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Research Article

Serosal application of carbachol to T84 cell monolayers mounted in an Ussing chamber caused an immediate increase in short circuit current (Isc) that peaked within 5 min and declined rapidly thereafter, although a small increase in Isc persisted for approximately 30 min. The increase in Isc was detectable with 1 microM carbachol; half-maximal with 10 microM carbachol; and maximal with 100 microM carbachol. Unidirectional Na+ and Cl- flux measurements indicated that the increase in Isc was due to net Cl- secretion. Carbachol did not alter cellular cAMP, but caused a transient increase in free cytosolic Ca2+ ([Ca2+]i) from 117 +/- 7 nM to 160 +/- 15 nM. The carbachol-induced increase in Isc was potentiated by either prostaglandin E1 (PGE1) or vasoactive intestinal polypeptide (VIP), agents that act by increasing cAMP. Measurements of cAMP and [Ca2+]i indicated that the potentiated response was not due to changes in these second messengers. Studies of the effects of these agents on ion transport pathways indicated that carbachol, PGE1, or VIP each increased basolateral K+ efflux by activating two different K+ transport pathways on the basolateral membrane. The pathway activated by carbachol was not sensitive to barium, while that activated by PGE1 or VIP was; furthermore, their action on K+ efflux are additive. Our study indicates that carbachol causes Cl- secretion, and that this action […]

Mechanism of Chloride Secretion Induced by Carbachol in a Colonic Epithelial Cell Line

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Abstract

Serosal application of carbachol to T_{84} cell monolayers mounted in an Ussing chamber caused an immediate increase in short circuit current (I_{sc}) that peaked within 5 min and declined rapidly thereafter, although a small increase in I_{sc} persisted for ~30 min. The increase in I_{sc} was detectable with 1 μM carbachol; half-maximal with 10 μM carbachol; and maximal with 100 μM carbachol. Unidirectional Na^+ and Cl^- flux measurements indicated that the increase in I_{sc} was due to net Cl^- secretion. Carbachol did not alter cellular cAMP, but caused a transient increase in free cytosolic Ca^{2+} ($[Ca^{2+}]_i$) from 117±7 nM to 160±15 nM. The carbachol-induced increase in I_{sc} was potentiated by either prostaglandin E_1 (PGE_1) or vasoactive intestinal polypeptide (VIP), agents that act by increasing cAMP. Measurements of cAMP and $[Ca^{2+}]_i$ indicated that the potentiated response was not due to changes in these second messengers. Studies of the effects of these agents on ion transport pathways indicated that carbachol, PGE_1, or VIP each increased basolateral K^+ efflux by activating two different K^+ transport pathways on the basolateral membrane. The pathway activated by carbachol was not sensitive to barium, while that activated by PGE_1 or VIP was; furthermore, their action on K^+ efflux are additive. Our study indicates that carbachol causes Cl^- secretion, and that this action may result from its ability to increase $[Ca^{2+}]_i$ and basolateral K^+ efflux. Carbachol's effect on Cl^- secretion is greatly augmented in the presence of VIP or PGE_1, which open a cAMP-sensitive Cl^- channel on the apical membrane, accounting for a potentiated response.

Introduction

Previous studies suggest that acetylcholine regulates intestinal ion transport (1–6). However, these studies were performed using intact mucosa or intestinal loops, and both tissues contain neuroendocrine elements (2, 3). In fact, findings that the cholinergic agent, carbachol, causes electrolyte absorption may be due to its ability to release adrenergic agonists from neural tissue in the gut and not due to a direct effect of carbachol on the intestinal epithelium (4).

In the present study, we sought to determine the direct cholinergic effect on intestinal epithelial transport by using a well-differentiated colonic epithelial cell culture, the T_{84} cell line. The advantages of studies with this cell line are that it contains a single cell type and is devoid of neural elements and peptide hormones. Furthermore, monolayers of this culture allow accessibility of both the apical and the basolateral surface, giving precise localization for receptors and transport pathways. Our previous studies indicate that the T_{84} cell line becomes well differentiated when grown to confluence on permeable supports. The resultant monolayers have a columnar epithelial appearance and retain receptor-mediated electrolyte transport mechanisms (7, 8). In particular, prostaglandin E_1 (PGE_1),[1] vasoactive intestinal polypeptide (VIP), carbachol, and A23187 cause increased short circuit current (I_{sc}), and Cl^- secretion (9–12). In addition, the Cl^- secretion depends on the Na^+, K^+, Cl^- co-transport, the Na^+, K^+-ATPase and K^+ efflux pathways on the basolateral membrane, as well as on a Cl^- transport pathway on the apical membrane (9–14). Because the Cl^- secretory processes are similar to those found in intact intestinal mucosa, we chose T_{84} cell monolayers to study the mechanism of carbachol-induced Cl^- secretion in intestine.

Methods

Growth and maintenance of T_{84} monolayers, transepithelial electrolyte transport studies, $^{36}Cl^-$ uptake studies, $^{86}Rb^+$ efflux studies, and measurement of cAMP follow similar procedures as described previously (9–11).

Free cytosolic calcium measurements. Monolayers were dispersed with 0.1% trypsin and 0.9 mM EDTA in Ca^{++}- and Mg^{++}-free phosphate-buffered saline solution for 15 min at 37°C, then washed with and incubated at 37°C in the cell culture medium in an untreated petri dish. In the untreated petri dish, the T_{84} cell would not attach but would recover from the trypsinization procedure. After 18 h, these dispersed cells were then loaded with Quin-2/AM in a solution containing 20 mM Hepes (pH 7.4), 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 10 mM glucose, 10 mM pyruvate, 10 mM ascorbate, 0.1% (wt/vol) bovine serum albumin (fraction V), 0.1% soybean trypsin inhibitor, and 50 mM Quin-2/AM. They were incubated for 30 min at 37°C and then washed of extracellular Quin-2/AM with the same solution without Quin-2/AM and resuspended in the solution without Quin-2/AM. For measurement of free cytosolic calcium ($[Ca^{2+}]_i$), Quin-2 fluorescence was measured in 2.0-ml aliquots of cell suspension in an Aminco-Bowman spectrofluoremeter with settings of 340 nM excitation and 490 nm emission, and slit widths of 5.5 nm for excitation and 22 nm for emission. The cell suspension was continually stirred, maintained at 37°C, and equilibrated for ~5 min before the addition of the various agents. $[Ca^{2+}]_i$ was calculated using the following formula (15–19): $[Ca^{++}]_i = 115 \text{ nM } (F - F_{min}/F_{max} - F)$.

All fluorescence values were measured relative to the $MnCl_2$-quenched signal (i.e., autofluorescence was subtracted from all values) that was determined as described below. F was the relative fluorescence measurement of the sample. To determine maximum fluorescence (F_{max}), cells were lysed with sonication in the presence of 25 mM $CaCl_2$, and

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1. *Abbreviations used in this paper:* I_{sc}, short circuit current; PGE_1, prostaglandin E_1; VIP, vasoactive intestinal polypeptide.

relative fluorescence was measured. After determination of F_{max}, 1.0 mM $MnCl_2$ was added to totally quench the Quin-2 signal. Minimum fluorescence (F_{min}) was calculated as $1/6 \times F_{max}$ (19).

Materials. All radionuclides and cAMP antisera were obtained from New England Nuclear, Boston, MA. Carbachol came from ICN, Irvine, CA. PGE_1 was from Upjohn Co., Kalamazoo, MI. VIP was a gift from Dr. J. Rivier, The Salk Institute, La Jolla, CA. Bumetanide was a gift from Dr. P. Feit of Leo Pharmaceutical Products, Ballerup, Denmark. Barium chloride dihydrate was purchased from J. T. Baker Chemical Co., Phillipsburg, NJ. Quinidine was from Sigma Chemical Co., St. Louis, MO. Quin-2/AM was from Behring Diagnostics, La Jolla, CA.

Statistical analysis. *t* tests were used as indicated (20).

Results

After 5 d or more in culture, T_{84} cells, grown on the permeable, collagen-coated Nucleopore filters, appear as a columnar epithelial monolayers with their basolateral membrane firmly attached to the collagen-coated surface, and their apical membrane facing the medium (8). These monolayers maintained a transepithelial resistance of $\sim$1.5 KΩcm^2. The collagen-coated Nucleopore filter, which served as the attachment support for the cells, had a resistance <4 Ωcm^2. Thus, the support 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 serosal side and apical membrane surface as mucosal side.

Stimulation of net Cl^- secretion across T_{84} monolayers by carbachol. The addition of 100 μM carbachol to the serosal bathing solution caused an immediate increase in I_{sc}, which reached the peak within a few minutes after the addition. Thereafter, the I_{sc} rapidly declined, reaching the baseline by $\sim$10 min (Fig. 1). Mucosal addition had little or no effect (data not shown). The action of carbachol could be totally inhibited by 100 μM atropine (data not shown). The response was dose dependent, with 1 μM carbachol causing detectable stimulation; 10 μM carbachol causing half-maximal stimulation; and 100 μM carbachol causing maximal stimulation (Fig. 2). 100 μM carbachol was therefore used in all subsequent studies.

Results of unidirectional Na^+ and Cl^- fluxes are summarized in Table I. Previous studies demonstrated that net fluxes and I_{sc} in the absence of added secretagogues remained constant at or near zero for >100 min (9–12), and thus, each monolayer served as its own control in this study. After the addition of 100 μM carbachol, serosal to mucosal flux of Cl^- increased, while mucosal to serosal flux remained stable, resulting in net Cl^- secretion. No effects were observed on the unidirectional or net Na^+ fluxes. Therefore, the carbachol-induced increase in I_{sc} results from net Cl^- secretion. Similar to the change in I_{sc}, Cl^- secretion induced by carbachol in the T_{84} cells was also transient, lasting only $\sim$10 min.

In isolated intestine, the effects of carbachol on Cl^- secretion usually last >10 min (3–5). Because intestinal mucosa contains other potential secretagogues, such as PGE_1 and VIP, we tested the possibility that the action of carbachol is potentiated by these agents using the I_{sc} to quantitate Cl^- secretion. In this study, we have demonstrated that the changes in I_{sc} reflect net Cl^- secretion induced by carbachol. In other studies, we have demonstrated that PGE_1- and VIP-induced I_{sc} also reflect net Cl^- secretion (11, 12), including the potentiated response by VIP and A23187. We have further confirmed this fact by measuring Cl^- secretion in the presence of both PGE_1 and carbachol (average I_{sc} increase in the first 10 min of 79±7 μA/cm^2 or 1.48±0.13 μeq/h·cm^2 approximated net Cl^- secretion, which was 1.65±0.26 μeq/h·cm^2). Carbachol (100 μM), PGE_1 (10 μM), and VIP (10 nM) each caused a peak increase in I_{sc} of 17±2, 22±3, and 22±6 μA/cm^2, respectively, at 5 min of incubation (n = 5 each). At 5 min incubation, the combination of 100 μM carbachol with 10 μM PGE_1 or 10 nM VIP caused a peak increase in I_{sc} of 52±7 and 68±9 μA/cm^2, respectively (n = 5 each). Thus, the response with carbachol plus PGE_1 or VIP was greater than the expected additive response ($P < 0.05$). More importantly, the potentiating response caused by PGE_1 persisted in monolayers preincubated with carbachol for >15 min (Fig. 3). Similarly, VIP potentiated the carbachol response even when the monolayers were preincubated with 100 μM carbachol for 30 min. The increase in I_{sc}

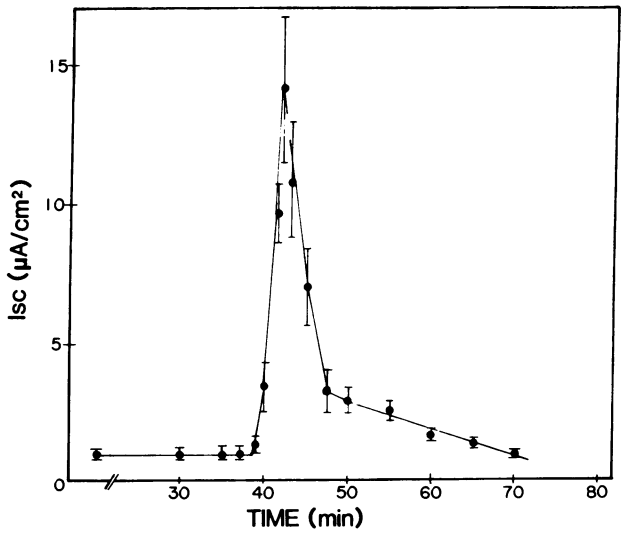

Figure 1. Time-course of the I_{sc} response to 10^{-4} carbachol. Results are expressed as mean±SE of six monolayers. Addition of carbachol to the serosal surface resulted in an immediate increase in the I_{sc}, and reached a peak within 5 min. Thereafter, the I_{sc} rapidly declined, but did not reach the baseline until $\sim$30 min after the addition.

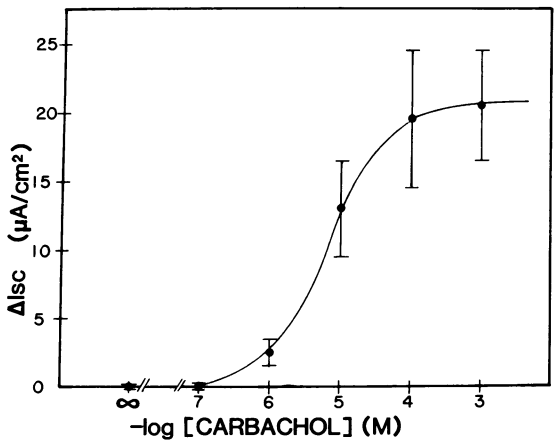

Figure 2. Graded dose effect of carbachol on the changes in I_{sc}. Carbachol was added to the serosal reservoir in concentrations shown. The peak I_{sc} that occured after the addition of carbachol was expressed as mean±SE of four monolayers in each group. Only one concentration was added to each monolayer.

Table I. Unidirectional Na^+ and Cl^- Fluxes

Period	Experimental condition	$J^{Na}_{m\to s}$	$J^{Na}_{s\to m}$	J^{Na}_{net}	$J^{Cl}_{m\to s}$	$J^{Cl}_{s\to m}$	J^{Cl}_{net}	I_{sc}	G
Effect of carbachol alone (n = 5)									
1	Control	0.26±0.03	0.32±0.09	−0.06±0.08	0.34±0.03	0.36±0.04	−0.02±0.04	0.01±0.01	0.58±0.06
2	10^{-4} M carbachol (5–10 min after the addition)	0.34±0.08	0.39±0.09	−0.05±0.15	0.36±0.07	0.64±0.10*	−0.28±0.16*	0.33±0.02*	0.61±0.06
3	10^{-4} M carbachol (10–25 min after the addition)	0.23±0.05	0.20±0.02	0.03±0.04	0.21±0.02	0.36±0.01	−0.15±0.02*	0.07±0.01*	0.61±0.06
Effect of PGE_1 plus carbachol (n = 4)									
1	Control	0.32±0.07	0.26±0.05	0.06±0.07	0.25±0.03	0.26±0.04	−0.01±0.04	0.17±0.02	0.59±0.04
2	10^{-6} M PGE_1 plus 10^{-4} M carbachol (5–10 min after the addition)	0.43±0.13	0.40±0.10	0.03±0.05	0.56±0.11*	2.21±0.33*	−1.65±0.26*	1.48±0.13*	1.08±0.04*
3	10^{-6} M PGE_1 plus 10^{-4} M carbachol (10–25 min after the addition)	0.26±0.04	0.33±0.06	−0.07±0.04	0.53±0.07*	1.44±0.12*	−0.91±0.15*	0.94±0.11*	0.86±0.04*

Results are expressed as mean of the average flux rate±SE of the number of paired monolayers indicated in parentheses in $\mu eq/h \cdot cm^2$, except for the conductance (G), which is in mS/cm^2. Period 1 is a 20-min flux period ending just before the addition of 10^{-4} M carbachol to the serosal bathing media (between 17 and 37 min after mounting). Period 2 is a 5-min flux period starting 5 min after the addition of carbachol (between 42 and 47 min after mounting). Period 3 is a 15-min flux period immediately after period 2 (between 47 and 62 min after mounting). There was an increase in I_{sc} after the addition of carbachol or PGE_1 plus carbachol, and Cl^- secretion could account for the changes in I_{sc}. The effect of carbachol on Cl^- secretion was more striking in the first 10 min after its addition. * Indicates that values are significantly different from control by paired t test ($P < 0.05$).

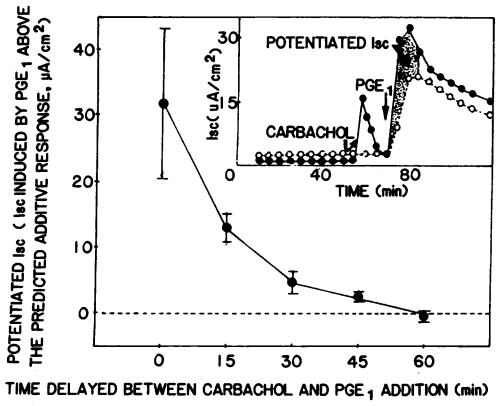

Figure 3. Potentiation of PGE_1 response to carbachol. The potentiated I_{sc} shown on the vertical axis is the average I_{sc} induced by PGE_1 in carbachol pretreated monolayers above the predicted additive response. The time interval between the addition of carbachol followed by PGE_1 is indicated on the horizontal axis. The average I_{sc} was measured during the 15-min period after the addition of PGE_1. From this value were subtracted the PGE_1-induced I_{sc} during the same period when the monolayers were not pretreated with carbachol and the average I_{sc} induced by carbachol alone during this period. In the *inset* in the upper right hand corner is a figure representing this calculation. In this figure, carbachol was added to one monolayer (●) 15 min before the addition of PGE_1 to both monolayers. Open circles indicate the effect of PGE, without carbachol pretreatment. The shaded area represents the potentiated response. We did not include the values for carbachol alone in this figure because they were near basal values. The results are expressed as mean±SE of four monolayers at each time point. The concentrations of carbachol and PGE_1 were 10^{-4} and 10^{-6} M, respectively. The effect of PGE_1 on carbachol-pretreated monolayers remained greater than the predicted additive response for >15 min.

with 10 nM VIP in these carbachol-pretreated monolayers was 20±1 $\mu A/cm^2$, while the increase by 10 nM VIP in nonpretreated monolayers was 14±1 $\mu A/cm^2$. These results indicate that carbachol potentiates the action of PGE_1 and VIP on I_{sc} even after the effect of carbachol alone on I_{sc} dissipates. These findings may explain the more sustained action of carbachol in whole mucosa.

Inhibition of carbachol-stimulated I_{sc} by bumetanide or barium. Previous studies demonstrated the existance of the Na^+, K^+, Cl^- co-transport pathway and the K^+ channel, as well as their participation in the Cl^- secretory process induced by cAMP (PGE_1 or VIP) or Ca^{++} ionophore (A23187) in T_{84} monolayers (9–14). In addition, these studies also indicated that bumetanide and barium inhibit the Na^+, K^+, Cl^- cotransport system and a K^+ channel, respectively. To identify the electrolyte transport pathways involved in the Cl^- secretory process induced by carbachol, the effect of carbachol on the I_{sc} was tested in the presence of these inhibitors. Bumetanide was used as an inhibitor for the Na^+, K^+, Cl^- co-transport pathway, while barium was as an inhibitor for the K^+ channels. Cl^- channels were not tested because we could not identify a suitable blocker for the Cl^- channels in this cell line. As demonstrated previously for PGE_1 and VIP (9, 11), serosal application of 0.3 mM bumetanide inhibited Cl^- secretion induced by carbachol (Fig. 4). This result suggests that the Na^+, K^+, Cl^- co-transport serves as the Cl^- uptake pathway on the basolateral membrane. In contrast to PGE_1 or VIP (10, 11), the effect of carbachol was not inhibited by 6 mM $BaCl_2$, but was inhibited by quinidine in a dose-dependent manner. Other putative K^+ channel blockers, including tetraethylammonium chloride (10 mM), 4-aminopyridine (10 mM), and apamin (1 μM) had no effect on Cl^- secretion induced by carbachol (data not shown). Such results indicate that the basolateral K^+ channel activated by PGE_1 and VIP is not activated by carbachol.

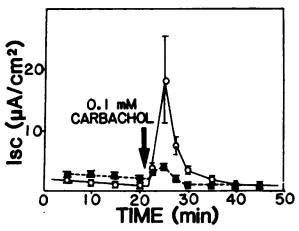

Figure 4. Inhibition of carbachol-induced I_{sc} by bumetanide. Comparison of I_{sc} responses to 10^{-4} M carbachol in control (○) and bumetanide- (●) pretreated monolayers. 100 μM bumetanide was added to the serosal side 5 min after mounting, and carbachol was added at 20 min. The results are expressed as mean±SE of seven monolayers in the bumetanide group and three in the control group. Bumetanide almost completely inhibited the response to carbachol.

Lack of influence by carbachol on Cl^- transport across the apical membrane. Previous studies demonstrated the existence of a Cl^- transport pathway on the apical membrane of the T_{84} cells that is stimulated by PGE_1 or VIP, but not by A23187. Kinetic studies and preliminary patch clamp studies suggest that this pathway is a Cl^- channel (reference 13, Frizzell, R. A., personal communication). According to these earlier studies, the activation of this Cl^- exit pathway by peptides or neurotransmitters can be detected by a $^{36}Cl^-$ uptake technique (13). In the present study, carbachol had no effect on $^{36}Cl^-$ uptake across the apical membrane, while both PGE_1 and VIP did increase $^{36}Cl^-$ uptake (Figs. 5 and 6). Furthermore, the PGE_1- and VIP-induced increases in $^{36}Cl^-$ uptakes were not altered by carbachol (Fig. 6). Similar results were obtained in the absence of bumetanide or ouabain. At 1, 2.5, and 5 min, $^{36}Cl^-$ uptakes in the presence and absence of 10^{-4} M carbachol were 2.0±0.9, 3.8±1.5, and 3.3±1.1 nmol/mg protein and 1.8±0.7, 3.8±0.5, and 3.6±0.9 nmol/mg protein, respectively. Carbachol also did not alter the increase in $^{36}Cl^-$ uptakes induced by VIP or PGE_1 under this experimental condition. At 5 min, Cl^- uptakes were 6.9±1.9 and 5.9±1.1 μmol/mg protein in the presence of 10^{-8} M VIP and 10^{-6} M PGE_1, respectively. Similar results of 6.6±1.6 and 6.0±1.1 μmol/mg protein, respectively, were obtained in the presence of 10^{-4} M carbachol. These results suggest that the effect of carbachol on Cl^- secretion is not a result of the opening of the cAMP-sensitive Cl^- channels on the apical membrane, but rather a result of an increase in the driving force that may operate more efficiently once the cAMP-sensitive Cl^- channel is opened. The lack of carbachol effect should be interpreted with some cautions. Our assay, which is suitable for detecting the opening of the cAMP-sensitive Cl^- channels, may not detect the opening of Ca^{++}-sensitive Cl^- channels.

Evidence for carbachol-induced K^+ efflux on the basolateral membrane and its additive effect to PGE_1-induced K^+ efflux. In a variety of epithelia, including T_{84} monolayers, agents that increase Cl^- secretion also cause an increase in basolateral membrane conductance to K^+, which appears to be required to sustain the Cl^- secretory process (10, 21, 22). To test whether carbachol stimulates K^+ efflux across the basolateral membrane, monolayers were preloaded with $^{86}Rb^+$ (as a tracer for K^+) and mounted in the Ussing chamber. This allowed us to measure $^{86}Rb^+$ efflux across both the apical and basolateral surfaces and the I_{sc}, which reflects Cl^- secretion, simultaneously. The results are shown in Table II. At the basal state, the rate of $^{86}Rb^+$ efflux

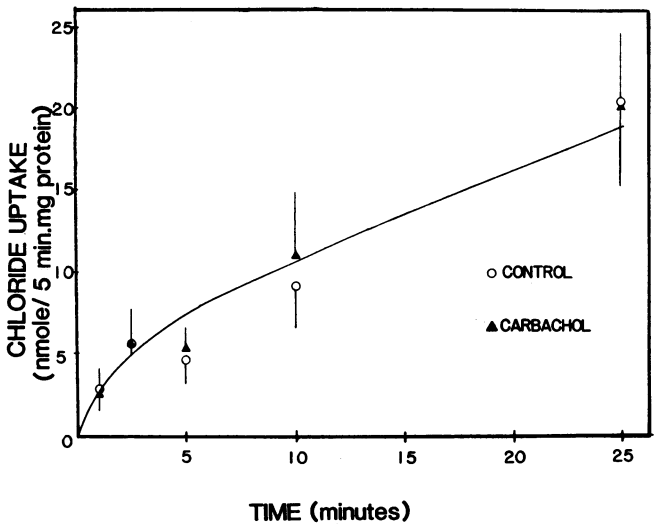

Figure 5. Lack of effect of carbachol on $^{36}Cl^-$ uptake across the apical membrane. Confluent monolayers grown on permeable supports similar to those used in the Ussing chamber were preincubated in KCl and sucrose-ouabain buffer as described previously (13). $^{36}Cl^-$ uptake across the apical membrane 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, and 1 μCi/ml $^{36}Cl^-$ (final concentration, 6 mM) with or without 10^{-4} M carbachol. An identical buffer, without added $^{36}Cl^-$, was added simultaneously to the basolateral side. Uptakes were carried out at the time intervals indicated on the horizontal axis at room temperature before terminating by dunk washing in four successive 1-liter containers of Mg gluconate-sucrose buffer. Results are the mean±SE of six monolayers at each time point. Carbachol had no effect on Cl^- uptake under this experimental condition.

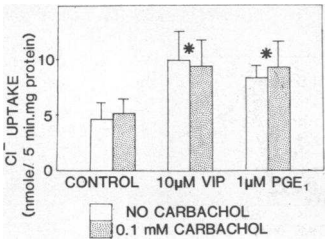

Figure 6. Lack of effect of carbachol on PGE_1 or VIP-stimulated $^{36}Cl^-$ uptake across the apical membrane. Conditions for uptake studies are as described in Fig. 5. Results are mean±SE of Cl^- uptake from five monolayers in each group. Results at 5 min, when carbachol's action was at its peak, are shown. Astericks indicate $P < 0.05$ by unpaired t test as compared with control without VIP or PGE_1. Carbachol does not alter the increased uptake stimulated by PGE_1 or VIP.

into the mucosal bath was ~10–20-fold smaller than that into the serosal bath. The addition of carbachol (added to both sides) dramatically increased the rate of $^{86}Rb^+$ efflux into the serosal bath for ~10 min. The rate of $^{86}Rb^+$ efflux into the mucosal bath also increased during this initial 10 min, although the magnitude of increase is quite small. These increases in $^{86}Rb^+$ efflux occurred simultaneously with the increase in I_{sc}. The increase in basolateral membrane $^{86}Rb^+$ efflux rate by carbachol was not inhibited by prior addition of 3 mM $BaCl_2$, which completely inhibited PGE_1 or VIP-stimulated $^{86}Rb^+$ efflux. The results suggest that carbachol stimulated K^+ efflux across the basolateral membrane via a K^+ transport mechanism that is distinct from that involved in the action of PGE_1 or VIP.

We then compared the magnitude of $^{86}Rb^+$ efflux and the I_{sc} (which reflects Cl^- secretion) induced by carbachol, PGE_1, or their combinations (Fig. 7). The carbachol-induced $^{86}Rb^+$ efflux was relatively large in magnitude as compared with that

Table II. $^{86}Rb^+$ Efflux in Response to Carbachol

Period	Experimental condition	$^{86}Rb^+$ efflux rate constant		I_{sc}	G
		Mucosal	Serosal		
		h^{-1}	h^{-1}	$\mu eq/h \cdot cm^2$	mS/cm^2
Control (n = 3)					
1	No addition	0.07±0.01	0.59±0.11	0.03±0.01	0.6±0.2
2	No addition	0.03±0.01	0.58±0.13	0.06±0.03	0.6±0.1
3	No addition	0.03±0.01	0.48±0.02	0.07±0.03	0.6±0.1
10^{-4} M carbachol (n = 11)					
1	No addition	0.09±0.01	0.50±0.02	0.07±0.02	0.8±0.1
2	Carbachol	0.12±0.02*	1.99±0.10*	0.40±0.10*	0.8±0.1
3	Carbachol	0.05±0.01	0.61±0.05	0.13±0.03	0.6±0.1
3 mM Ba^{++} + 10^{-4} M carbachol (n = 3)					
1	Ba^{++}	0.07±0.01	0.39±0.05	0.04±0.01	0.7±0.1
2	Ba^{++} + carbachol	0.16±0.03*	1.98±0.17*	0.56±0.15*	0.9±0.1
3	Ba^{++} + carbachol	0.06±0.01	0.63±0.05	0.17±0.04	0.6±0.1

The results are expressed as mean±SE of the number of experiments indicated in parentheses. The results were analyzed during three time intervals: period 1, 0–20 min; period 2, 20–30 min; and period 3, 30–45 min. $BaCl_2$, when used, was added within 5 min after mounting, while carbachol was added at 20 min. $^{86}Rb^+$ effluxes were increased by carbachol with serosal effluxes being ~10–20-fold greater than mucosal effluxes. The carbachol-induced $^{86}Rb^+$ effluxes were not affected by Ba^{++}. * Indicates significant difference from control values during the same time interval ($P < 0.05$) by unpaired t test.

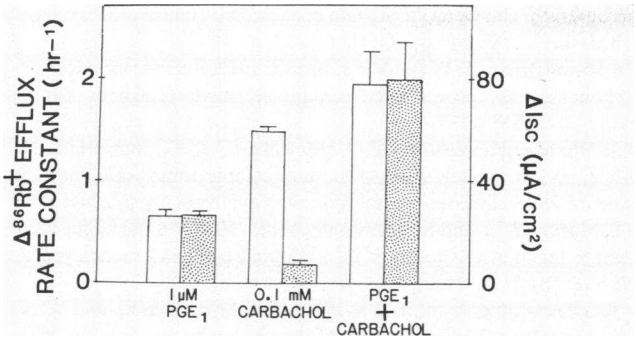

Figure 7. Comparison of $^{86}Rb^+$ efflux across the basolateral membrane and I_{sc}. Results are expressed as mean±SE of 6–8 monolayers. Average changes in rate constant of $^{86}Rb^+$ efflux per hour in the first 10 min after the addition of carbachol and/or PGE_1 are expressed as clear bars, while changes in I_{sc} represent changes in the rate of Cl^- secretion, and are shown as solid bars. Basal rate constants for $^{86}Rb^+$ efflux in the first 20 min were 0.48±0.04, 0.56±0.06, and 0.43±0.02 in PGE_1, carbachol, and PGE_1 + carbachol groups, respectively, while basal I_{sc} were 3±1, 4±2, and 1±1 $\mu A/cm^2$, respectively. These basal values were subtracted from the observed responses to obtain the results shown in the figure.

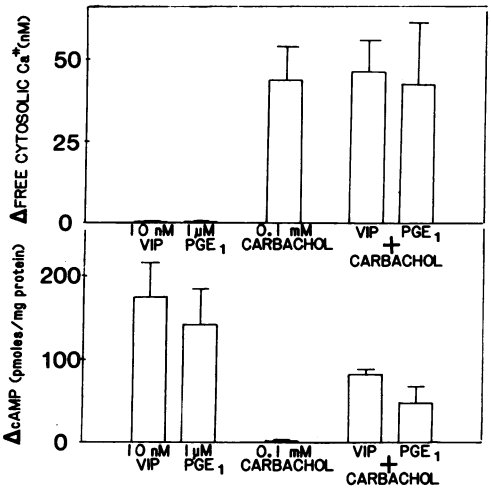

Figure 8. cAMP and free cytosolic Ca^{++} in response to carbachol, VIP, and PGE_1. cAMP was measured in monolayers similar to those using for Ussing chamber studies using New England Nuclear radioimmunoassay kits, while free cytosolic calcium was measured in dispersed T_{84} cells as described in Methods. The results are expressed as mean±SE of the changes in cAMP or free cytosolic Ca^{++} from 3 to 10 monolayers. For ΔcAMP, the changes were calculated from the cAMP level 15 min after stimulation by the agents indicated minus the basal cAMP, which was 5±1 pmol/mg protein. For free cytosolic Ca^{++}, the values were calculated as the difference between the basal concentration and the peak concentration after the addition of the agent indicated. The average baseline level of free cytosolic calcium in these five groups was from 112±12 to 125±11 nM. The increase in free cytosolic Ca^{++} induced by carbachol was not altered by VIP or PGE_1. Similarly, the increase in cAMP by VIP or PGE_1 was not altered by carbachol.

induced by PGE_1, while its effect on I_{sc} was relatively small. In contrast, the effect of PGE_1 on I_{sc} was greater than that for carbachol. $^{86}Rb^+$ efflux induced by the combination of carbachol and PGE_1 was additive, further supporting the presence of two different types of K^+ efflux pathways, one activated by PGE_1 and another activated by carbachol. On the other hand, the increase in I_{sc} induced by the combination of carbachol and PGE_1 was greater than the additive response (potentiated). The above findings suggest that stimulation of K^+ efflux across the basolateral membrane alone by carbachol, without the opening of the cAMP-sensitive Cl^- exit pathway on the apical surface, is a relatively ineffective means to stimulate Cl^- secretion. Furthermore, these findings suggest that PGE_1 and VIP potentiate the action of carbachol by opening the apical Cl^- exit pathway, making Cl^- secretion by carbachol more effective.

cAMP and free cytosolic Ca^{++} measurements. To further explore the mechanisms of action of carbachol, PGE_1, and VIP, and the mchanism of action of potentiation, we measured the effects of these agents on cAMP and free cytosolic Ca^{2+} ($[Ca^{2+}]_i$). The results are summarized in Fig. 8. Carbachol had no effect on cellular cAMP, while VIP and PGE_1 both significantly increased cellular cAMP. The increase in cAMP by PGE_1 or VIP was not altered by carbachol. Carbachol increased $[Ca^{2+}]_i$ from 117±7 to 160±15 nM within 1 min of addition. The increase was transient, such that $[Ca^{2+}]_i$ returned to the basal concentration in ~5 min. The effect of carbachol was totally inhibited by 100 μM atropine (data not shown). Both PGE_1 and VIP had no effect on $[Ca^{2+}]_i$, and neither one augmented the carbachol-induced changes in $[Ca^{2+}]_i$. As a matter of fact, carbachol diminished the increments in cAMP concentration caused by VIP and PGE_1. These results suggest that carbachol's action is mediated by an increase in $[Ca^{2+}]_i$, while VIP's and PGE_1's actions are mediated by cAMP. In addition, the ability of VIP or PGE_1 to potentiate carbachol's effect on I_{sc} cannot be explained by changes in either $[Ca^{2+}]_i$ or cAMP alone.

Discussion

The use of a cultured intestinal cell line allows for the determination of the cellular mechanism involved in the action of cholinergic agents as well as the interaction of cholinergic agents with other proposed secretagogues. The present study indicates that carbachol stimulates Cl^- secretion from T_{84} cell monolayers. The response of T_{84} monolayers resembles that occurring with isolated small intestine of animals and man, except that the effect is more sustained in isolated intestine (1–6). The difference may be due to the fact that intact mucosa contains neuroendocrine elements that also have effects on electrolyte absorption and secretion. Therefore, the results obtained with isolated intestine in part reflect the effect of endogenous agents. This notion is supported by the findings in the present study that carbachol pretreatment augmented the action of PGE_1 and VIP.

Because carbachol stimulates an increase in $[Ca^{2+}]_i$, and because A23187, the calcium ionophore, also causes Cl^- secretion (12), our results suggest that carbachol's action on Cl^- secretion is due at least in part to its effect on $[Ca^{2+}]_i$. The ability of carbachol to cause Cl^- secretion appears to involve Cl^- uptake across the basolateral membrane via the Na^+, K^+, Cl^- transport carrier and activation of a basolateral K^+ transport pathway. The activation of the latter pathway results in efflux of cellular K^+. The K^+ efflux hyperpolarizes the cell to create a favorable electrical gradient for Cl^- exit across the apical surface. That basolateral K^+ efflux is linked to apical Cl^- secretion is substantiated by findings that K^+ channel blockers, such as barium, inhibit Cl^- secretion. Carbachol did not activate the apical Cl^- channel in contrast to agents that act by increasing cellular cAMP such as PGE_1 or VIP. The inability of carbachol to activate the apical Cl^- channel may explain why carbachol is less effective in causing

Cl^- secretion than PGE_1 or VIP, although their effects on K^+ efflux and Na^+, K^+, Cl^- cotransport are similar.

Our study indicates that another difference between carbachol and VIP or PGE_1 is their effect on basolateral K^+ pathways. The K^+ pathway activated by carbachol is distinct from that activated by VIP and PGE_1, as indicated by the following findings. First, carbachol's effect on $^{86}Rb^+$ efflux is additive to that for VIP or PGE_1. Second, barium, a K^+ channel blocker, inhibits $^{86}Rb^+$ efflux stimulated by PGE_1 or VIP, but has no effect on $^{86}Rb^+$ efflux stimulated by carbachol.

Our findings suggest that the ability of PGE_1 or VIP to potentiate the action of carbachol on Cl^- secretion results from the differing effects of these agents on the ion pathways rather than on an increase in either cAMP or $[Ca^{2+}]_i$. As indicated above, carbachol does not open the cAMP-sensitive Cl^- channel on the apical membrane, so that Cl^- secretion is limited, even though it is very effective in stimulating basolateral K^+ efflux. With the addition of PGE_1 or VIP to carbachol-stimulated cells, the cAMP-sensitive Cl^- channel on the apical membrane opens so that carbachol's effect on basolateral K^+ efflux can be effectively linked to Cl^- secretion. In other words, PGE_1 and VIP augment the action of carbachol on Cl^- secretion by opening the apical Cl^- gate, which is a rate-limiting step in carbachol's action, while their effect on K^+ efflux remains additive to that induced by carbachol, because they activate different K^+ transport pathways. The end result is the potentiated response on Cl^- secretion. Because the intestine contains prostaglandins, other arachidonic acid metabolites, and VIP (23–25), this integration between PGE_1 or VIP and carbachol may also explain the more sustained response observed with cholinergic stimulus of intestinal mucosa compared with the more transient response we observed with T_{84} monolayers.

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