Vasoactive Intestinal Polypeptide-induced Chloride Secretion by a Colonic Epithelial Cell Line

Direct Participation of a Basolaterally Localized Na^+,K^+,Cl^- Cotransport System

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Abstract

We have used a well-differentiated human colonic cell line, the T_{84} cell line, as a model system to study the pathways of cellular ion transport involved in vasoactive intestinal polypeptide (VIP)-induced chloride secretion. A modified Ussing chamber was used to study transepithelial Na^+ and Cl^- fluxes across confluent monolayer cultures of the T_{84} cells grown on permeable supports. In a manner analogous to isolated intestine, the addition of VIP caused an increase of net Cl^- secretion which accounted for the increase in short circuit current (I_{sc}). The effect of VIP on I_{sc} was dose dependent with a threshold stimulation at 10^{-10} M VIP, and a maximal effect at 10^{-8} M. Bumetanide prevented or reversed the response to VIP. Inhibition by bumetanide occurred promptly when it was added to the serosal, but not to the mucosal bathing media. Ion replacement studies demonstrated that the response to VIP required the simultaneous presence of Na^+, K^+, and Cl^- in the serosal media. Utilizing cellular ion uptake techniques, we describe an interdependence of bumetanide-sensitive $^{22}Na^+$, $^{86}Rb^+$, and $^{36}Cl^-$ uptake, which is indicative of a Na^+,K^+,Cl^- cotransport system in this cell line. This transport pathway was localized to the basolateral membrane. Extrapolated initial velocities of uptake for each of the three ions was consistent with the electroneutral cotransport of 1 Na^+:1 K^+ (Rb^+):2 Cl^-. Our findings indicate that VIP-induced Cl^- secretion intimately involves a bumetanide-sensitive Na^+,K^+,Cl^- cotransport system which is functionally localized to the basolateral membrane.

Introduction

Furosemide, or its more specific analogue bumetanide, has been shown to block transepithelial chloride transport in a number of disparate epithelia, including intestine, trachea, and cornea (1–6). Frizzell et al. (1) have postulated that these so-called "loop diuretics" inhibit an electroneutral Na^+,Cl^- cotransport system on the serosal surface of Cl^- secreting epithelia, or the mucosal surface of Cl^- absorbing epithelia (1). The furosemide-sensitive Na^+,Cl^- cotransport system was postulated to act in concert with the Na^+,K^+ ATPase to drive Cl^- entry into the cells to a point above its electrochemical equilibrium. Chloride would then passively flow out of the cells through a postulated Cl^- channel on the opposite membrane. Furosemide- or bumetanide-sensitive ion transport is not limited to epithelial tissues, but seems to be ubiquitously distributed in many types of cells. Using cultured cell lines (MDCK, Ehrlich ascites) and erythrocytes (human and avian), in which precise measurements of unidirectional ion movements across the plasma membrane are possible, a number of investigators have identified this loop-diuretic-sensitive component of ion transport as an electroneutral $Na^+ + K^+ + 2Cl^-$ cotransport system (7–13). Recently, using the Cl^- absorbing epithelia from flounder intestine, Musch et al. (14) have noted a mucosal K^+ dependence of Cl^- absorption, thus directly implicating the involvement of a Na^+,K^+,Cl^- cotransport system in this phenomenon.

We have investigated the role of bumetanide-sensitive ion transport in transepithelial Cl^- secretion using a cultured epithelial cell line from human colon (the T_{84} cell line) which retains receptor-mediated vectorial electrolyte transport (15). Cultured epithelial cell lines from dog kidney, pig kidney, and toad bladder have been successfully utilized to study hormone-stimulated transepithelial transport after growth to confluence on permeable supports (16–21). Such preparations serve as model epithelia because the cells form tight junctions between each other and become polarized with distinct apical and basolateral membranes. The use of cultured cell lines as model epithelia has several disadvantages, the foremost of which is that the cells can be grown in vitro unlike normal epithelial cells, and thus presumably lack some mechanisms of normal growth control (16). However, this approach also possesses a number of distinct advantages, including the ability to perform both physiological and preparative biochemical studies with a homogeneous cell population. Such studies, in most cases, are not possible with intact epithelial tissues, since these preparations contain muscle, nerve, and other interstitial cells, as well as several different types of epithelial cells.

In the following report we have used two experimental approaches to study vasoactive intestinal polypeptide (VIP)-[1] induced electrolyte transport across T_{84} cell monolayers. Firstly, confluent T_{84} cell monolayers grown on a permeable support were mounted in a modified Ussing chamber for the study of active transepithelial ion transport and its response to VIP. Our results are consistent with the interpretation that VIP elicits a stimulation of transepithelial Cl^- secretion across T_{84} cell monolayers, which is similar to previous observations with isolated intestine from man and animal systems (22–25). Secondly, we have utilized cellular uptake of $^{22}Na^+$, $^{86}Rb^+$ (an

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1. *Abbreviations used in this paper:* I_{sc}, increase in short circuit current; M → S, mucosal to serosal; PD, potential difference; S → M, serosal to mucosal; G, tissue conductance; J, unidirectional flux rates; VIP, vasoactive intestinal polypeptide.

isotopic probe for K^+), and $^{36}Cl^-$ to confirm that Cl^- entry into the T_{84} cells, on the basolateral membrane, occurs via an electroneutral bumetanide-sensitive Na^+,K^+,Cl^- cotransport process.

Methods

Growth and maintenance of T_{84} cells. T_{84} cells were grown as monolayers in a 1:1 mixture of Dulbecco-Vogt modified Eagle's medium and Ham's F-12 medium supplemented with 15 mM Na^+-Hepes buffer, pH 7.5, 1.2 g $NaHCO_3$, 40 mg penicillin, 8 mg ampicillin, and 90 mg of streptomycin per liter, and 5% newborn calf serum (15, 26). Confluent monolayers were subcultured by trypsinization with 0.1% trypsin and 0.9 mM EDTA in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline.

Transepithelial electrolyte transport studies. The Ussing chamber was modified to allow maintenance of the integrity of the cell monolayers during the study. The modified chamber was designed to minimize turbulence created by the air lift system and to avoid edge damage to the monolayers. These modifications permitted the monolayer to remain intact for >3–4 h.

For the Ussing chamber experiments, 10^6 T_{84} cells were plated on a permeable support (1.98 cm^2 surface area) and maintained for 5–6 d before use. At this time, the transepithelial conductance of the preparations became stable. The supports were suspended over the bottom of a 100-mm culture dish to permit "bottom feeding" by laying them on top of a layer of glass beads. These supports were similar to the "filter-bottom dish" developed by Handler et al. (27) consisting of a rat tail collagen-coated polycarbonate Nucleopore filter (Nucleopore Corp., Pleasanton, CA, 5 μm pore size) glued to one open end of a Lexan ring. Crude rat tail collagen was solubilized by dissolving 1 g rat tail tendons in 100 ml 1% acetic acid. Further steps in preparation of rat tail collagen and procedures for collagen coating Nucleopore filters (Nucleopore Corp.) were as described by Cereijido et al. (28). After cell growth, the entire ring assembly was inserted into the Ussing chambers. No pressure was exerted directly on the monolayers and hence edge damage was avoided. Procedures after this point followed those described for isolated intestine by Binder and Rawlins (29). Mucosal and serosal reservoirs contained identical volumes of oxygenated Ringer's solution (pH 7.4, at 37°C) that contained (in millimolar): Na, 140; K, 5.2; Ca, 1.2; Mg, 1.2; Cl, 119.8; HCO_3, 25; H_2PO_4, 2.4; HPO_4, 0.4; and glucose, 10. Potential difference (PD) across the cell monolayer was measured by calomel electrodes in 3 M KCl and monitored with a potentiometer. Throughout the experiment, except for 5–10 s every 5 min while the PD was being recorded, spontaneous tissue PD was short circuited and nullified by an automatic voltage clamp (WPI, New Haven, CT) with Ag:$AgCl_2$ electrodes. Tissue conductance (G) was calculated from the PD and the imposed current according to Ohm's law. Unidirectional $^{22}Na^+$ and $^{36}Cl^-$ fluxes were carried out simultaneously in tissue pairs with similar conductance. Our preliminary experiments demonstrated that the unidirectional flux rates of $^{22}Na^+$ and $^{36}Cl^-$ were stable for up to 2.5 h, and that unidirectional $^{22}Na^+$ and $^{36}Cl^-$ fluxes under basal conditions varied directly with the conductance (all $r \geq 0.85$, $P < 0.005$).

Radionuclide uptake studies. For all radionuclide uptake experiments, except those shown in Table III, T_{84} cells were plated and grown to confluency in 35-mm culture dishes. Preincubation and assay procedures followed the Na^+ depletion conditions of Rindler et al. (30, 31). Monolayers were first washed and incubated in 140 mM KCl, 10 mM Tris-SO_4, pH 7.5, and 1.2 mM $MgSO_4$ for 1 h. The monolayers were then washed three times with 2 ml sucrose buffer (241 mM sucrose, 10 mM Tris-SO_4, pH 7.5, and 1.2 mM $MgSO_4$) and incubated for 1–1.5 h in the same buffer containing 0.5 mM ouabain. In Fig. 6, Tris-NO_3 and $Mg(NO_3)_2$ were substituted for Tris-SO_4 and $MgSO_4$, respectively. Uptakes were initiated by aspiration of the preincubation buffer and the addition of uptake buffer containing the given concentrations of inhibitors and 1–2 μCi/ml $^{86}Rb^+$, 0.5–2 μCi/ml $^{22}Na^+$, or 0.5–1 μCi/ml $^{36}Cl^-$. The ionic composition of the assay buffers are given in each of the figure or table legends. All uptakes were carried out at room temperature. All $^{36}Cl^-$ uptakes and those $^{86}Rb^+$ and $^{22}Na^+$ uptakes which were carried out simultaneously with $^{36}Cl^-$ uptakes (Fig. 5) were terminated by washing rapidly three times with 2 ml ice-cold $MgSO_4$-sucrose buffer (137 mM sucrose, 100 mM $MgSO_4$, and 10 mM Tris-SO_4, pH 7.5) followed by a 5-min incubation on ice and a final 2-ml wash. All other uptakes were terminated in a similar manner using ice-cold 100 mM $MgCl_2$ and 10 mM Tris-Cl, pH 7.5, as described previously (30, 31). This Mg wash procedure virtually eliminated extracellularly bound label while allowing retention of >90% intracellular label (30). The cellular radioactivity was extracted by incubation of the dishes with either 1 ml 0.5 N NaOH for 30 min or 1 ml H_2O overnight and counted with Betablend (Westchem, San Diego, CA) scintillation fluid. Zero time values (<5 s) were subtracted from all data. Representative monolayers were taken for determination of their protein content. Protein was determined by the method of Lowry et al. (32) using bovine serum albumin as a standard.

Unilateral (mucosal or serosal) uptakes followed a similar protocol, except that monolayers were maintained on permeable supports identical to those used in the Ussing chamber studies, and all preincubation and assay buffers contained 1.2 mM Ca^{++}. Monolayers were not Na^+-depleted but simply preincubated in 241 mM sucrose buffer with or without 0.5 mM ouabain. Rings were washed and placed in 35-mm culture dishes which had five drops of glue symmetrically placed and dried so as to raise the monolayers off the culture dishes. After the preincubation period, either the solution inside (mucosal) or outside (serosal) the ring was aspirated and replaced with 0.4 ml (mucosal) or 1.6 ml (serosal) uptake buffer containing the desired inhibitors and radionuclide. Ouabain (0.5 mM) was also included in the uptake buffer when indicated. After 3 min, the entire ring was washed using either the $MgSO_4$ sucrose wash procedure (for $^{36}Cl^-$ uptake studies) or $MgCl_2$ wash procedure (for all other uptake studies) as described above, except that 3–4 ml buffer was used with each wash. Cells were lysed with 0.5 ml 0.5 N NaOH and counted as described above.

Materials. All the radionuclides were obtained from New England Nuclear, Boston, MA. VIP was provided by Dr. Jean Rivier, Dr. Marvin Brown, and Dr. Wylie Vale at The Salk Institute, La Jolla, CA, and dissolved in deionized distilled water. Bumetanide was a gift from Dr. P. W. Feit of Leo Pharmaceutical Products, Ballerup, Denmark. Amiloride was generously provided by Merck Sharp and Dohme, West Point, PA. Bumetanide and amiloride were prepared as 100–500-mM solutions in dimethyl sulfoxide and stored at −20°C. Dimethyl sulfoxide concentrations of up to 1% had no effect on the rate of radionuclide uptake. Dulbecco-Vogt modified Eagle's media and Ham's F_{12} media were purchased from Gibco Laboratories, Grand Island, NY, and filter sterilized before use. Newborn calf serum was purchased from Irvine Scientific, Irvine, CA.

Results

Transepithelial electrolyte transport studies. T_{84} cells when grown on the permeable, collagen-coated nucleopore membranes and suspended over the culture dish to permit "bottom feeding" formed a confluent monolayer with a high electrical resistance. After 5 to 6 d in culture, the cells appeared as a columnar epithelial monolayer with their basolateral membrane firmly attached to the collagen-coated surface and their microvillus membrane facing the medium (Madara, J., and K. Dharmsathaphorn, manuscript in preparation). These monolayers maintained a transepithelial resistance (R) of ~1.5 $k\Omega \cdot cm^{-2}$. Transepithelial electrolyte transport studies were carried out in a modified Ussing chamber. For these Ussing chamber studies, we have denoted the basolateral membrane as serosal and microvillus membrane as mucosal surface, respectively. The collagen-coated nucleopore membrane which

served as the attachment support for the cells had a resistance of <4 $\Omega \cdot cm^{-2}$; thus, it contributed insignificantly to the transepithelial resistance. As shown in the following sections, these monolayer preparations retained stable conductances and constant rates of transcellular electrolyte transport for over 2.5 h when mounted in the modified Ussing chambers and bathed with an oxygenated glucose-Ringer's solution.

(a) *VIP-stimulated increase in short circuit current (I_{sc}) is reversed by serosal addition of bumetanide.* As illustrated in Fig. 1, addition of 10^{-8} M VIP to the serosal bathing solution caused an increase in the I_{sc}. Mucosal addition of VIP had no effect (data not shown). Maximal response was reached ~15 min after the addition of VIP, and the effect persisted for over 1 h. This increase in I_{sc} induced by VIP was reversed by bumetanide. While the mucosal addition of bumetanide had little or no effect, the addition of bumetanide to the serosal side resulted in an immediate reversal of the VIP-induced I_{sc}, which was similar to that observed upon the addition of bumetanide to both the mucosal and serosal bathing solutions. Pretreating the monolayers with bumetanide inhibited the response to VIP (data not shown).

The effect of VIP was dose dependent, with a threshold concentration of 10^{-10} M VIP inducing an alteration in the I_{sc}, and a maximal effect observed at 10^{-8} M (Fig. 2 *A*). 10^{-8} M VIP was used for the experiments illustrated in Fig. 1 and in all subsequent studies. The effect of bumetanide was also dose dependent. Identical effects were observed when bumetanide was added to both the serosal and mucosal bathing media or when bumetanide was added to the serosal side alone (Fig. 2 *B*). Mucosal addition of bumetanide, at concentrations below 10^{-4} M, had no effect on the VIP-stimulated I_{sc}. Indeed, even at 10^{-4} M, the mucosal addition of bumetanide caused only a slow and partial reversal of the I_{sc}. The time course of this response differed dramatically from that observed upon the serosal addition of 10^{-4} M bumetanide (see Fig. 1), and may represent leakage of the drug across the monolayers.

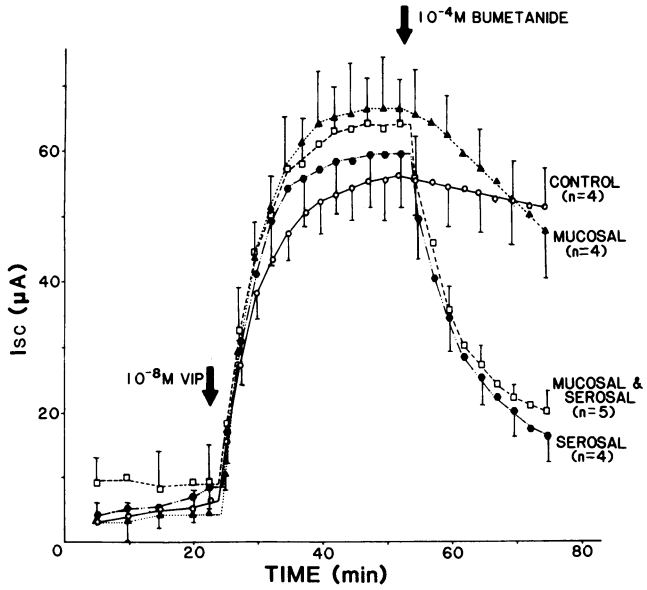

Figure 1. Reversal of VIP-stimulated I_{sc} by the serosal addition of bumetanide. T_{84} cell monolayers grown on permeable supports were mounted in the modified Ussing chambers. VIP (10^{-8} M) was added at 22.5 min to the serosal side. 10^{-4} M bumetanide was added at 57.5 min to the mucosal (▲), serosal (●) or to both sides (□) of the monolayers. Controls received an equivalent addition of dimethyl sulfoxide (○). Values are expressed as mean±SE in μA/monolayer. The number of experiments (*n*) is indicated in parentheses.

(b) *VIP-stimulated I_{sc} represents an increase in net Cl^- secretion across T_{84} monolayers.* As shown in Fig. 3, which illustrates a representative experiment for a single set of paired monolayers, the addition of 10^{-8} M VIP caused an increase in both the mucosal to serosal (M → S) and the serosal to mucosal (S → M) movement of Cl^-, while having little or no effect on the transepithelial transport of Na^+. Unidirectional flux rates (J) were determined from the slopes of the lines. A series of control experiments where unidirectional flux of Na^+ and Cl^- were measured in the absence of VIP and/or bumetanide confirmed that basal flux rates, and the transepithelial conductances, remained constant over the time period for these studies (2.5 h). Unidirectional flux rates of Na^+ and Cl^- in either the S → M or M → S directions were of similar magnitudes (see Table I). Hence, the basal rates for $J_{net}^{Cl^-}$ and $J_{net}^{Na^+}$ did not differ significantly from zero.

The results from six paired monolayers are illustrated in Fig. 4. After the addition of VIP (10^{-8} M), the increase in net Cl^- secretion (illustrated by the open bars) totally accounted for the change in I_{sc}. Changes in the I_{sc} and net Cl^- secretion had an excellent correlation ($r = 0.90$, $P < 0.001$). No significant effects were observed on net Na^+ flux (illustrated by the solid bars). Addition of bumetanide (to the serosal side of the monolayers) reversed both net Cl^- secretion and I_{sc} to values near zero while having little or no effect on net Na^+ flux. The unidirectional ion fluxes from these six paired monolayers are summarized in Table I. No changes in M → S or in S → M Na^+ movements were observed after addition of VIP or the subsequent addition of bumetanide. After addition of VIP, both $J_{S\rightarrow M}Cl^-$ and $J_{M\rightarrow S}Cl^-$ increase; however, the increase in the S → M movement of Cl^- was consistently greater, and resulted in net Cl^- secretion. Subsequent addition of bumetanide reduced unidirectional Cl^- movement in both mucosal to serosal and serosal to mucosal directions, which resulted in the inhibition of net Cl^- secretion. The increase in conductance observed after the addition of VIP was not reversed by the subsequent addition of bumetanide.

(c) *Ion replacement studies.* The inhibition of Cl^- secretion by bumetanide, described above, suggests that Na^+, Cl^-, and possibly K^+ are required for the Cl^- uptake step in VIP's action and that this process is localized to the basolateral membrane of the T_{84} cells. To verify the involvement of Na^+, K^+, and Cl^-, ion replacement studies were carried out, and results of these studies are summarized in Table II. VIP's action was partially reversed when the Ringer's solution was bilaterally replaced by either a Na^+-, K^+-, or Cl^--free Ringer's solution. Exclusion of K^+ from the serosal bathing solution alone partially reversed VIP-stimulated I_{sc} while mucosal exclusion had no effect. The exclusion of either Na^+, K^+, or Cl^- before the addition of VIP also partially inhibited the action of VIP (data not shown). These ion replacement studies indicate that the Cl^- entry step for VIP-induced Cl^- secretion requires the simultaneous presence of Na^+, K^+, and Cl^-, which suggests that the Cl^- entry step on the basolateral membrane is either a Na^+,K^+,Cl^- cotransport system or Na^+,Cl^- cotransport operating in close association with an active Na^+,K^+ ATPase. The Ussing chamber technique cannot, however, differentiate between these two possibilities, since removal of serosal K^+ also inactivates the Na^+,K^+ ATPase. Furthermore,

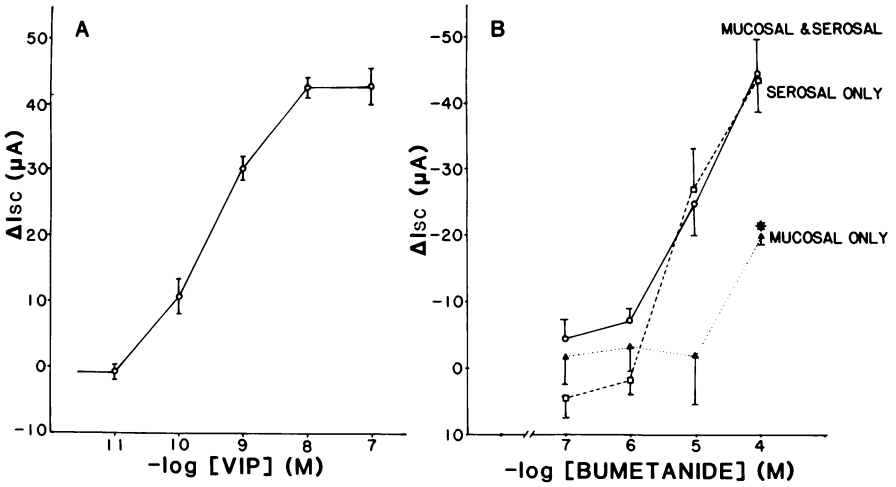

Figure 2. Dose dependencies for VIP-stimulation of I_{sc} (*A*) and for bumetanide inhibition of the VIP-stimulated I_{sc} (*B*) across T_{84} monolayers. (*A*) Graded dose effect of VIP on T_{84} monolayers grown on permeable supports. VIP was added to the serosal side of monolayers 22.5 min after mounting in the Ussing chambers. The ΔI_{sc} values represent the net increase in I_{sc} observed 25 min after the addition of 10^{-11} M–10^{-7} M VIP, and are expressed as the mean±SE of 4–5 experiments in μA/monolayer. (*B*) Graded dose effect of bumetanide on VIP induced I_{sc} across T_{84} cell monolayers. 10^{-8} VIP was added to the serosal side of monolayers mounted in the modified Ussing chambers at 22.5 min after mounting. The mean±SE for VIP response was 49±10 μA/monolayer. Varying concentrations of bumetanide were added at 47.5 min to the mucosal (▲), serosal (□), or to both mucosal and serosal (○) bathing solutions. The change in I_{sc} (ΔI_{sc}) shown in the figure was recorded 25 min after the addition of bumetanide. Values are expressed as mean±SE for 3–6 experiments in μA/monolayer.

we have observed in the T_{84} cells that inhibition of the Na^+,K^+ ATPase by ouabain causes the active transepithelial Cl^- transport process to cease (as indicated by the reversal of VIP-induced I_{sc}; data not shown).

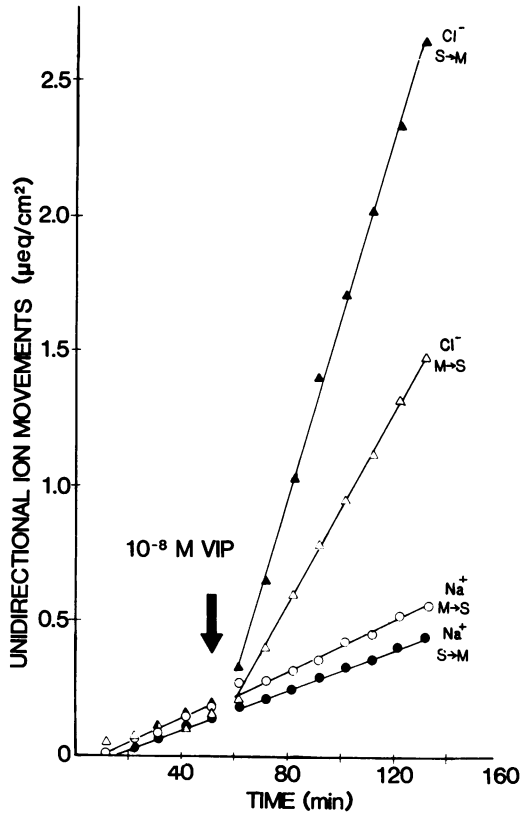

Figure 3. VIP stimulates unidirectional transport of Cl^- across T_{84} cell monolayers while having no effect on unidirectional Na^+ transport. This figure depicts the results for a representative pair of T_{84} monolayers grown on the permeable support; the monolayers in this experiment had an initial conductance of 0.5 ms/cm² and ion flux was determined as described in Methods. VIP (10^{-8} M) was added 52 min after the addition of $^{22}Na^+$ and $^{36}Cl^-$ to the chambers. Samples were obtained at 10-min intervals. △, M → S Cl^- movement; ▲, S → M Cl^- movement; ○, M → S Na^+ movement; and ●, S → M Na^+ movement.

Radionuclide uptakes by T_{84} monolayers. The Ussing chamber technique detects the ions that move across both the basolateral and brush border membranes. This net transepithelial flux activity is dependent on an intact active transport process. In contrast, ion uptake across the plasma membrane and into the cell measures the movement of ions across the brush border and/or basolateral membrane. Ion uptake into the cell can be measured in the presence of ouabain or other inhibitors of transport pathways and allows discernment of passive or facilitated transport processes in the absence of a net, transepithelial flux activity.

(a) *Verification of the existence of a bumetanide-sensitive Na^+,K^+,Cl^- cotransport system in T_{84} cells.* Results presented in Fig. 5 indicate that Na^+, Rb^+ (K^+), and Cl^- uptake into T_{84} cells are interdependent and highly sensitive to bumetanide. In the presence of both K^+ and Cl^-, approximately half of the $^{22}Na^+$ uptake is inhibitable by bumetanide (Fig. 5 *A*). Bumetanide inhibitable $^{22}Na^+$ uptake requires the presence of both K^+ and Cl^-. In the absence of either K^+ or Cl^-, $^{22}Na^+$ uptake was inhibited to the same extent as that in the presence of bumetanide; addition of bumetanide had no further effect on $^{22}Na^+$ uptake when K^+ or Cl^- were absent. Similar results were observed for $^{86}Rb^+$ (as a tracer for K^+) and $^{36}Cl^-$ uptakes (Fig. 5, *B* and *C*). $^{86}Rb^+$ uptake was not observed unless both Na^+ and Cl^- were present. Conversely, $^{36}Cl^-$ uptake was inhibited by removal of Na^+ and K^+ from the uptake buffer. Both $^{86}Rb^+$ and $^{36}Cl^-$ uptake were highly bumetanide sensitive. The interdependence of Na^+, K^+, and Cl^- uptake in the presence of ouabain to inhibit Na^+,K^+ ATPase activity suggests the presence of a bumetanide-sensitive Na^+,K^+,Cl^- cotransport in this cell line.

Since $^{22}Na^+$ uptake was only partially inhibited, even in the presence of saturating concentrations of bumetanide and ouabain, the existence of Na^+ transport pathways other than Na^+,K^+,Cl^- cotransport and the Na^+,K^+ ATPase was suggested. We found that the remaining Na^+ uptake, which is K^+ independent was also amiloride sensitive. We further observed that this amiloride inhibitable portion of Na^+ uptake was pH dependent, which suggested that it was a Na^+/H^+ antiporter (data not shown). Similar systems have been described on a number of other epithelia (31, 33, 34). However, since amiloride has little or no effect on VIP-induced I_{sc} and Cl^- secretion, this transport system was not studied further.

Table I. Unidirectional Sodium and Chloride Fluxes in the T_{84} Cell Monolayers

	J_{M-S}^{Na}	J_{S-M}^{Na}	J_{net}^{Na}	J_{M-S}^{Cl}	J_{S-M}^{Cl}	J_{net}^{Cl}	I_{sc} M–S	I_{sc} S–M	G_{M-S}	G_{S-M}
	μeq/h-cm²	*μeq/h-cm²*	*μeq/h-cm²*	*μeq/h-cm²*	*μeq/h-cm²*	*μeq/h-cm²*	*μeq/h-cm²*	*μeq/h-cm²*	*ms/cm²*	*ms/cm²*
Control	0.31±0.05	0.30±0.08	0.01±0.03	0.35±0.05	0.42±0.10	−0.07±0.05	0.02±0.005	0.04±0.02	0.64±0.08	0.65±0.06
VIP (10^{-8} M) (serosal)	0.31±0.05	0.34±0.05	−0.03±0.01	1.30±0.15*	2.13±0.06*	−0.83±0.09*	1.05±0.11*	1.01±0.07*	1.02±0.08*	1.01±0.05*
VIP (10^{-8} M) and bumetanide (10^{-4} M) (serosal)	0.41±0.09	0.39±0.07	0.02±0.02	0.53±0.07	0.62±0.09	−0.09±0.02	0.17±0.03	0.13±0.02	0.96±0.15	1.08±0.13

Asterisks indicate significant statistical differences as compared with control or VIP and bumetanide, except for conductance that was different from control only. (All P values <0.01 by t tests.) We have in another study (49) demonstrated that under basal state, unidirectional fluxes across the T_{84} monolayers of $^{22}Na^+$ and $^{36}Cl^-$ were extremely stable for more than 2 h. The addition of 10^{-8} M VIP caused a persistent and stable increase of transepithelial conductance associated with an increase in Cl^- movement bidirectionally, while Na^+ movement was not affected in either direction (Fig. 3). Na^+ and Cl^- flux results were obtained from the same monolayers. The results are expressed as mean±SE of six paired monolayers. Control represents electrolyte fluxes between 12 and 52 min after mounting. 10^{-8} VIP was then added to the serosal bathing solution and electrolyte fluxes measured between 62 and 102 min. Following this period, 10^{-4} M bumetanide was added serosally. Thus, the serosal bathing solution contained both VIP and bumetanide, and electrolyte fluxes again measured between 112 and 152 min.

To determine the stoichiometry of bumetanide-sensitive ion cotransport, the uptake of each of its three substrate ions, Na^+, Cl^-, and Rb^+ (as an analogue of K^+) was determined in parallel using an identical assay buffer containing either $^{22}Na^+$, $^{86}Rb^+$, or $^{36}Cl^-$. In these experiments, Rb^+ was substituted for K^+ in order to eliminate any "isotope" effect caused by using $^{86}Rb^+$ as a tracer for K^+.[2] Uptakes were carried out at 45-s intervals over 3 min in the presence of 0.5 mM ouabain to prevent efflux of Na^+ by the Na^+,K^+ ATPase and 0.5 mM amiloride to inhibit Na^+/H^+ exchange, with or without 0.1 mM bumetanide (Fig. 6). Cl^- uptake proceeded in a linear fashion for up to 3 min, while the rate of Na^+ uptake decreased over time, and Rb^+ uptake accelerated. One explanation for the acceleration of Rb^+ uptake over time may be apparent trapping in the intracellular compartment due to a high internal concentration of K^+. The rates of bumetanide-sensitive uptake for each of the three ions were extrapolated to zero time by polynomial regression of the rates of uptake during the four time intervals using the smoothing method of Jacquez (35). Under these experimental conditions, the initial velocity of bumetanide sensitive Na^+, Rb^+, and Cl^- uptakes were 9.8±1.4, 11.5±1.7, and 21.2±2.5 nmol/min per mg protein, respectively. These uptake rates approached a ratio of 1 Na^+:1 Rb^+:2 Cl^-, similar to the Na^+,K^+,Cl^- cotransport systems reported in other cell types (7–13, 36, 37).

To support that in our Ussing chamber we were dealing with the same bumetanide-sensitive transport system, we tested the graded dose effect of bumetanide for both the Ussing chamber and radionuclide uptake studies. The dose-response curve for bumetanide inhibition of VIP-stimulated I_{sc} closely paralleled the dose-response curve for bumetanide inhibition of Na^+ dependent $^{86}Rb^+$ uptake (Fig. 7). The dose of bumetanide which resulted in 50% inhibition of $^{86}Rb^+$ uptake was 2×10^{-6} M, as compared with 7×10^{-6} M for inhibition of VIP-induced I_{sc}. The discrepancy may be explained by the large experimental error in the Ussing chamber experiments and by changes in the affinity of bumetanide for the cotransporter by temperature or small differences in Na^+, K^+, or Cl^- concentrations for the two experimental conditions (37, 38).

(b) *Localization of the Na^+,K^+,Cl^- cotransport to the serosal side of T_{84} cells.* In the experiments described above, the uptake of $^{22}Na^+$, $^{86}Rb^+$, or $^{36}Cl^-$ may occur via either the brush border or basolateral membrane, since omission of calcium in the incubation buffer allowed the basolateral surface of the cells, as well as the brush border surface, to be exposed to the uptake media. As described earlier, bumetanide inhibition

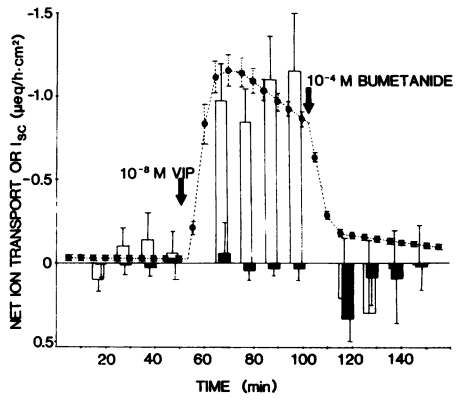

Figure 4. Time course of net Na^+ and Cl^- transport and correlation of net Cl^- secretion with the I_{sc}. The mean±SE from six paired monolayers are illustrated. The circles and dotted line represent the I_{sc}; clear bars represent net Cl^- transport; and solid bars represent net Na^+ transport measured over 10-min intervals. I_{sc} and flux rates are expressed as $\mu eq\ h^{-1}\ cm^{-2}$. Values above the line indicate net secretion, and values below the line net absorption. The unidirectional flux rates for these data are summarized in Table I.

2. Unlike MDCK cells, where Rb^+ and K^+ have nearly identical affinities for the cotransporter (30), in T_{84} cells, Rb^+ has a slightly higher affinity than K^+, as measured by the apparent K_a values for Rb^+- or K^+-stimulation of bumetanide-sensitive $^{22}Na^+$ uptake. At 14 mM Na^+ and 140 mM Cl^-, the apparent K_a values for Rb^+ and K^+ were 9.3 and 13.9 mM, respectively. The apparent V_{max} values were identical.

Table II. Reversal of VIP-induced I_{sc} by Ion Substitution

Ringer's solution	ΔI_{sc} induced by 10^{-8} M VIP	ΔI_{sc} caused by the ion substitution	ΔI_{sc} when the substituted ion is returned
	μA	μA	μA
Both mucosal and serosal substitution			
Control, n = 12	32±4	−1±3	−1±2
Na^+ free, n = 14	30±5	−21±4*	18±6*
K^+ free, n = 14	35±5	−22±4*	14±4*
Cl^- free, n = 13	35±5	−29±4*	11±3*
Mucosal substitution alone			
K^+ free, n = 6	32±9	3±2	
Serosal substitution alone			
K^+ free, n = 6	31±4	−23±8*	

VIP, 10^{-8} M, was added to both sides of the monolayer 22.5 min after mounting. The maximal response to VIP occurred within 25 min after its addition. After observing the maximal response to VIP, the bathing media was changed to either Na^+-, K^+-, or Cl^--free media, as indicated. Controls received replacement with regular Ringer's solution containing all three ions. Values represent mean±SE in μA per monolayer 25 min after ion replacement.
* Significantly different as compared with control ($P < 0.05$) by t test.

of Cl^- secretion across T_{84} cell monolayers in the Ussing chamber system appeared to be localized to the serosal or basolateral membrane side. To determine the site for localization of the bumetanide-sensitive Na^+,K^+,Cl^- cotransport system, uptake studies were carried out on cell monolayers grown on

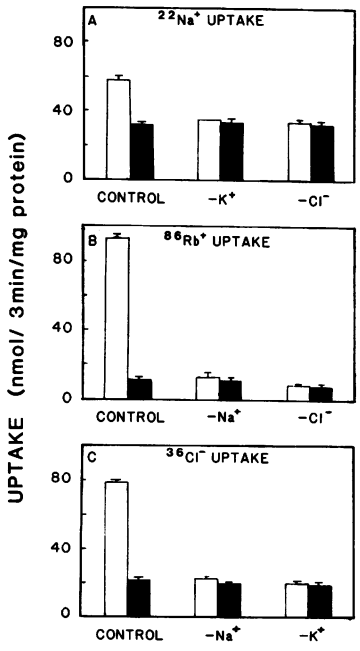

Figure 5. Interdependence of bumetanide-sensitive $^{22}Na^+$, $^{86}Rb^+$, and $^{36}Cl^-$ uptake. Clear bars represent uptake in the absence of bumetanide, and solid bars represent uptake in the presence of 0.2 mM bumetanide. T_{84} monolayers attached to 35-mm culture dishes were preincubated as described in Methods. In the control experiments, uptake buffer contained 35 mM Na^+ gluconate, 35 mM K^+ gluconate, 70 mM *N*-methylglucamine Cl^-, 10 mM Tris-SO_4, pH 7.5, and 1.2 mM $MgSO_4$. In the ion substitution experiments, when Na^+, K^+, or Cl^- were excluded from the uptake buffers, sucrose was added to maintain isotonicity. Experiments depicted in each panel were carried out on different days with different sets of confluent monolayers.

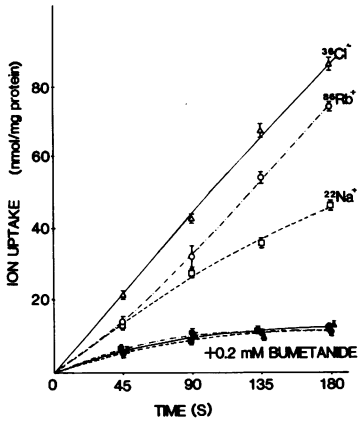

Figure 6. Time course of bumetanide-sensitive $^{22}Na^+$, $^{86}Rb^+$, and $^{36}Cl^-$ uptake by T_{84} cell monolayers. A single set of confluent monolayers was preincubated as described in Methods with the final preincubation buffer containing both 0.5 mM ouabain and 0.5 mM amiloride. Uptake buffer contained 70 mM choline chloride, 35 mM $NaNO_3$, 35 mM $RbNO_3$, 10 mM Tris-NO_3, pH 7.5, and 1.2 mM $Mg(NO_3)_2$, with trace amounts of either $^{86}Rb^+$ (○, ●), $^{22}Na^+$ (□, ■), or $^{36}Cl^-$ (△, ▲). Uptakes were carried out in the presence (●, ■, ▲) or absence (○, □, △) of 0.1 mM bumetanide for the given time intervals and terminated using the $MgSO_4$-sucrose wash, as described in Methods. Each point represents the mean±SD of triplicate determinations. Zero time values (1–2 nmol/mg protein) have been subtracted from all data.

permeable supports identical to those used for the Ussing chamber studies. Either the brush border or basolateral surface was exposed to the radionuclide in the uptake media.. As summarized in Table III, preferential bumetanide-sensitive uptakes of $^{22}Na^+$, $^{86}Rb^+$, and $^{36}Cl^-$ were observed only from the serosal side, thus localizing the bumetanide-sensitive Na^+,K^+,Cl^- cotransport pathway to the basolateral membrane and confirming the sidedness for bumetanide inhibition of I_{sc} and Cl^- secretion observed in the Ussing chamber studies. Similar basolateral localization was observed for ouabain-sensitive $^{86}Rb^+$ uptake, which was indicative of Na^+,K^+ ATPase.

Discussion

We have previously shown that the T_{84} human colonic cell line maintains morphological characteristics of well-differentiated epithelial cells and can respond physiologically to secretagogues and antisecretagogues with alterations in transcellular electrolyte transport (15, 26). We have recently reported that confluent T_{84} cell monolayers retain polarity, form tight junctions between cells, and grow to confluence with the basolateral surface attached to the growth substratum and their microvillus

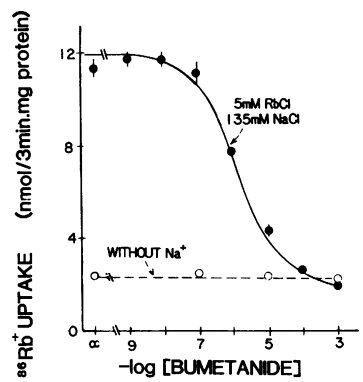

Figure 7. Dose-dependent effect of bumetanide on Na^+-dependent $^{86}Rb^+$ uptake. Confluent monolayers were preincubated as described in Methods. Uptakes were carried out for 3 min with varying concentrations of bumetanide in 135 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, and 10 mM Tris-SO_4, pH 7.5, and 2 μCi/ml $^{86}Rb^+$ (●). Uptakes in the absence of Na^+ (○) were carried out in an identical buffer, except that NaCl was replaced with choline Cl. Uptakes were terminated using the $MgCl_2$ wash procedure. Values shown represent the mean of triplicate determinations±SD.

Table III. Sidedness of Ion Uptake

	Serosal uptake (nmol/3 min per monolayer)			Mucosal uptake (nmol/3 min per monolayer)		
	Without bumetanide	With bumetanide	Bumetanide sensitive	Without bumetanide	With bumetanide	Bumetanide sensitive
^{22}Na	45.8±1.8	11.3±1.3	34.5±2.2	4.4±2.1	4.0±2.5	0.4±3.3
^{86}Rb	69.2±9.9	3.0±1.0	66.2±9.9	5.2±2.6	1.4±0.5	3.8±2.6
^{36}Cl	64.7±18.3	11.7±2.6	53.0±18.4	8.8±2.3	6.3±0.8	2.5±2.4
	Without ouabain	With ouabain	Ouabain sensitive	Without ouabain	With ouabain	Ouabain sensitive
^{86}Rb	81.5±2.7	63.8±5.6	17.7±6.2	3.4±0.1	2.6±0.4	0.8±0.4

Monolayer cultures of T_{84} cells on permeable supports were prepared as described in Methods and were similar to those used for Ussing chamber studies. After 8 to 12 d in culture, the rings were washed with sucrose buffer (241 mM sucrose, 10 mM Tris-NO_3, pH 7.5, 1.2 mM $Mg[NO_3]_2$, 1.2 mM $Ca[NO_3]_2$), then incubated 1 h with the same buffer containing 0.5 mM ouabain. The transcellular epithelial resistance after 2 h in sucrose-ouabain buffer was still >800 $\Omega \cdot cm^2$. Uptakes were initiated by adding uptake buffer containing the appropriate isotope with or without the indicated inhibitor to either the serosal or mucosal side of the monolayers. After 3 min, uptakes were terminated by washing the entire tissue four times with ice-cold $MgCl_2$-Tris wash buffer. For bumetanide-sensitive uptakes, the uptake buffer consisted of 70 mM choline chloride, 35 mM $NaNO_3$, 35 mM $RbNO_3$, 1.2 mM $Mg(NO_3)_2$, 1.2 mM $Ca(NO_3)_2$, 10 mM Tris-NO_3, pH 7.5, and 0.5 mM ouabain. For ouabain-sensitive $^{86}Rb^+$ uptake, preincubations were carried out in an identical manner, except that ouabain was omitted from half the monolayers and the assay buffer consisted of 135 mM NaCl, 5 mM RbCl, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, and 10 mM Tris-Cl, pH 7.5, with or without 0.5 mM ouabain. The values shown represent the mean of triplicate determinations ±SD. The difference, representing the drug-sensitive portion of the uptake, is shown in the table along with the standard error of the measurement. Tissues for each set of experiments were prepared and assayed on different days.

membrane facing the media (15). In this report, we demonstrate that the addition of VIP to T_{84} cell monolayers mounted in a modified Ussing chamber apparatus stimulates transcellular electrogenic secretion of Cl^-. This response is similar to the phenomenon observed in isolated intestine from animal and man (22–25). The similarity of the physiological response has encouraged us to use the T_{84} cell line as a model system to study the mechanism of VIP-stimulated Cl^- secretory process.

Our results clearly show the presence of a Na^+,K^+,Cl^- cotransport system in the T_{84} cells. The studies strongly suggest that VIP-induced Cl^- secretion requires a functional Na^+,K^+,Cl^- cotransport system localized in the basolateral membrane for the Cl^- uptake step. This conclusion is drawn from the following observations reported herein. (*a*) In the Ussing chamber system, we have observed that the VIP-stimulated I_{sc} and Cl^- secretion are reversed or prevented by the serosal application of bumetanide, an established and specific inhibitor of Na^+,Cl^- cotransport or Na^+,K^+,Cl^- cotransport. (*b*) In the presence of ouabain to inactivate the Na^+,K^+ ATPase, the bumetanide-sensitive uptake of $^{22}Na^+$, $^{36}Cl^-$, and $^{86}Rb^+$ (used as a tracer for K^+) required the simultaneous presence of all three substrate ions, Na^+, Cl^-, and K^+. Omission of any one ion reduced the level of uptake to that observed in the presence of bumetanide. The stoichiometry for the interdependent uptake of the three substrate ions approached a ratio of 1 Na^+:1 K^+:2 Cl^-. This is identical to that previously reported for electroneutral Na^+,K^+,Cl^- cotransport systems in several epithelia as well as in erythrocytes and cultured fibroblasts (8–13, 36, 37). (*c*) Radionuclide uptake studies on monolayers grown on a permeable support verified the localization of the bumetanide-sensitive Na^+,K^+,Cl^- cotransport system to the basolateral membrane (Table III). (*d*) Inhibition of both transcellular Cl^- secretion, as well as ion uptake by the cells, show a similar dose-response to bumetanide (Figs. 2 and 7). This is seen despite the differing conditions used in the two experimental approaches. These results, obtained from both the Ussing chamber and cellular ion uptake studies, strongly suggest that the Cl^- entry step involved in VIP-induced Cl^- secretion is a Na^+,K^+,Cl^- cotransport system localized on the basolateral membrane. These results also support the model of Frizzell et al. (1), with the exception that a Na^+,K^+,Cl^- cotransporter, rather than a Na^+,Cl^- cotransporter, is localized to the basolateral membrane of Cl^- secreting epithelia.

The presence of a Na^+,K^+,Cl^- cotransport on the basolateral membrane is the opposite of what was found in the flounder intestine (14), where it is present on the brush border membrane. However, flounder intestine is a Cl^- absorbing epithelium without a Cl^- secretory capacity, while our cell line appears to be a Cl^- secreting epithelium. Cl^- secretion has been reported to be inhibited by the presence of furosemide in the serosal bathing media in several secretory epithelia (1–6). The absorptive and secretory capacity of different epithelia may, therefore, depend on the localization of the Na^+,K^+,Cl^- cotransport to either the brush border or basolateral membrane. Studies in other epithelia which secrete Cl^-, e.g., the trachea, cornea, ciliary body, or shark rectal gland, have produced findings that are also compatible with our proposed mechanism for Cl^- secretion, and suggest that similar Cl^- transport mechanisms may be shared by many Cl^- secreting epithelia (39–47).

There were some differences between the effects of VIP on transepithelial electrolyte fluxes across the T_{84} cell monolayers to that previously reported using isolated intestinal preparations (22, 23). Firstly, the graded dose effect of VIP on the T_{84} monolayers was at least 10–100-fold more potent, as compared to that reported previously with isolated intestine. The increased sensitivity to VIP may be a result of the monolayer presenting no barrier to VIP, except the relatively thin and porous collagen-coated Nucleopore filter; thus, more VIP may be reaching the basolateral membrane where the receptors are located (48). Indeed, we have demonstrated a close correlation between ^{125}I-VIP binding, cAMP production, and the increase in I_{sc} in response to VIP (49). In the isolated intestine, VIP, a

relatively large molecule with a molecular weight of 3326, may have difficulty penetrating the lamina propria and the remaining muscle layers. Another postulate is that isolated intestine may be presensitized by endogenous VIP, which is estimated to be ~100–200 pmol/g intestine (50). Support for this idea is the fact that normally isolated intestine always exhibits a relatively high spontaneous PD and I_{sc}. If our monolayer is similar to the normal epithelial cells, its spontaneous PD and I_{sc}, which are near zero, may be a result of the monolayer not being exposed to endogenous VIP and other peptide hormones or neurotransmitters. Secondly, the lack of a VIP effect on transcellular Na^+ transport contrasts to the observations with isolated intestine and is likely a result of the lack of paracellular transport of Na^+ which would be expected to increase after the stimulated Cl^- secretion. This may be due to the very high transepithelial resistance of the T_{84} cell monolayers which is ~1.5 kΩ/cm^2. It should be pointed out that although isolated intestine has a lower overall transepithelial resistance, it is likely that its resistance varies greatly from the villous to the crypt region, with the low resistance region being limited to a small area of the crypt (51). Our results suggest that Cl^- is the primary ion being secreted by the cell upon VIP stimulation. They also suggest that the process of Cl^- secretion is transcellular. The increase in conductance with selective increase in bidirectional flux of Cl^- is likely a result of the opening of Cl^- transport pathways, e.g., Cl^- channels, which can catalyze transcellular Cl^- flux in both directions. A selective increase in paracellular Cl^- fluxes, as suggested in some other studies (52–56), is also compatible with the results, although it is difficult to explain why unidirectional Na^+ flux did not change. Under normal circumstances, the negative electrical charge of the tight junction area should allow better movement of a positively charged ion across the junction.

Regulatory mechanisms involved in VIP-induced Cl^- secretion remain to be elucidated. We have recently reported that VIP increases intracellular cAMP in this cell line (49). Another human colonic carcinoma cell line, $CaCO_2$, has also been reported to secrete Cl^- in response to dibutyryl cAMP; however, the magnitude of the response is small (57). The findings described in this study suggest that VIP increases the coupled entry of Na^+, K^+, and Cl^- across the basolateral membrane, but the study does not indicate whether this coupled entry step is primarily regulated by VIP or cAMP. Indeed, bumetanide-sensitive Rb^+ transport does not appear to be regulated by VIP in this cell line (58). VIP may well regulate other ion transport pathways which result in an increase in the Na^+,K^+,Cl^- cotransport activity. For example, an increase in brush border Cl^- permeability via Cl^- channels will lower cellular Cl^-, thus creating a favorable ion gradient for net uptake by the Na^+,K^+,Cl^- cotransport system. Therefore, Na^+,K^+,Cl^- cotransport on the basolateral membrane, which is involved in VIP-induced Cl^- secretion in the T_{84} cells, may or may not be the pathway primarily activated in VIP's action. The increase in Cl^- permeability has been observed with cAMP mediated secretion in a variety of epithelia (39–41), and may serve as the primary process regulated by VIP, with the increase in Na^+,K^+,Cl^- cotransport activity being secondary. Recently, using the T_{84} cells, we have observed the regulation by VIP of a Ba^{++} sensitive K^+ efflux pathway on the basolateral membrane (58, 59); we have also demonstrated that this K^+ efflux pathway is intimately involved in VIP-induced Cl^- secretion (59). It is possible that only the Cl^- or K^+ channels may be the primary process activated by VIP; alternatively, both channels may be activated simultaneously and independently. The interrelationship of these two transport pathways and the Na^+,K^+,Cl^- cotransport in VIP-induced secretion remains to be clarified.

The cultured T_{84} cell monolayers, besides serving as a model for the study of the mechanism of transepithelial Cl^- secretion and its regulation, may also serve as an intestinal secretory model system for other types of investigation. Utilization of the cell line may make it possible to investigate the interaction of other cellular functions with the epithelium, especially those related to the electrolyte transport processes. A few possible applications of the cell line include the study of intestinal secretion in response to mast cell and/or lymphocytes and their cell products, or the study of the attachment of microorganisms which induce secretion. The effect of various compounds on the paracellular and transcellular pathways can also be tested. Hopefully, the application of the T_{84} cultured colonic epithelial cell line will allow better understanding of some aspects of the physiology and pathophysiology related to the gastrointestinal system.

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