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Active Chloride Secretion by Rabbit Colon: Calcium-Dependent Stimulation by Ionophore A23187

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Summary. Addition of Ca ionophore, A23187, to the solution bathing the mucosal surface of descending rabbit colon resulted in a reversal of active Cl absorption to active Cl secretion, a twofold increase in short-circuit current and a 40% increase in tissue conductance without affecting the rate of active Na absorption. These alterations in electrolyte transport are quantitatively similar to those previously observed in response to cyclic 3′,5′-AMP (cAMP) (R.A. Frizzell, M.J. Koch & S.G. Schultz, *J. Membrane Biol.* **27**:297, 1976). When medium Ca concentration was reduced to 10^{-6} M, the secretory response to A23187 was abolished but the response to cAMP was unaffected. The ionophore did not influence the cAMP levels of colonic mucosa. Addition of cyclic AMP to colonic strips preloaded with ^{45}Ca elicited a reversible increase in Ca efflux from the tissue. These results suggest that an increase in intracellular Ca concentration stimulates colonic electrolyte secretion and that the secretory response to cAMP may be due, at least in part, to a release of Ca from intracellular stores.

In recent years evidence has accumulated suggesting that the activation of physiologic processes by extracellular stimuli may be mediated by both calcium and cyclic nucleotides. Interactions between these intracellular messengers appear to determine the coordinated response which characterizes cellular activation in a variety of tissues [2, 28, 29]. In most instances, the effects of Ca and cyclic 3′,5′-AMP (cAMP) are complementary, as in the stimulation of fluid secretion by fly salivary gland [25], activation of renal gluconeogenesis [19], stimulation of amylase secretion by exocrine pancreas [36], and release of insulin from endocrine pancreas [20]; while in others, their effects are antagonistic [2].

One experimental approach toward identifying the possible involvement of Ca as an intracellular messenger has employed the divalent cation ionophore, A23187. This agent enhances divalent cation uptake or exchange across a variety of artificial and biological membranes and therefore has been used extensively as a tool for studying the influence

of alkali metal cations on biological systems [30]. In this regard, A23187 has been found to mimic the physiologic response of a variety of tissues to extracellular stimuli or exogenous cAMP [2, 29].

Recent studies of ion transport by descending rabbit colon [15] have demonstrated that active Cl secretion by this tissue is elicited by cAMP. The results of the present study indicate that the effects of A23187 on ion transport are virtually identical to those of the cyclic nucleotide and suggest that changes in intracellular Ca may be responsible for the secretory response.

Materials and Methods

Descending colon was obtained from male white rabbits (2–3 kg) which had been sacrificed with pentobarbital. A "partial mucosal strip" preparation (*see* Fig. 1, ref. 15) was employed for studies of bidirectional Na and Cl fluxes under short-circuit conditions using paired tissues from the same animal as previously described. The effects of A23187 on unidirectional Na or Cl fluxes were determined following a 40-min control period of flux determinations and sufficient time (approx. 20 min) to achieve a steady state of tracer flux. In all instances, flux values for individual tissues represent the mean of at least four determinations at 10-min intervals. The short-circuit current (I_{sc}) was continuously monitored except for brief periods when the spontaneous transepithelial potential difference (ψ_{ms}) was recorded; the electrical conductance of the epithelium (G_t) was determined from the ratio (I_{sc}/ψ_{ms}), and expressed in mmhos/cm^2. This procedure is justified by previous observations [15] indicating that the tissue behaves as an ohmic resistor over the range of spontaneous values of ψ_{ms} encountered in this study. In some experiments, the effects of A23187 or cAMP on the short-circuit current alone were determined. A protocol similar to that outlined above was followed except that unidirectional fluxes of Na and Cl were not measured.

The standard electrolyte solution used in most studies contained (mM): Na, 140; Cl, 124; HCO_3, 21; K, 5; HPO_4, 2.4; H_2PO_4, 0.6; Ca, 1.2; Mg 1.2; glucose, 10; and had a pH of 7.4 at 37 °C when gassed with 95% O_2, 5% CO_2. Solutions of reduced free Ca concentration were obtained by addition of 1.2 mM ethyleneglycol-bis (β-amino ethyl ether)-N,N′-tetraacetic acid (EGTA) to the standard medium. Stock solutions of A23187 were prepared by dissolving the ionophore in absolute ethanol. The final concentration of ethanol in the incubation media did not exceed 1% and in most studies was 0.1%. Several preliminary experiments indicated that addition of ethanol alone, at these concentrations, had no effect on I_{sc}, G_t or the bidirectional fluxes of Na or Cl. These results also indicated that the procedure employing a control flux period followed by an additional flux period in the presence of ionophore is justified. As noted previously [15], bidirectional fluxes of Na and Cl across this tissue are stable for at least 3 hr *in vitro*.

Tissue cAMP levels were determined in duplicate by the method of Gilman [17] as previously applied to rabbit ileal mucosa [22], and are expressed as pmoles of cAMP per mg tissue wet weight. A "mucosal strip" preparation was employed in which the colonic mucosa was stripped-free of serosal musculature using glass microscope slides as described for rabbit ileum [31]. This approach results in a preparation which is functionally indistinguishable from the "partial mucosal strip" which retains the *muscularis mucosa (unpublished observations)*.

Segments of the "mucosal strip" preparation were also employed for determinations of Ca efflux. Tissues were mounted on individual holding devices which permitted gassing with 95% O_2, 5% CO_2 from below so that a fine stream of bubbles continually rose over the tissue surface. Paired tissues were preloaded with ^{45}Ca for 1 hr in the same isotope-containing medium. Each tissue was then transferred sequentially to individual, 2-ml aliquots of isotope-free medium at 10-min intervals. During the period from 50–110 min following removal from the loading solution, experimental tissues were exposed to media containing 7.5 mM cAMP while control tissues were continually exposed to the standard electrolyte solution. Following a total efflux period of 160 min, tissues were placed in pyrex tubes and ashed overnight in a muffle furnace. The ash was dissolved in 0.1 N HCl and counted by liquid scintillation techniques, together with aliquots of the efflux media employing appropriate quench correction. An efflux rate coefficient for each 10-min period was calculated as the per cent of ^{45}Ca which left the tissues during each time interval according to the relation [5]:

$$ERC_i = \frac{\Delta C_i}{Cm_i \Delta t} \times 100$$

where ΔC_i is the activity lost from the tissue during each time interval, Δt, and Cm_i is the mean activity of the tissue between t_i and $t_i + \Delta t$. The rate coefficient for Ca efflux from experimental tissues was expressed as a percentage of that obtained from paired control tissues not exposed to cAMP.

Ionophore A23187 was the generous gift of Dr. R. Hamill, Eli Lilly & Co., Indianapolis, Indiana. Cyclic AMP was obtained from Sigma Chemical Co., ^{22}Na and ^{36}Cl from ICN Pharmaceuticals, Inc., and ^{45}Ca from New England Nuclear. All values are expressed as the mean $\pm$ SEM. Differences were evaluated using the Student's t-test; values of $p < 0.05$ were considered significant.

Results

Effect of A23187 on Ion Transport by Rabbit Colon

The results of recent studies indicate that under normal conditions the spontaneous electrical potential difference and short-circuit current (I_{sc}) across descending rabbit colon are determined solely by the rate of active Na absorption. Table 1 illustrates these findings; under control conditions J^{Na}_{net} and I_{sc} are in excellent agreement. As discussed previously [15] and as shown in Table 1, control, the observed rate of active Cl absorption, is balanced by an unmeasured ion flux J_r which can probably be attributed to HCO_3 secretion. Thus, the movements of Cl and HCO_3 across this tissue appear to be mediated by a Na-independent, anion exchange process which is electrically silent.

Addition of A23187 to the mucosal solution alone resulted in active Cl secretion which was entirely attributable to an increase in the unidirectional Cl flux from serosa-to-mucosa. At the same time, the short-circuit current across the tissue approximately doubled and tissue conductance increased by 40%. In contrast, A23187 had no effect on the rate of

Table 1. Effects of A23187 on sodium and chloride fluxes across rabbit colon

J_{ms}^{Na}	J_{sm}^{Na}	J_{net}^{Na}	J_{ms}^{Cl}	J_{sm}^{Cl}	J_{net}^{Cl}	I_{sc}	J_r	G_t
Control (4)								
3.4±0.6	1.5±0.3	1.9±0.6	6.3±0.4	4.9±0.4	1.4±0.4	2.2±0.4	1.7±0.7	4.6±0.2
+A23187 (0.1 μg/ml)								
3.4±0.7	1.6±0.3	1.8±0.6	6.1±0.3	7.5±0.4[a]	−1.4±0.3[a]	4.6±0.4[a]	1.4±0.7	6.5±0.3[a]

J_{ms}^{i} designates the unidirectional ion flux from mucosa-to-serosa; J_{sm}^{i} the unidirectional flux from serosa-to-mucosa; $J_{net}^{i} = J_{ms}^{i} - J_{sm}^{i}$. All values are in μEquiv/cm² hr, except G_t which is expressed in mmhos/cm². $J_r = I_{sc} - J_{net}^{Na} + J_{net}^{Cl}$. Fluxes in the presence of A23187 were determined following an initial 40-min control flux period and a 20-min preincubation in the presence of ionophore. Number of experiments given in parentheses.

[a] Significant difference from control value, $p < 0.05$.

active Na absorption. Thus, the increase in I_{sc} observed in the presence of ionophore is primarily due to stimulation of an electrogenic Cl secretory process. In preliminary experiments, the concentration of A23187 employed for the studies summarized in Table 1 (0.1 μg/ml) was found to be the smallest concentration of ionophore which produced a maximal increment in I_{sc}. Levels of 5 μg/ml generally resulted in a reduced response. A similar reduction in the response to higher levels of A23187 was observed by Wollheim *et al.* [37] for ionophore-induced insulin release from cultured pancreatic islets. Addition of 0.1 μg/ml A23187 to both the mucosal and serosal bathing media produced no greater increase in I_{sc} than addition to the mucosal solution alone. The effects of adding ionophore to the serosal solution alone were not evaluated.

These effects of A23187 are quantitatively similar to the previously reported response of this tissue to exogenous cAMP [15]. Indeed, the sole discrepancy observed when comparing the effects of these agents lies in the residual ion flux which was not significantly influenced by the ionophore (Table 1), but was reduced by the cyclic nucleotide. However, it is possible that A23187 (as cAMP) does reduce HCO_3 secretion but that other unidentified ion movements contribute to the observed discrepancy between the I_{sc} and the net Na and Cl fluxes in the presence of ionophore. In the absence of direct measurements of HCO_3 secretion by colonic mucosa, a firm conclusion regarding the effects of A23187 on Cl-HCO_3 exchange cannot be drawn. Continued HCO_3 secretion in the presence of A23187 would suggest that active Cl secretion elicited by ionophore results from stimulation of a *de novo* Cl secretory mechanism without involving the anion exchange process which normally mediates Cl absorption.

Effect of Extracellular Calcium on Response to A23187 and cAMP

The effect of reducing the concentration of free Ca in the bathing media on the secretory response of the tissue to A23187 (as judged by the increase in I_{sc}) is illustrated in Fig. 1. In the presence of the standard electrolyte solution, the ionophore-induced increase in I_{sc} reached a maximal value 10 min following addition of A23187 to the mucosal solution. However, when the free Ca concentration in the bathing medium was reduced with EGTA, the ionophore had no effect on I_{sc}. The calculated free Ca concentration under these conditions is approximately 10^{-6} M [23]. Restoration of the free Ca concentration to 10^{-3} M by addition of $CaCl_2$ to the bathing solutions resulted in an immediate secretory response; I_{sc} rose to levels similar to those observed when A23187 was added to the standard electrolyte solution. Thus, the response to A23187 is dependent upon a critical free Ca concentration in the solutions bathing the tissue.

Similar experiments were performed by simply omitting Ca from the bathing solutions; the Ca concentration of the media, measured by fluorometric titration [7] was approximately 20 μM. Addition of A23187 under these conditions had no effect on the I_{sc}. Subsequent addition of Ca to a final concentration of 10^{-3} M resulted in a secretory response similar to that shown in Fig. 1 (data not shown). However, further reductions in the free Ca concentration of the standard electrolyte

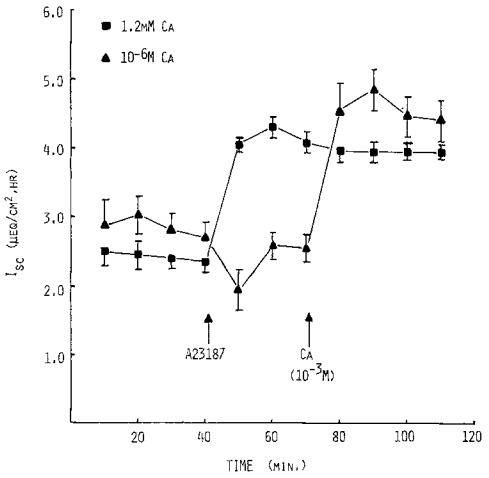

Fig. 1. Effect of A23187 on the short-circuit current across rabbit colon. Bathing media contained either 1.2 mM Ca or 1.2 mM Ca plus 1.2 mM EGTA (free Ca concentration, 10^{-6} M) as indicated above. A23187 added to the mucosal solution alone (final concentration, 0.1 μg/ml) at the time indicated. $CaCl_2$ (final concentration, 10^{-3} M) added to EGTA-containing media only

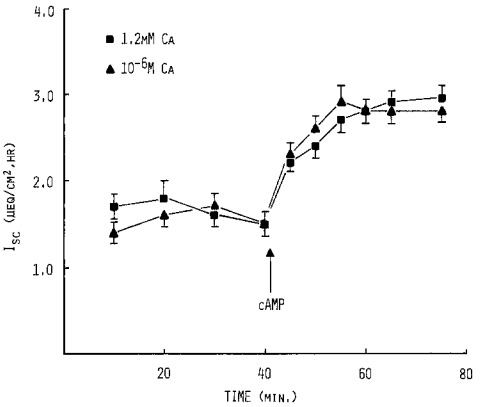

Fig. 2. Effect of cAMP on the short-circuit current across rabbit colon. *See* legend, Fig. 1 for solution composition. cAMP added to both bathing media (7.5 mM, final concentration) at the time indicated

solution could not be carried out due to the collapse of tissue resistance observed with any excess of EGTA concentration over that of Ca. Since Ca is thought to be an integral component of junctional complexes in epithelia [34], it is likely that this decline of tissue resistance with excess EGTA results from a removal of Ca from junctional regions which largely determine the passive conductance properties of the tissue under normal conditions [15].

The results of similar experiments, performed using cAMP as the secretory stimulus, are illustrated in Fig. 2. In contrast to the results obtained with the ionophore, cAMP elicited equal increases in the I_{sc} of tissues exposed to control or EGTA-containing media. Thus, the effects of the cyclic nucleotide are independent of the free Ca concentration of the bathing solutions over this range.

Effect of A23187 on cAMP Content

The cAMP levels of colonic mucosa were determined under conditions identical to those of the experiments illustrated in Fig. 1. The tissues were taken for analysis at times corresponding to the maximal increase in short-circuit current which resulted from ionophore or Ca addition, and the results are given in Table 2. The cAMP levels observed in the presence and absence of A23187 do not differ significantly at either 1.2 mM or 10^{-6} M free Ca concentration; nor was a significant difference observed when the free Ca concentration of media containing EGTA

Table 2. Effect of A23187 and theophylline on cAMP levels of colonic mucosa

	cAMP (pmoles/mg)
Control (1.2 mM Ca)	0.71 ± 0.04
+A23187 (0.1 μg/ml)	0.71 ± 0.05
Control (10^{-6} M Ca)	0.61 ± 0.05
+A23187 (0.1 μg/ml)	0.64 ± 0.05
+ Ca (10^{-3} M)	0.80 ± 0.09
Control (1.2 mM Ca)	0.69 ± 0.06
+ Theophylline (10 mM)	1.38 ± 0.10[a]

See text for details. Mean values in each group from six determinations.
[a] Significant difference from control value, $p < 0.05$.

and A23187 was returned to 10^{-3} M ($p > 0.1$). The response to theophylline is shown in the lower portion of Table 2. Theophylline, which elicits a secretory response identical to that observed with cAMP (data not shown), increased tissue cAMP levels twofold.

Effect of cAMP on Ca Exchange by Rabbit Colon

The effect of cAMP on Ca efflux from strips of colonic mucosa preloaded with ^{45}Ca is illustrated in Fig. 3; the results of a typical experiment are shown. A threefold increase in the efflux rate coefficient for

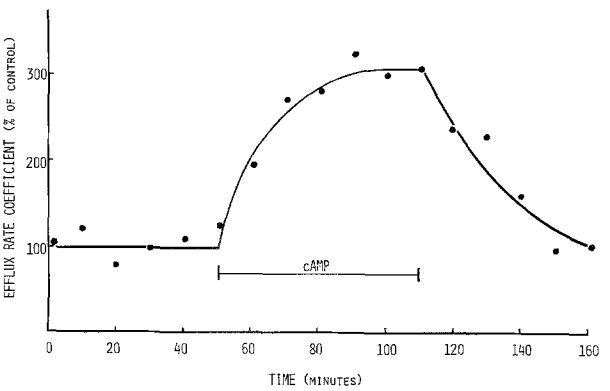

Fig. 3. Effect of cAMP on the rate coefficient for Ca efflux from strips of colonic mucosa. *See* text for details. Experimental tissues exposed to cAMP-containing media (7.5 mM) during the period indicated

Ca was observed in experimental tissues during exposure to the cyclic nucleotide. The time-course of the rise and fall of the efflux rate coefficient in response to cAMP varied among tissues from different animals; however, a similar magnitude of increase was observed in all studies and the efflux rate coefficient consistently returned to control values when tissues were returned to cAMP-free media.

Discussion

Addition of the ionophore, A23187, to the solution bathing the mucosal surface of isolated descending rabbit colon reproduces, in almost all respects, the effects of cAMP on electrolyte transport by this tissue. This response is characterized by an increase in short-circuit current and transepithelial electrical conductance, and a reversal of active Cl absorption to active Cl secretion. As with cAMP, the reversal of net Cl movement in the presence of ionophore is attributable to a significant increase in the unidirectional flux of Cl from serosa-to-mucosa. Despite the similarity of the responses to cAMP and ionophore, A23187 did not influence the cAMP levels of colonic mucosa, so that the ionophore-induced secretion is not mediated by an increase in mucosal cAMP.

The secretory response to A23187 requires a critical level of Ca concentration in the bathing media; reducing the free Ca concentration to 10^{-6} M with EGTA abolished the ionophore-induced increase in I_{sc}. In this regard, the effects of cAMP and A23187 differ, inasmuch as the response to exogenous cAMP was not affected by a similar reduction in external free Ca concentration. Thus, although A23187 displays affinity for other divalent cations (in particular, Mg [30]), addition of a relatively specific chelator of Ca to the bathing solutions abolished the secretory response to ionophore. In all probability, then, the stimulation of Cl secretion elicited by A23187 results from an elevation of free cytoplasmic Ca concentration. The studies of Babcock *et al.* [1] suggest that the ionophore is capable of increasing cytosolic Ca by facilitating Ca entry across the plasma membrane and/or by releasing Ca from mitochondria. The results of the present study favor the former alternative in this instance because the secretory response requires an adequate free Ca concentration in the bathing media, and presumably a gradient for Ca entry into the cell. However, the possibility that an effective level of cytoplasmic Ca results from both ionophore-stimulated entry and release from intracellular stores cannot be excluded.

A similar requirement for Ca in the bathing medium has been observed for a variety of systems where A23187 has been found to mimic the response to added cAMP or theophylline. Thus, catecholamine release from the adrenal medulla [16], insulin secretion by isolated pancreatic islets [37], amylase secretion by exocrine pancreas [36], fluid secretion by fly salivary gland [27], and electrolyte secretion by rabbit ileum [4] are processes which can be stimulated by cAMP or A23187, but the effects of ionophore are reduced or abolished in the absence of external Ca. Of particular relevance to the present study are the findings of Bolton and Field [4]. These investigators demonstrated that the effects of A23187 on electrolyte transport by rabbit ileum were similar to those of cAMP or theophylline, and that the ionophore had no effect on endogenous cAMP levels of ileal mucosa. In addition, removal of Ca from the bathing media abolished the secretory response of ileum to A23187 but not to theophylline. Although the underlying explanation for these parallel findings may be similar, it should be stressed that the characteristics of electrolyte transport in these tissues and their alterations by cAMP differ significantly. Thus, the cyclic nucleotide either abolishes active Na absorption [22] or promotes active Na secretion by rabbit ileum [32] whereas cAMP has no effect on Na transport by rabbit colon [15]. In rabbit ileum, cAMP promotes active Cl secretion due to an increase in serosa-to-mucosa Cl flux and a decrease in mucosa-to-serosa Cl flux [13], whereas its effects on Cl transport by rabbit colon can be entirely attributed to an increase in the serosa-to-mucosa flux. The electrical conductance across rabbit ileum is decreased but that across rabbit colon is increased. Frizzell *et al.* [14, 15] have suggested possible explanations for the different responses of ileum and colon to cAMP. Nonetheless, the important point is that, regardless of underlying transport mechanisms, the effects of cAMP in both tissues are closely mimicked by A23187.

The stimulation of active Cl secretion elicited by cAMP or A23187 in rabbit colon appears to result from stimulation of a current-generating Cl secretory process which is independent of active Na absorption. The results of previous studies [15] suggested that the cAMP-induced increment in short-circuit current could be attributed solely to the rate of active Cl secretion, and the results given in Table 2 indicate that A23187 has no effect on the bidirectional fluxes of Na across the tissue. The physiologic role of this secretory process in mammalian colon has not been identified at present. Waldman and Makhlouf [35] have observed a cAMP-mediated decrease in colonic fluid transport in the presence

of vasoactive intestinal polypeptide and secretin. However, it is not yet clear that this response represents a physiologically important control over electrolyte transport by the large intestine. Entry of excess bile salts into the colonic lumen is known to result in a significant electrolyte and water secretion [21]. Several investigators have suggested that bile salts elicit colonic secretion by stimulating cAMP production in rat [3] and rabbit [11] colonic mucosa. The results of the present study suggest that agents which are capable of increasing membrane permeability for Ca could result in colonic electrolyte secretion that is not mediated by an increase in intracellular cAMP. Thus, it is possible that the effect of bile salts may in some way be related, at least in part, to a "detergent action" which increases the permeability of the mucosal membrane permitting Ca to enter the cells down a steep electrochemical gradient for this ion. Studies on the dependence of the effect of bile salts on free Ca concentrations in the luminal solution might assist in resolving this possibility.

The mechanism by which an elevation of cytoplasmic Ca concentration might induce active Cl secretion is unknown; however, several possibilities exist. The finding that A23187 had no effect on cAMP levels of colonic mucosa seems to rule out the possibility that Ca significantly influenced adenylate cyclase or phosphodiesterase activities under the conditions of these studies. The ionophore also stimulates secretion by fly salivary gland [27] and rabbit ileum [4] without altering intracellular cAMP. However, a Ca-dependent stimulation of some component of the cAMP-dependent pathway remains a possibility. For example, the active form of muscle phosphorylase b kinase (the phosphoprotein product of cAMP-dependent phosphorylase b kinase kinase activity) is activated by Ca [24].

The effect of A23187 could be mediated by an increase in cyclic 3′,5′-GMP (cGMP) levels. Guanylate cyclase is a predominantly cytoplasmic enzyme which is Ca-dependent, and A23187 has been shown to increase cGMP levels in a number of tissues [9, 10, 33]. However, in many systems studied to date, the effects of cGMP generally oppose those of cAMP [18]. Thus, in rabbit ileal mucosa, norepinephrine results in a transient increase in cGMP levels [8] and the response of this tissue to catecholamines is an increase in NaCl absorption, whereas cAMP and A23187 promote electrolyte secretion [4, 13]. A similar lack of correlation between cGMP levels and physiologic response has recently been reported by Fain *et al.* [12] with regard to glycogenolysis in isolated rat liver cells. At concentrations which did not affect cAMP, both insulin

and glucagon were found to increase cGMP levels; however, glucagon promoted glycogenolysis while insulin inhibited glycogenolysis.

Finally, Ca may act as a direct activator of the transport process responsible for Cl secretion. The secretory response to cAMP is not affected by reducing the external free Ca concentration to 10^{-6} M, and a reversible increase in Ca efflux is observed in the presence of the cyclic nucleotide. Similar results have been obtained in several epithelia where Ca has been implicated as an intracellular mediator of "stimulus-secretion coupling". Cyclic AMP increases Ca efflux from fly salivary gland [26], isolated kidney cells [6] and isolated pancreatic acinar cells [10]. These findings suggest that cAMP itself may elicit electrolyte secretion in rabbit colon by releasing Ca from intracellular stores. However, direct evidence for a cAMP-mediated rise in cytoplasmic free Ca concentration has not been presented and the identity of the storage site(s) which might be affected by cAMP is unknown at the present time.

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