phorbol esters alone stimulates Cl\(^{-}\) permeabilities. In marked contrast, we and others have found that short-term exposure to phorbol esters alone had no direct effects on Cl\(^{-}\) secretion in T84 cells but attenuated cAMP-activated Cl\(^{-}\} secretion (11, 20). Furthermore, long-term treatment with PDB induced downregulation of PKC activity in T84 cells (20). Both NCM460 cells and the primary transverse colo cyanopy cultures exhibited enhanced Cl\(^{-}\} permeability with short-term PDB treatment, similar to the results of our studies on the distal human colonicocytes. However, long-term treatment with submaximal doses of PDB shows desensitization to further PDB stimulation at 24 h. This is most probably due to downregulation of PKC as reported for T84 cells by Matthews et al. (11) and our own unpublished observations. The desensitizing effect of prolonged exposure to PDB begins to dissipate by 48 h both in NCM460 cells (Table 1) and T84 cells (20). This observation remains to be correlated with PKC activity.

Histamine and serotonin are present in large amounts in the colon (12, 14), and histamine and cholinergic agonists are known to act by releasing Ca\(^{2+}\} from intracellular stores in T84 cells (26). We had previously demonstrated that serotonin and neureotensin stimulate Cl\(^{-}\} channels and the Na\(^{+}\}-K\(^{+}\)-2Cl\(^{-}\} cotransporter in rabbit (19) and distal human (15) colonicocytes. In contrast, although histamine largely activates DPC-sensitive Cl\(^{-}\} transport in human distal colonicocytes (15), it activates only the Na\(^{+}\}-K\(^{+}\}-2Cl\(^{-}\} cotransporter in rabbit distal colonicocytes (19), suggesting species-specific differences. In the present study, the responses of NCM460 cells to serotonin and histamine are very similar to those seen in primary cultures of transverse human colonicocytes (Table 2), emphasizing the advantage of studying nontransformed colonic epithelial cells. Another striking feature of NCM460 cells is that, unlike T84 cells, the activation of Cl\(^{-}\} permeabilities by Ca\(^{2+}\} -dependent secretagogues is not Na\(^{+}\} sensitive. These results imply that activation of K\(^{+}\} channels may not be required for Ca\(^{2+}\} -dependent Cl\(^{-}\} transport in all colonicocyte preparations and reinforce the advantage of studying nontransformed colonic cell lines, such as NCM460.

The present studies demonstrate that the immortalized NCM460 colonicocytes establish resistance and demonstrate second messenger-mediated Cl\(^{-}\} permeabilities very similar to human transverse colonic crypt cells in primary culture. With the exception of the striking quantitative differences in response to STa, we found no major differences between these two cell types in the responses to second messengers. Together with our recent findings that NCM460 colonicocytes, like the transverse colonic crypts, possess transcripts for the NHE-1 and NHE-2 isoforms, but not for NHE-3 isoform, these studies validate the NCM460 colonicocytes as a good model of the transverse colonic crypts. The fact that the origin of these cells is known makes NCM460 cells an invaluable tool for dissecting the cellular basis of ion transport in the human colon.

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