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Research Article

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Chloride Secretory Mechanism Induced by Prostaglandin E_1 in a Colonic Epithelial Cell Line

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Abstract

Confluent T_{84} monolayers grown on permeable supports and mounted in a modified Ussing chamber secrete chloride (Cl^-) in response to prostaglandin E_1. The threshold stimulation was observed at 10^{-9} M and a maximal effect at 10^{-6} M. Unidirectional flux studies showed an increase in both serosal to mucosal and mucosal to serosal Cl^- fluxes with 10^{-6} M prostaglandin E_1; the increase in serosal to mucosal Cl^- flux exceeded the increase in mucosal to serosal flux, resulting in net Cl^- secretion. Na^+ transport was not affected in either direction and the changes in net Cl^- flux correlated well with the changes in short circuit current. To identify the electrolyte transport pathways involved in the Cl^- secretory process, the effect of prostaglandin E_1 on ion fluxes was tested in the presence of putative inhibitors. Bumetanide was used as an inhibitor for the basolaterally localized Na^+,K^+,Cl^- cotransport system whose existence and bumetanide sensitivity have been verified in earlier studies (Dharmsathaphorn et al. 1984. *J. Clin. Invest.* 75:462–471). Barium was used as an inhibitor for the K^+ efflux pathway on the basolateral membrane whose existence and barium sensitivity were demonstrated in this study by preloading the monolayers with $^{86}Rb^+$ (as a tracer for K^+) and simultaneously measuring $^{86}Rb^+$ efflux into both serosal and mucosal reservoirs. Both bumetanide and barium inhibited the net chloride secretion induced by prostaglandin E_1 suggesting the involvement of the Na^+,K^+,Cl^- cotransport and a K^+ efflux pathways on the basolateral membrane in the Cl^- secretory process. The activation of another Cl^- transport pathway on the apical membrane by prostaglandin E_1 was suggested by Cl^- uptake studies. Our findings indicate that the prostaglandin E_1-stimulated Cl^- secretion, which is associated with an increase in cyclic AMP level, intimately involves (*a*) a bumetanide-sensitive Na^+,K^+,Cl^- cotransport pathway that serves as a Cl^- uptake step across the basolateral membrane, (*b*) the stimulation of a barium-sensitive K^+ efflux mechanism on the basolateral membrane that most likely acts to recycle K^+, and (*c*) the activation of a Cl^- transport pathway on the apical membrane that serves as a Cl^- exit pathway.

Introduction

Prostaglandins and other arachidonic acid metabolites may play an important role in the regulation of ion transport in the intestine. These compounds are synthesized in the intestinal mucosa and appear to be increased in inflammatory bowel disease (1–3). In isolated intestine, prostaglandins cause an increase in water and electrolyte secretion associated with an increase in mucosal cyclic AMP levels (4–7). This study utilized a cultured colonic cell line as a model system to investigate the mechanisms and the transport pathways involved in the chloride (Cl^-) secretory process stimulated by prostaglandin E_1 The advantages of using a cultured cell line result from the uniformity of the model, which contains only a single cell type. This allows elimination of the influence of the preexisting peptide hormones or neurotransmitter. Further, the equal accessibility of both apical and basolateral membranes allows localization of the transport pathways involved in the transport process. We have previously shown that this epithelial cell line becomes very well differentiated when grown to confluency on a permeable support. The cell monolayers have a columnar epithelial appearance and retain many receptor-mediated electrolyte transport processes, including the response to prostaglandin E_1, vasoactive intestinal polypeptide (VIP),[1] carbachol, etc. (8, 9). Several transport pathways have been identified in this cell line and their participation in the Cl^- secretory process induced by VIP, calcium ionophore A23187, or carbachol has been demonstrated (10–15). These include the Na^+,K^+,Cl^- cotransport and one or several K^+ efflux pathways on the basolateral membrane, together with and a Cl^- conductance pathway on the apical membrane. Because the response to prostaglandin E_1 in this cell line resembles that observed in the intact intestine of animals and humans, we have chosen this cell line as a model system to study the mechanism of Cl^- secretion induced by prostaglandin E_1.

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 of $NaHCO_3$, 40 mg of penicillin, 8 mg of ampicillin, and 90 mg of streptomycin per liter, and 5% newborn calf serum. 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 as reported earlier (10).

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 7 d prior to 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. (16), consisting of a rat tail collagen-coated polycarbonate Nuclepore filter (Nuclepore Corp., Pleasanton, CA, 5-μm pore size), glued to one open end of a Lexan ring. Crude rat tail

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1. *Abbreviations used in this paper:* I_{sc}, short circuit current; PD, potential difference; VIP, vasoactive intestinal polypeptide.

collagen was solubilized by dissolving 1 g of rat tail tendons in 100 ml of 1% acetic acid. Further steps in preparation of rat tail collagen and procedures for collagen coating Nuclepore filters were as described by Cereijido et al. (17). 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 et al. (18). Mucosal and serosal reservoirs contained identical volumes of oxygenated Ringer's solution (pH 7.4, at 37°C), containing (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 (W-P Instruments, Inc., New Haven, CT) with $Ag/AgCl_2$ electrodes. Tissue conductance 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 $r \geq 0.85$, $P < 0.005$).

$^{86}Rb^+$ efflux studies. For this set of experiments, monolayers were grown on collagen-coated Nuclepore filters, under conditions identical to those used in the ion flux studies described above. Prior to use, the monolayers were equilibrated overnight with $^{86}Rb^+$ by adding 0.35 μCi/ml $^{86}Rb^+$ to the culture media (~4.3 mM K^+) and sterile filtering. The preincubated monolayers were washed rapidly in two changes of Ringer's solution, mounted in the Ussing chambers, and voltage clamped as described earlier. The washing and mounting procedure took ~15 s. Serial samples were taken simultaneously from the serosal and mucosal reservoirs (every 8 min for this study) with buffer replacement. 3 mM $BaCl_2$ or 0.3 mM bumetanide was added at 20 min as indicated. 1 μM prostaglandin E_1 was added at 40 min. All additions were made to both the mucosal and serosal reservoirs. The data were corrected for sampling volume (0.5 ml of a 5-ml reservoir) and previous sampling loss due to buffer replacement. The results for serosal and mucosal $^{86}Rb^+$ efflux were analyzed independently using the closed three-compartment system with the assumption that $^{86}Rb^+$ acts as a perfect tracer for K^+ and the rate of return flux back into the cell from either compartment was negligible (19). This assumption is reasonable for the time period of the experiment.[2] The apparent rate constants for serosal (k_s) and mucosal (k_m) efflux were determined for each sampling time using the relationships:

$$k_s = \frac{\Delta Q}{\Delta t} \cdot \frac{1}{Po - R - Q};$$

$$k_m = \frac{\Delta R}{\Delta t} \cdot \frac{1}{Po - R - Q},$$

where Po is the initial amount of $^{86}Rb^+$ in the cell, and R and Q are the amount of isotope in the mucosal and serosal compartments, respectively. Po was determined experimentally as the total amount of $^{86}Rb^+$ in replicate tissues and as the total amount in the experimental tissues at the end of the experiment plus the amount in the bathing media. The calculated intracellular K^+ of the monolayers which was estimated to be ~639 nmol (derived from $^{86}Rb^+$ counts of the monolayers as compared to $^{86}Rb^+$ counts in the culture media with [K^+] of 4.3 mM) and extracellular K^+ in the bathing reservoir which was 26 μmol (5 ml of solution with [K^+] of 5.2 mM).

$^{36}Cl^-$ uptake studies. Confluent monolayers were grown on collagen-coated Nuclepore filters of 1.98 cm^2 surface area identical to those utilized in the Ussing chamber studies. The monolayers were washed three times with 2 ml of KCl buffer (containing 140 mM KCl, 20 mM Tris-SO_4, pH 7.5, 1 mM Mg gluconate, and 1 mM Ca gluconate) and incubated for 1.5 h at 37°C in the same buffer. They were then washed three times with 2 ml sucrose buffer (containing 241 mM sucrose, 20 mM Tris-SO_4, pH 7.5, 1 mM Mg gluconate, and 1 mM Ca gluconate) and incubated in the same buffer containing 0.5 mM ouabain and 0.1 mM bumetanide for another hour at room temperature. Both the serosal and mucosal surfaces were so treated. The uptakes were carried out by replacing the preincubation buffer with uptake buffer containing 140 mM Na gluconate, 20 mM Tris-SO_4, pH 7.5, 1 mM Mg gluconate, 1 mM Ca gluconate, 0.5 mM ouabain, 0.1 mM bumetanide, and 1 μCi/ml $^{36}Cl^-$ (final [Cl] = 4.3 mM) with or without 10^{-6} M prostaglandin E_1. This buffer was added to one side of the monolayers, while an identical buffer without $^{36}Cl^-$ was added simultaneously to the opposite side. Uptakes were carried out for 3 min before terminating by dunk-washing in four successive 1-liter containers of $MgSO_4$ sucrose buffer (containing 20 mM Tris-Hepes, 100 mM $MgSO_4$, and 137 mM sucrose).

Extraction and measurement of cAMP. Cells were grown to confluency (7 d) in 35-mm culture plates. To disrupt the tight junctions and allow better access to the basolateral membrane, the cells were washed twice with 2 ml of Ca^{++}-free Ringer's solution containing 10 mM glucose and then incubated at 37°C for 1 h in 1 ml of the same buffer. This solution was aspirated and replaced with 0.7 ml Ringer's solution containing 10 mM glucose, 1.2 mM Ca^{++}, and varying concentrations of prostaglandin E_1 or prostaglandin E_1 and/or the inhibitor for transport pathway of interest. The cells were incubated in these solutions at 37°C in a 5% CO_2 atmosphere, to approximate conditions used in the Ussing chamber studies, for the duration of 15 min. The reaction was stopped with the addition of 1.4 ml of iced 95% ethanol. (We have demonstrated in preliminary studies that practically all of the cyclic AMP was confined to the intracellular compartment and similar results were obtained with monolayers mounted in the Ussing chamber as compared to this method of incubation.) Samples were kept at 4°C for at least 15 min, then centrifuged at 1,500 g for 20 min. Methods then followed those described by Harper and Brooker (20). Briefly, duplicate 25-μl samples of supernatant were added to 100 μl of 50 mM acetate (pH 6.2). Samples were acetylated with 5 μl of a mixture of triethylamine/acetic anhydride, 2:1. 100 μl of ^{125}I-cyclic AMP and 100 μl of cyclic AMP antiserum were added, and the samples incubated at 4°C for 18 h. 2 ml of iced 50 mM acetate (pH 6.2) were added to each tube and samples were centrifuged at 1,500 *g* for 20 min. The supernatant was decanted and the precipitate was counted for radioactivity. A standard curve was done for each assay by adding known amounts of cyclic AMP to buffer solution and processing these standards in the same manner as experimental samples. Representative monolayers were taken for determination of their protein content by the method of Lowry et al. (21) using bovine serum albumin as a standard.

Materials. All radionuclides and cyclic AMP antisera were obtained from New England Nuclear, Boston, MA. Prostaglandin E_1 was purchased from The Upjohn Co., Kalamazoo, MI. Bumetanide was a gift from Dr. P. W. Feit of Leo Pharmaceutical Products, Ballerup, Denmark. Barium chloride dihydrate was purchased from J. T. Baker Chemical Company, Phillipsburg, NJ, and anthracene-9-carboxylic acid from Aldrich Chemical Company, Milwaukee, WI. Stock solutions of bumetanide and anthracene-9-carboxylic acid were made in dimethyl sulfoxide as 10 mM

2. Backflux of $^{86}Rb^+$ into the cells can be ignored for two reasons. First, at all times, the rate constants relating uptake and efflux are a function of the "size" of the compartments, i.e., the total amount of K^+ in each compartment. Because the K^+ contained in the cellular compartment is ~40-fold smaller than either extracellular compartment, then as long as the size of the cellular compartment does not change radically, the rate constant for $^{86}Rb^+$ uptake is 40-fold smaller than the rate constant for efflux. Second, during the times of the experiment, although as much as 60% of the label leaves the cell, the specific activity in either extracellular compartment is still at least 30-fold smaller than that in the cellular compartment. The rate of $^{86}Rb^+$ uptake, which is equal to the product of the rate constant for uptake and the specific activity in the extracellular compartment, is negligibly small (<1/1,000th) compared to the rate of $^{86}Rb^+$ efflux. Therefore, the differential equations describing the three-compartment system (31, 36) can be reduced to those describing simple decay.

and 1 M solutions, respectively, while prostaglandin E_1 was dissolved in ethanol. Preliminary studies indicate that dimethyl sulfoxide up to 50 μl and ethanol up to 25 μl per 5 ml, the amount used to dissolve prostaglandin E_1 or specific inhibitors, did not affect unidirectional Na^+ or Cl^- fluxes.

Statistical analysis. Student's *t* tests and analysis of variance were used as indicated (22).

Results

After 5 d or more in culture, T_{84} cells, grown on permeable, collagen-coated Nuclepore filters appear as a columnar epithelial monolayer with their basolateral membrane firmly attached to the collagen-coated surface, and their apical membrane facing the medium (9). These monolayers maintained a transepithelial resistance of $\sim$1.5 k$\Omega \cdot cm^2$. The collagen-coated Nuclepore membrane, which served as the attachment support for the cells, had a resistance $<4\Omega \cdot cm^2$; thus it contributed insignificantly to the transepithelial resistance. For electrolyte transport studies carried out in the modified Ussing chamber, we have denoted the basolateral membrane side as the serosal side and the apical membrane surface as the mucosal side.

Stimulation of net Cl^- secretion across T_{84} monolayers by prostaglandin E_1. The addition of 10^{-6} M prostaglandin E_1 to the serosal bathing solution caused an immediate increase in short circuit current (I_{sc}) which reached the peak 10–15 min after the addition. Thereafter, the I_{sc} gradually declined, although it remained significantly elevated throughout the study period. Mucosal addition had little or no effect except at high concentrations ($\geq 10^{-6}$ M) where a small increase in I_{sc} was observed (data not shown). The response to prostaglandin E_1 was dose dependent with the threshold stimulation occurring at 10^{-9} M, and maximal response at 10^{-6} M (Fig. 1). Because a maximal response was observed with 10^{-6} M prostaglandin E_1, this concentration was used in all subsequent studies.

Unidirectional flux results are summarized in Table I with the time course of the changes in I_{sc} and net fluxes illustrated in Fig. 2. Net flux and I_{sc} in the absence of added secretagogues remained at or near zero for >100 min (Fig. 2 *A*). After the addition of 10^{-6} M prostaglandin E_1, the increase in net chloride secretion was observed (illustrated by the solid bars in Fig. 2 *B*)

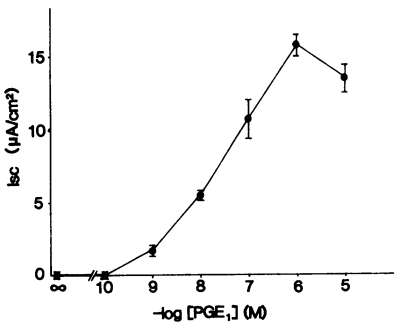

Figure 1. Graded dose effect of prostaglandin E_1 on the changes in I_{sc}. T_{84} monolayers were grown on permeable supports with a surface area of 1.98 cm^2. At the basal state the monolayers has a spontaneous PD and I_{sc} close to 0. Prostaglandin E_1 (PGE_1) were added to the serosal side 35 min after mounting. The I_{sc} values represent the actual I_{sc} 15 min after the addition of prostaglandin E_1 at the concentration indicated and are expressed as the mean±SE of four to six experiments in microamperes per square centimeter. Only one addition was made to each monolayer.

and could totally account for the change in I_{sc}. Changes in the I_{sc} and net Cl^- secretion had an excellent correlation ($r = 0.90$; $P < 0.01$). No significant effects were observed on net Na^+ flux (illustrated by the open bars in Fig. 2 *B*). Examination of the unidirectional fluxes showed no changes in mucosal-to-serosal or in serosal-to-mucosal Na^+ movement after the addition of prostaglandin E_1. In contrast, both serosal-to-mucosal and mucosal-to-serosal Cl^- fluxes increased, with the increase in the serosal-to-mucosal direction being consistently greater, resulting in net Cl^- secretion (Table I).

Inhibition of prostaglandin E_1-stimulated Cl^- secretion by bumetanide. A recent study verified the existence of a basolaterally localized Na^+,K^+,Cl^- cotransport system in the T_{84} cell; the study also demonstrated that bumetanide, a loop diuretic, inhibited the Na^+,K^+,Cl^- cotransport system in this cell line (10). Therefore, bumetanide was used to test the involvement of the Na^+,K^+,Cl^- cotransport system in the Cl^- secretory process. Bumetanide inhibited the action of prostaglandin E_1 in a dose-dependent manner when it was added to the serosal bathing media (Fig. 3). A significant inhibitory effect was observed at 10^{-6} M. The inhibition by bumetanide was not complete, at concentrations as high as 10^{-3} M, $\sim$15% of the peak I_{sc} induced by prostaglandin E_1 persisted. The time course of response to prostaglandin E_1 in the presence of bumetanide was much shorter in duration, as compared to that observed in the absence of bumetanide. The results of unidirectional and net fluxes are summarized in Table I and Fig. 2 *C*. Bumetanide (0.3 mM) selectively reduced the increase in serosal-to-mucosal Cl^- flux stimulated by prostaglandin E_1 resulting in a 80% decrease in net Cl^- secretion with a similar decrease in I_{sc}. Bumetanide by itself had no effect on the basal Na^+ or Cl^- fluxes under our experimental condition. It should be noted that, in the presence of bumetanide, prostaglandin E_1 still increased the transepithelial Cl^- permeability as reflected by the increased bidirectional Cl^- fluxes. The increased transepithelial Cl^- permeability occurred despite the cessation of net Cl^- secretion. The inhibitory effect of bumetanide was reversible; the I_{sc} rose significantly when the monolayers were washed and the bathing media were replaced with media containing 10^{-6} M prostaglandin E_1 (without bumetanide). Bumetanide also reversed the action of prostaglandin E_1 when added after prostaglandin E_1 elicited a response (data not shown). The effect of bumetanide was observed only with serosal application. Mucosal addition of bumetanide at 10^{-5} or less had no effect.

Inhibition of prostaglandin E_1-stimulated Cl^- secretion by barium. Barium, a putative K^+ channel blocker, has been found to inhibit Cl^- secretion in a number of epithelia (23, 24). We found that $BaCl_2$ inhibited the increase in I_{sc} induced by prostaglandin E_1 in a dose-dependent manner when added serosally (Fig. 3), while mucosal addition had little or no effect. A threshold inhibition is observed with 10^{-4} M. At a concentration of 10^{-3} M, >90% of the effect of prostaglandin E_1 was inhibited. The results of unidirectional and net fluxes of Na^+ and Cl^-, summarized in Table I and Fig. 2 *D*, confirm that barium inhibited net Cl^- secretion. Similar to bumetanide, barium by itself had no effect on basal I_{sc} or on unidirectional Na^+ and Cl^- fluxes under our experimental condition. Also similar to bumetanide, barium did not inhibit the increase in Cl^- permeability induced by prostaglandin E_1, in spite of its ability to inhibit net Cl^- secretion. Unidirectional Cl^- fluxes in both directions were augmented by prostaglandin E_1 regardless of the presence of barium. The inhibitory effect of barium on the action of prostaglandin

Table I. Unidirectional Sodium and Chloride Fluxes across T_{84} Cell Monolayers: Inhibition of Prostaglandin-induced Chloride Secretion by Inhibitors of Transport Pathways

Experimental groups	Addition	Flux period	Flux $J^{Na}_{s\to m}$	$J^{Na}_{m\to s}$	J^{Na}_{net}	$J^{Cl}_{s\to m}$	$J^{Cl}_{m\to s}$	J^{Cl}_{net}	I_{sc}	G
			$\mu eq/h \cdot cm^2$	$\mu eq/h \cdot cm^2$	$\mu eq/h \cdot cm^2$	$\mu eq/h \cdot cm^2$	$\mu eq/h \cdot cm^2$	$\mu eq/h \cdot cm^2$	$\mu eq/h \cdot cm^2$	mS/cm^2
Control ($n = 6$)	No addition	1	0.37±0.05	0.50±0.09	0.13±0.06	0.44±0.07	0.54±0.11	0.10±0.11	0.03±0.01	0.75±0.11
	No addition	2	0.41±0.08	0.50±0.09	0.10±0.03	0.55±0.10	0.56±0.11	0.01±0.05	0.02±0.01	0.82±0.13
	No addition	3	0.39±0.07	0.52±0.08	0.13±0.07	0.51±0.09	0.61±0.08	0.10±0.09	0.03±0.01	0.80±0.12
Prostaglandin E_1 ($n = 6$)	No addition	1	0.42±0.09	0.42±0.06	0.0±0.07	0.52±0.09	0.49±0.07	−0.03±0.09	0.02±0.01	0.72±0.09
	No addition	2	0.42±0.08	0.41±0.05	−0.01±0.08	0.55±0.09	0.49±0.07	−0.06±0.08	0.04±0.01	0.76±0.09
	Prostaglandin E_1	3	0.40±0.06	0.40±0.06	0.0±0.06	1.88±0.08*	1.10±0.08*	−0.78±0.06*	0.64±0.05*	0.98±0.05*
Bumetanide and prostaglandin E_1 ($n = 5$)	No addition	1	0.45±0.14	0.44±0.11	−0.01±0.05	0.56±0.14	0.47±0.14	−0.09±0.04	0.01±0.01	0.73±0.20
	Bumetanide	2	0.51±0.10	0.46±0.10	−0.05±0.05	0.58±0.15	0.47±0.11	−0.11±0.06	0.01±0.01	0.97±0.31
	Bumetanide and prostaglandin E_1	3	0.66±0.23	0.58±0.17	−0.08±0.10	1.10±0.29*‡	0.96±0.25*	−0.16±0.03‡	0.16±0.03*‡	1.43±0.38*
$BaCl_2$ and prostaglandin E_1 ($n = 6$)	No addition	1	0.39±0.07	0.41±0.08	0.02±0.05	0.45±0.10	0.42±0.05	−0.03±0.07	0.01±0.01	0.77±0.16
	$BaCl_2$	2	0.43±0.07	0.38±0.05	−0.05±0.04	0.51±0.10	0.48±0.07	−0.03±0.05	0.01±0.01	0.71±0.13
	$BaCl_2$ and prostaglandin E_1	3	0.37±0.09	0.41±0.06	0.04±0.06	1.19±0.21*‡	1.14±0.31*	−0.05±0.13‡	0.11±0.01*‡	0.84±0.10
Anthracene-9-COOH and prostaglandin E_1 ($n = 6$)	No addition	1	0.36±0.08	0.44±0.07	0.08±0.05	0.47±0.09	0.48±0.10	0.01±0.03	0.03±0.01	0.71±0.11
	Anthracene-9-COOH	2	0.27±0.04	0.33±0.04	0.06±0.03	0.41±0.07	0.39±0.04	−0.03±0.05	0.05±0.01	0.68±0.06
	Anthracene-9-COOH and prostaglandin E_1	3	0.49±0.11	0.42±0.04	−0.07±0.07	0.85±0.12*‡	0.65±0.06‡	−0.12±0.09‡	0.20±0.04*‡	0.87±0.09

Results are expressed as mean±SE; the number of paired monolayers for each experimental group (n) is indicated in parenthesis. Periods 1, 2, and 3 represent the average to two consecutive 10-min flux periods starting 17 min, 47 min, and 77 min after mounting the addition isotope to the Ussing chambers, respectively. Additions, if made, were at 40 and 70 min for the first and second addition, respectively. The concentration of prostaglandin E_1 was 1 μM; bumetanide, 0.3 mM; $BaCl_2$, 3 mM; and anthrancene-9-carboxylic acid, 10 mM. * Significant differences ($P < 0.05$) as compared to controls. ‡ Significant differences as compared to prostaglandin E_1 experiments. Abbreviations: G, conductance; J, flux; m, mucosal; s, serosal.

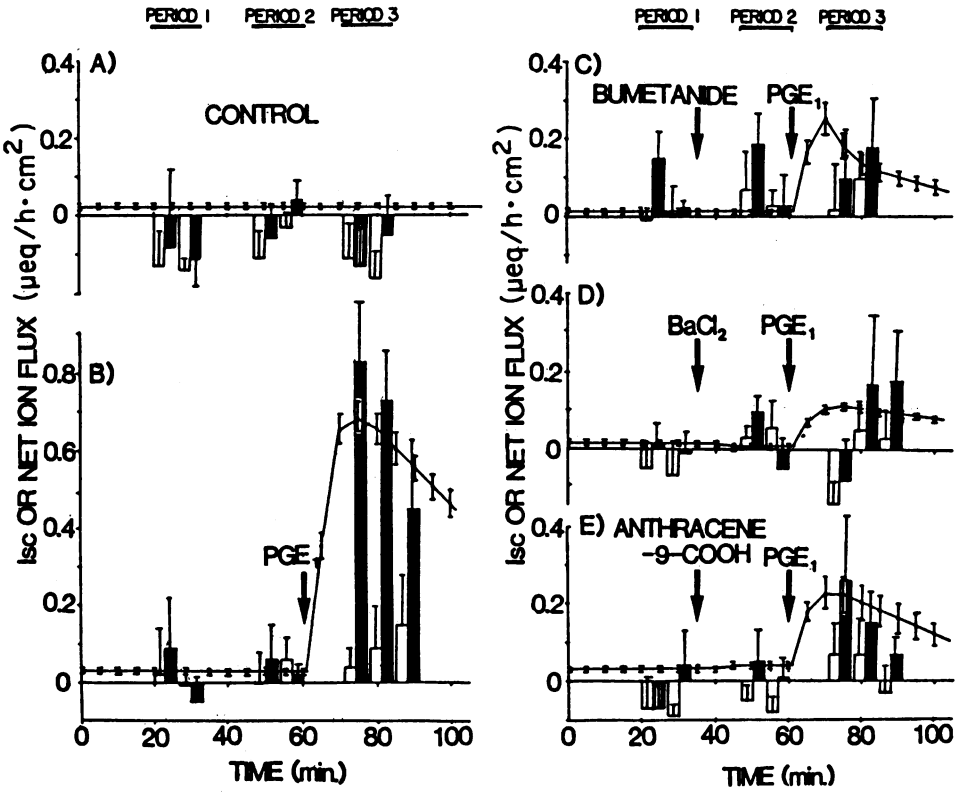

Figure 2. Time course of net Na^+ and Cl^- transport and correlation of net Cl^- secretion with the I_{sc}. (*A*) Control; (*B*) 10^{-6} M prostaglandin E_1 (PGE_1) alone; (*C*) 10^{-6} M prostaglandin E_1 in the presence of 0.1 mM bumetanide; (*D*) 10^{-6} M prostaglandin E_1 in the presence of 3 mM $BaCl_2$; (*E*) 10^{-6} M prostaglandin E_1 in the presence of 10 mM anthracene-9-carboxylic acid. The means±SE from the same paired monolayers in Table I, which summarizes unidirectional flux rates, are illustrated. The circles and line represent the I_{sc}; solid bars represent net Cl^- transport, and open bars represent net Na^+ transport measured over 10-min intervals. I_{sc} and flux rates are expressed in microequivalents per hour per square centimeter. Values above the line indicate net secretion, and values below the line, net absorption. The time periods covered by periods 1, 2, and 3 in Table I are indicated by lines at the top of the figure.

E_1 was reversible. Barium also reversed the action of prostaglandin E_1 when barium was added after prostaglandin E_1 elicited a response (data not shown).

Evidence for prostaglandin E_1-induced K^+ efflux on the basolateral membrane and its inhibition by barium. In a variety of epithelia, agents that increased Cl^- secretion also caused an increase in basolateral membrane conductance to K^+. In order to verify the existence of a K^+ efflux pathway and test whether barium or bumetanide inhibited this process, monolayers were preloaded with $^{86}Rb^+$ (as a tracer for K^+) and mounted in the Ussing chamber. This method allowed the measurement of $^{86}Rb^+$ efflux across both the apical and basolateral surfaces while Cl^- secretion was simultaneously monitored. The apparent first-order rate constants, along with the mean I_{sc} (which reflected the net Cl^- secretion) and conductance of the monolayer for each of three time intervals in which additions were made, are shown in Table II. At the basal state, the rate of $^{86}Rb^+$ efflux into the mucosal bath was ~20-fold smaller than that into the serosal bath. Basal $^{86}Rb^+$ efflux rates were not decreased by 3 mM barium or 0.3 mM bumetanide. The addition of prostaglandin E_1 (which was added to both sides) increased the rate of $^{86}Rb^+$ efflux into the serosal bath by approximately twofold, whereas the rate of $^{86}Rb^+$ efflux into the mucosal bath was not affected. The increase in basolateral membrane $^{86}Rb^+$ efflux rate was totally inhibited by prior addition of 3 mM $BaCl_2$ to the bathing media. Net Cl^- secretion induced by prostaglandin E_1 in these experiments was also inhibited by $BaCl_2$ as indicated by the I_{sc}. Somewhat unexpectedly, 0.3 mM bumetanide also inhibited $^{86}Rb^+$ efflux induced by prostaglandin E_1.

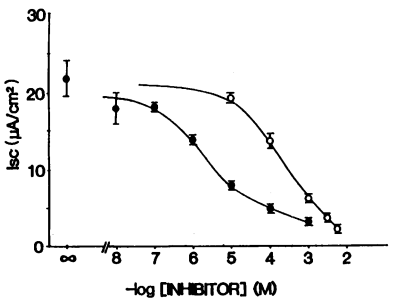

Figure 3. Graded dose effect of bumetanide and barium for inhibition of prostaglandin E_1-stimulated I_{sc}. Varying concentrations of bumetanide (●) or barium (○) were added at 30 min after mounting. After that, 10^{-6} M prostaglandin E_1 was added to the serosal side of the monolayers mounted in the Ussing chamber at the concentration indicated at 50 min (20 min after the addition of the inhibitors). The means±SE of the peak I_{sc} observed within 15 min after addition are expressed in microamperes per square centimeter. Three to four experiments were carried out in each group, only one concentration of the inhibitor was tested in each monolayer.

Inhibition of prostaglandin E_1-stimulated Cl^- secretion by anthracene-9-carboxylic acid. Anthracene-9-carboxylic acid was used in this study as a possible Cl^- channel blocker, as reported by Welsh (25). Results summarized in Table I and Fig. 2 *E* demonstrate that 10^{-2} M anthracene-9-carboxylic acid inhibited the effect of prostaglandin E_1 on unidirectional and net Cl^- fluxes. Partial inhibitory action was observed with 10^{-3} M. Both mucosal and serosal addition were equally effective. However, as noted below, anthracene-9-carboxylic acid also inhibited the increase in cyclic AMP production caused by prostaglandin E_1. This nonspecific action of anthracene-9-carboxylic acid complicated the interpretation of the results.

Sidedness of prostaglandin E_1-stimulated $^{36}Cl^-$ uptake. Although the study utilizing a putative Cl^- channel blocker, anthracene-9-carboxylic acid, was inconclusive, unidirectional Na^+ and Cl^- flux measurements clearly demonstrated a selective in-

Table II. $^{86}Rb^+$ Efflux from T_{84} Monolayers Mounted in Ussing Chambers

	Period	Addition	$^{86}Rb^+$ efflux rate constant		I_{sc}	G
			Mucosal	Serosal		
			h^{-1}	h^{-1}	$\mu A/cm^2$	mS/cm^2
Control	1	No addition	0.040±0.014	0.473±0.019	1.5±0.3	0.7±0.1
	2	No addition	0.046±0.012	0.587±0.019	0.9±0.2	0.5±0.1
	3	No addition	0.034±0.001	0.588±0.027	0.9±0.2	0.4±0.1
Bumetanide	1	No addition	0.046±0.019	0.569±0.048	1.4±0.6	0.7±0.2
	2	Bumetanide	0.037±0.014	0.533±0.045	0.5±0.1	0.5±0.1
	3	Bumetanide	0.043±0.006	0.671±0.063	0.5±0.1	0.5±0.1
$BaCl_2$	1	No addition	0.034±0.005	0.492±0.072	1.4±0.2	0.6±0.0
	2	Ba^{++}	0.025±0.006	0.494±0.051	0.5±0.1	0.5±0.1
	3	Ba^{++}	0.041±0.009	0.578±0.082	0.5±0.1	0.4±0.1
Prostaglandin E_1	1	No addition	0.031±0.002	0.486±0.050	0.9±0.2	0.6±0.1
	2	No addition	0.034±0.006	0.599±0.050	0.5±0.1	0.4±0.1
	3	Prostaglandin E_1	0.071±0.009	1.218±0.135*	20.4±1.9*	0.7±0.1
Bumetanide and prostaglandin E_1	1	No addition	0.031±0.007	0.479±0.066	1.4±0.2	0.6±0.1
	2	Bumetanide	0.026±0.007	0.564±0.097	0.9±0.2	0.5±0.1
	3	Bumetanide and prostaglandin E_1	0.056±0.006	0.688±0.061	4.2±0.6	0.7±0.1
$BaCl_2$ and prostaglandin E_1	1	No addition	0.029±0.002	0.574±0.054	1.4±0.2	0.6±0.1
	2	Ba^{++}	0.020±0.005	0.578±0.058	1.4±0.2	0.5±0.1
	3	Ba^{++} and prostaglandin E_1	0.030±0.002	0.637±0.034	3.7±0.2	0.7±0.1

The results were analysed during three time intervals. Period 1, 0–24 min; period 2, 24–40 min; and period 3, 48–80 min. Within each time frame, the points were fit to straight lines by the method of least squares. The slope of the line, representing the apparent constant for $^{86}Rb^+$ efflux is shown in the table for both apical (mucosal) and basolateral (serosal) effluxes together with the changes in I_{sc} and conductance (G). The results are the mean±SE of three experiments. The concentration of bumetanide was 0.3 mM; $BaCl_2$, 3mM, and prostaglandin E_1, 1 μM. * Significant differences ($P < 0.05$).

crease in Cl^- flux across T_{84} monolayers in both directions in response to prostaglandin E_1. These findings suggest that prostaglandin E_1 activates a Cl^- transport pathway. Unilateral $^{36}Cl^-$ uptakes were carried out as another independent method to confirm the opening of a Cl^- transport pathway by prostaglandin E_1 and to localize this pathway to the apical or basolateral membrane. The applicability of this $^{36}Cl^-$ uptake method has been demonstrated in a recent study (12). We found that VIP, whose action is very similar to prostaglandin E_1, stimulated $^{36}Cl^-$ efflux as well as $^{36}Cl^-$ uptake via a Cl^- transport pathway which was apically localized. This Cl^- transport pathway in the T_{84} cells was not inhibited by bumetanide or 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid but was inhibited by *n*-phenyl anthranilic acid and most likely represents the "Cl^- channel" reported in the literature. $^{36}Cl^-$ uptake in the presence of bumetanide reflects the activity of this pathway. The results shown in Fig. 4 indicated that, at the basal state, both apical and basolateral membrane Cl^- permeability were observed; however, the increase in Cl^- permeability activated by prostaglandin E_1 was localized to the apical membrane alone. This increase in Cl^- permeability on the apical membrane by prostaglandin E_1 was not inhibited by bumetanide, and most likely represented the site for electrogenic Cl^- exit in the Cl^- secretory process stimulated by prostaglandin E_1.

Effect of prostaglandin E_1 and specific inhibitors on cyclic AMP production. To test whether the action of prostaglandin E_1 was associated with an increase in cyclic AMP levels, we measured the cyclic AMP in response to prostaglandin E_1 (Table III). Cellular cyclic AMP production increased dramatically when the T_{84} cells were incubated with prostaglandin E_1, confirming that the action of prostaglandin E_1 in the T_{84} cells was similar to those reported for isolated intestine. To test whether the inhibitory effects of bumetanide, barium, and anthracene-9-carboxylic acid were independent of the cyclic AMP levels, cellular cyclic AMP production in response to 10^{-6} M prostaglandin E_1 were measured in the presence and absence of these inhibitors. The presence of bumetanide and $BaCl_2$, at the concentrations that inhibit Cl^- secretion, did not prevent the increase in cyclic AMP produced by prostaglandin E_1. Bumetanide did reduce the level of cyclic AMP produced in response to prostaglandin E_1. However, a similarly low level of cyclic AMP (stimulated by a lower concentration of prostaglandin E_1) readily caused Cl^- secretion, suggesting that the inhibitory effect on Cl^- secretion occurs at a step distal to the production of cyclic AMP. However, cyclic AMP production was totally inhibited by anthracene-9-carboxylic acid and *n*-phenyl anthranilic acid, indicating that these compounds were not specific.

Discussion

The use of cultured cell lines as model systems may allow better elucidation of the cellular mechanisms involved in the electrolyte

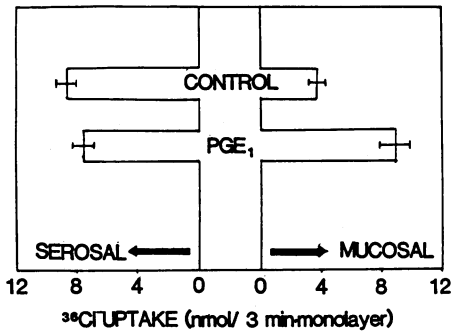

Figure 4. Confluent monolayers grown on permeable supports similar to those utilized in the Ussing chamber studies were preincubated in KCl and sucrose-ouabain buffer as described in Methods. Both the serosal and mucosal surfaces were so treated. $^{36}Cl^-$ uptake was carried out in uptake buffer consisting of 140 mM Na gluconate, 20 mM Tris-Hepes, 1 mM Ca gluconate, 1 mM Mg gluconate, 0.5 mM ouabain, 0.1 mM bumetanide, 1 μCi/ml ^{36}Cl (final concentration = 4.3 mM), with or without 10^{-6} M prostaglandin E_1 (PGE_1). An identical buffer, without added $^{36}Cl^-$, was added simultaneously to the opposite side. Uptakes were carried out for 3 min at room temperature before terminating by dunk-washing in four successive 1-liter containers of Mg gluconate-sucrose buffer. Results are the means±SE of triplicate determinations.

secretory processes. Cultured cells possess a number of distinct advantages and disadvantages (26, 27). The advantages stem from the homogeneity of the cultured cell population which, in contrast to intact epithelial tissues, contains only a single cell type. Among the disadvantages is the concern that cultured cell lines, which lack certain mechanisms of normal growth control, may also be different from normal cells in other aspects. Thus, cultured cell lines should be useful for the study of certain physiologic mechanisms only if they possess similar properties as compared to those observed in intact organs.

Table III. Cyclic AMP Production by T_{84} Cells in Response to Prostaglandin E_1

Experiment (15 min)	cAMP level
	pmol per mg protein
Control	5±1
10^{-8} M PGE_1	14±1*
10^{-7} M PGE_1	25±3*
10^{-6} M PGE_1	100±4*
10^{-6} M PGE_1 + DMSO	142±6*
10^{-6} M PGE_1 + 0.3 mM bumetanide	44±2*‡
10^{-6} M PGE_1 + 0.1 mM bumetanide	53±4*‡
10^{-6} M PGE_1 + 3 mM $BaCl_2$	109±10*
10^{-6} M PGE_1 + 10 mM anthracene-9-carboxylic acid	6±1‡
10^{-6} M PGE_1 + 10 mM *n*-phenyl anthranilic acid	10±1‡

Cyclic AMP production in response to prostaglandin E_1 (PGE_1) in T_{84} cells in the presence and absence of specific inhibitors. T_{84} cells were preincubated for 15 min with the indicated agents as described in Methods. The results are expressed as mean±SE of four experiments in picomoles per milligram of protein. Bumetanide, barium, or anthracene-9-carboxylic acid by itself did not affect basal cyclic AMP production. Dimethyl sulfoxide (DMSO) up to 10 μl/ml, the amount needed to dissolve anthracene-9-carboxylic acid and *n*-phenyl anthranilic acid did not inhibit cyclic AMP production induced by PGE_1.
* Significant difference as compared to control.
‡ Significant difference as compared to results obtained with 10^{-6} M PGE_1.

In this report, we have demonstrated that T_{84} monolayers responded to prostaglandin E_1 in a manner similar to those occurring with isolated intestine of animals and humans (4–7); and therefore, the cell line may serve as a useful model system to study the mechanism of action of prostaglandin E_1. In the T_{84} cells, prostaglandin E_1 caused an increase in both serosal-to-mucosal and mucosal-to-serosal Cl^- movements with the increase in the serosal-to-mucosal direction being consistently greater, resulting in net Cl^- secretion. The only differences as compared to isolated intestine include (*a*) the fact that Na^+ fluxes were not also affected by prostaglandin E_1, and (*b*) the graded dose effect of prostaglandin E_1 was at least 10–100-fold more potent, as compared to that reported previously with isolated intestine. The finding that Na^+ fluxes did not change actually is similar to that observed in isolated colon and support the belief that Cl^- is the ion primarily regulated by prostaglandin E_1. The lack of absorptive cells and our ability to short circuit the monolayers better, together with the high resistance of the monolayers, might make passive paracellular movement of Na^+ more restricted as compared to isolated small intestine. Regarding the increased sensitivity to prostaglandin E_1, this may be a result of the monolayer presenting less barrier to prostaglandin E_1, as compared to isolated intestine. Because the only barrier was a relatively thin and porous collagen-coated Nuclepore filter, more prostaglandin E_1 might reach the basolateral membrane where it could activate the Cl^- secretory function of the cells. A similar increase in sensitivity of T_{84} cells to other secretagogues, such as VIP, has been observed in an earlier study (10). Another postulate is that isolated intestine might be presensitized or down-regulated by endogenous prostaglandin E_1. Support for this idea is the finding that indomethacin, which blocks endogenous prostaglandin synthesis, increased the sensitivity of isolated intestine to prostaglandins by about 10-fold (6). The relatively transient response to prostaglandin E_1 is also compatible with a possible down-regulation.

Measurement of cyclic AMP confirmed that the effect of prostaglandin E_1 was associated with an increase in cellular cyclic AMP. A similar increase in cyclic AMP production was also observed with VIP, which caused Cl^- secretion identical to prostaglandin E_1 in the T_{84} cell line (10, 28). The results suggest that both prostaglandin E_1 and VIP cause net Cl^- secretion via the same cyclic AMP-mediated mechanism, similar to those observed in isolated intestine (7, 29). Our studies did not address the question why the response to prostaglandin E_1 appeared to be less sustained as compared to VIP. One explanation is that the level of cyclic AMP maximally produced by prostaglandin E_1 was lower.

The major aims of our study were to identify the transport pathways involved in the Cl^- secretory process activated by prostaglandin E_1. The homogeneity of our cell model together with the accessibility to both apical and basolateral membrane provides unique advantages for this type of study. We selected to test the involvement of three transport pathways, including the Na^+,K^+,Cl^- cotransport pathway, the K^+ channels, and the Cl^- channels, because other studies in the T_{84} cell imply their involvement in the Cl^- secretory process induced by VIP, carbachol, and calcium ionophore A23187 (10–15).

By the use of bumetanide, we have implicated the involvement of the Na^+,K^+,Cl^- cotransport pathway on the basolateral membrane. The results also suggest that this transport pathway serves as the Cl^- uptake step in the Cl^- secretory process, and is required to sustain the action of prostaglandin E_1. Na^+,K^+,Cl^- cotransport has been observed in a variety of cells and is inhibitable by bumetanide (30–37). In a recent study, the existence of a bumetanide-sensitive Na^+,K^+,Cl^- cotransport pathway has been demonstrated in the T_{84} cells and localized to the basolateral membrane (10). In the present study, we have shown that serosal application of bumetanide inhibited net Cl^- secretion induced by prostaglandin E_1. That bumetanide inhibition occurred only when it was present on the serosal surface is compatible with the notion that the cotransport pathway serves as a Cl^- uptake step across the basolateral membrane, and that this pathway is localized to the basolateral membrane (10). It should be noted that inhibition by bumetanide was incomplete. This might be due to incomplete blockage of the cotransport system by bumetanide. Alternate explanations include the possibility that Na^+,K^+,Cl^- cotransport might not be directly regulated by prostaglandin E_1, but rather serves to bring Cl^- into the cell in the presence of a favorable electrochemical gradient, i.e., after Cl^- is secreted from the cells. The sensitivity of Cl^- secretion to loop diuretic has been observed in a variety of epithelia (38–44) and suggests that similar Cl^- uptake mechanisms may be shared by many epithelial cell types that secrete Cl^-.

A sequence of studies confirms the critical role of K^+ recycling in the Cl^- secretory process. Firstly, by measuring Na^+ and Cl^- fluxes, we have observed that Cl^- secretion was inhibited by serosal application of barium. Secondly, utilizing $^{86}Rb^+$ efflux technique, we demonstrated the existence of a K^+ efflux pathway on the basolateral membrane. Thirdly, by the same technique, we showed that the activity of this K^+ efflux pathway was stimulated by prostaglandin E_1. Finally, we proved that barium inhibited the increase in K^+ efflux by prostaglandin E_1 across the basolateral membrane. Participation of K^+ channels in the Cl^- secretory process has been suggested in a number of studies. Barium, an inhibitor for a number of K^+ channels (45–50), has been found to inhibit electrogenic Cl^- secretion in frog and piglet gastric mucosa (23, 24). Increased basolateral membrane permeability has also been observed, and attributed to K^+, during Cl^- secretion in other epithelial cells (51, 52).

Activation of a Cl^- exit pathway by prostaglandin E_1 has been implicated both indirectly and directly in the present study. The persistent increase in bidirectional Cl^- fluxes by prostaglandin E_1 regardless of the presence of bumetanide or barium indirectly suggests the activation of another Cl^- transport pathway besides the Na^+,K^+,Cl^- cotransport system. Utilizing $^{36}Cl^-$ uptake techniques, we detected a Cl^- transport pathway on the apical membrane which is insensitive to bumetanide and stimulated by prostaglandin E_1. Under the same experimental conditions, this Cl^- transport pathway in the T_{84} cells has been demonstrated to function in both uptake and efflux directions (12). Verification that this Cl^- transport pathway represented the Cl^- channel was beyond the scope of this study, as our experimental techniques could not directly differentiate between a "channel" and a "carrier." The findings in this study compare favorably with those observed in other epithelia, i.e., the intestine, trachea, and gallbladder, which suggests that cyclic AMP or prostaglandin E_1 increase Cl^- permeability of the apical membrane (51–54). In this study, we have observed that the Cl^- secretory process was inhibited by anthracene-9-carboxylic acid similarly to Cl^- secretion in the trachea (25). However, the finding that anthracene-9-carboxylic acid inhibited cyclic AMP production, which mediated the Cl^- secretory process, complicated the results. Our study indicates the need for cautious interpretation of the action of this putative Cl^- channel blocker.

The interrelation between these transport pathways in the Cl^- secretory process remains to be fully elucidated. Na^+,K^+,ATPase pump activity appeared to be needed for the net Cl^- secretory process, as ouabain effectively inhibited net Cl^- secretion (data not shown). Our studies suggest that the driving force for net Cl^- secretion, provided by the Na^+,K^+,ATPase pump, is closely associated with the Na^+,K^+,Cl^- cotransport and the K^+ efflux pathways, all located basolaterally. Inhibition of Na^+,K^+,Cl^- cotransport or K^+ recycling by bumetanide or barium, respectively, resulted in an inhibition of net Cl^- secretion. Our studies also provide evidence that prostaglandin E_1 activates or opens a Cl^- transport pathway, presumably the Cl^- channel on the apical membrane, and suggest that this activation occurs rapidly and independently of the Na^+,K^+,Cl^- cotransport and K^+ efflux mechanisms. An opening of the Cl^- channels, which by itself may not increase Cl^- secretion, allowed an effective Cl^- secretory process to occur in the presence of the proper driving force. In addition, other factors such as cell volume and electrical properties of the cell may also be important.

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