Synergism between Calcium-Mediated and Cyclic AMP-Mediated Activation of Chloride Secretion in Isolated Guinea Pig Distal Colon

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Abstract Synergistic effects of Ca\(^{2+}\)-mediated secretagogues and cyclic AMP-mediated secretagogues on Cl\(^{-}\) secretion by guinea pig distal colon were studied in vitro using Ussing chambers. Short-circuit current (\(I_{sc}\)) and bidirectional fluxes of \(^{22}\)Na\(^{+}\) and \(^{36}\)Cl\(^{-}\) were measured. Bethanechol (10\(^{-4}\) M, a Ca\(^{2+}\)-mediated secretagogue) caused an increase in \(I_{sc}\), which was enhanced 4–20 fold when the tissue was pretreated with prostaglandin E\(_2\) (10\(^{-7}\)–10\(^{-6}\) M), vasoactive intestinal polypeptide (5 x 10\(^{-9}\)–10\(^{-8}\) M) (cyclic AMP-mediated secretagogues), or 8-bromocyclic AMP (5 x 10\(^{-4}\)–2 x 10\(^{-3}\) M). Measurement of \(^{36}\)Cl flux showed that the increase in \(I_{sc}\) in the presence of both 8-bromocyclic AMP and bethanechol resulted principally from increased Cl\(^{-}\) secretion. On the other hand, the net absorptive flux of \(^{22}\)Na was not influenced under these conditions. Potentiation of the increase in \(I_{sc}\) was also elicited by the combination of A23187 (5 x 10\(^{-6}\) M) and 8-bromocyclic AMP (10\(^{-3}\) M). The results are consistent with the notion that simultaneous activation of the cyclic AMP-mediated and Ca\(^{2+}\)-mediated systems produces a synergistic increase in colonic Cl\(^{-}\) secretion.

Key words: Ca\(^{2+}\), colon, cyclic AMP, intestinal secretion, potentiation.

Electrogenic Cl\(^{-}\) transport from the serosa into the intestinal lumen is the primary event of intestinal secretion (Frizzell et al., 1979; Heintze et al., 1983). Various substances are capable of activating the process through the mediation of either intracellular cyclic nucleotide generation, such as cyclic AMP and cyclic GMP, or an increase in cytosolic free Ca\(^{2+}\) (for reviews, see Donowitz and Welsh, 1986, 1987). It is interesting to investigate whether these two pathways are independent or interact with each other. Multiple types of relationships between the
Ca\(^{2+}\) and cyclic AMP messenger system have been demonstrated in regulating cellular functions in a variety of tissues (RASMUSSEN, 1981; RASMUSSEN and BARRETT, 1984).

Recently, the synergistic interaction between Ca\(^{2+}\)-mediated and cyclic AMP-mediated secretagogues has been demonstrated in Cl\(^-\) secretion by the colonic cancer cell line T-84 (CARTWRIGHT et al., 1985; DHARMSATHAPHORN and PANDOL, 1986) and guinea pig ileum (COOKE et al., 1987). The aim of the present study was to investigate whether such synergistic interaction occurs in normal colonic mucosa in vitro. Some of the results of this study have been published in abstract form (YAJIMA et al., 1987).

MATERIALS AND METHODS

1. Tissue preparation. Female Hartley strain guinea pigs weighing 300-400 g were maintained on a pelleted diet (Type GM-1, Funabashi Farm, Chiba) ad libitum with free access to water until the time of the experiments. The animals were stunned by a blow to the head and bled to death. A 15-20 cm segment of the distal colon was obtained from 5 cm proximal to the anus. The colon was opened longitudinally into a flat sheet and the mucosa was separated from the underlying connective tissue and musculature using glass microscope slides. Histological studies revealed that the plane of division was between the muscularis mucosae and the submucosa (data not shown). The tissue segment was mounted vertically between Ussing-type chambers that provided an exposed area of 0.5 cm\(^2\). The volume of the bathing solution in each chamber was 10 ml and its temperature was kept at 37°C with a water jacket connected to a warmed bath.

The bathing solution contained (mM): NaCl, 119; NaHCO\(_3\), 21; K\(_2\)HPO\(_4\), 2.4; KH\(_2\)PO\(_4\), 0.6; CaCl\(_2\), 1.2; MgCl\(_2\), 1.2; glucose, 10. The solutions were gassed with a mixture of 95% O\(_2\) and 5% CO\(_2\). For high-K\(^+\) solution, 100 mM KCl was added in place of 100 mM NaCl (total K\(^+\) concentration: 105.4 mM).

2. Short-circuit current and flux measurements. The short-circuit current (I\(_{sc}\)) was measured using an automatic voltage clamping device (DVC-1000, W-P Instrument, New Haven, CT, U.S.A.) that compensates for solution resistance between the potential measuring electrodes. The transepithelial potential was recorded through 3 M KCl-agar bridges connected to a pair of calomel half cells, and the transepithelial current was applied across the tissue via a pair of Ag/AgCl electrodes that was kept in contact with the mucosal and serosal bathing solution using a pair of 3 M KCl-agar bridges. All the experiments were done under short-circuit conditions and the transepithelial conductance (G\(_t\)) was determined intermittently from the change in current required to clamp transepithelial potential from zero to 2.5 mV briefly (2 s), using Ohm’s law. The I\(_{sc}\) is referred to as positive when current flows from mucosa to serosa.

In most cases, we performed several kinds of measurements on one preparation. The tissue was washed with fresh solution several times and 20–30 min was
allowed before the start of the next measurement.

Unidirectional fluxes of Cl\(^{-}\) and Na\(^{+}\) were measured separately with \(^{36}\)Cl and \(^{22}\)Na. The mucosal-to-serosal flux and the serosal-to-mucosal flux were determined using adjacent tissues. The difference in \(G_t\) between the pair of tissues was within 30\%. Thirty min were allowed for equilibration after the bathing solution of one side of the tissue was labeled with \(^{36}\)Cl or \(^{22}\)Na. Six samples (0.5 ml) were taken from the unlabeled side at 10-min intervals and replaced with an equal volume of the unlabeled solution. The drugs were added 20 min after the start of sampling. Just prior to the first sampling, 20 \(\mu\)l was taken from the labeled side. The radioactivity was counted by liquid scintillation procedures.

In all experiments, tetrodotoxin (10\(^{-7}\) M) and indomethacin (10\(^{-6}\) M, except one experiment) were added to prevent possible influences by tonic nerve activity and by endogenous production of prostaglandins, respectively. When the effects of A23187 were examined, 10\(^{-5}\) M of indomethacin was used, since A23187 has been shown to strongly activate prostaglandin synthesis in the colon (Smith and McCabe, 1984; Suzuki, unpublished observation).

3. Drugs. Bethanechol chloride, atropine sulfate, indomethacin, prostaglandin E\(_2\), A23187, and 8-bromocyclic AMP were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Vasoactive intestinal polypeptide (VIP) was obtained from Protein Institute (Osaka). Tetrodotoxin and bumetanide (Lunetoron\(^{R}\)) were obtained from Sankyo Seiyaku (Tokyo). \(^{36}\)Cl and \(^{22}\)Na were purchased from NEN Products (Boston, MA, U.S.A.).

All these drugs were applied to the serosal bathing solution from an appropriate stock solution. Prostaglandin E\(_2\), A23187, and indomethacin were dissolved in ethanol and the others were dissolved in water.

4. Statistics. The results are given as mean ± S.E. unless otherwise specified \((n = \) number of tissue preparations). Measurements for each experimental condition were done on not more than two tissue preparations obtained from one animal and the same measurement was repeated using other animals. Thus, the number of animal examined was equal to or more than half of the \(n\) values in all experiments. The statistical significance was evaluated using the paired or unpaired Student’s \(t\)-test and a value of \(p < 0.05\) was considered significant. Paired samples were those obtained from the same tissue.

RESULTS

Electrical properties of the tissues attained a stable value within 30 min after the start of incubation. The \(I_{sc}\) and \(G_t\) under basal conditions were \(-5.7 ± 3.1 \ \mu\)A·cm\(^{-2}\)·h\(^{-1}\) and \(7.4 ± 2.2 \ \text{mS}·\text{cm}^{-2}\), respectively (S.D., \(n = 24\)).

1. Potentiation of \(I_{sc}\) response to bethanechol by pretreatment with VIP, prostaglandin \(E_2\), or 8-bromocyclic AMP

Bethanechol, a cholinergic muscarinic agonist, elicited an increase in \(I_{sc}\), which
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was, in some cases, followed by a slight and slow decline (Fig. 1a). An increase in $G_i$ was associated with this $I_{sc}$ change, though it was apparent only at high concentrations of bethanechol ($10^{-4}$–$10^{-3}$ M).

When the tissue was pretreated with VIP or prostaglandin E₂, the response to bethanechol was markedly enhanced, as shown in Fig. 1b and c. The presence of VIP ($5 \times 10^{-9}$–$10^{-8}$ M) or prostaglandin E₂ ($10^{-7}$–$10^{-6}$ M) potentiated the bethanechol-induced increase in $I_{sc}$ 7–11 fold ($n=3$) or 4–20 fold ($n=4$), respectively. The $G_i$ increment by bethanechol was also augmented in the presence of VIP or prostaglandin E₂ (data not shown).

The $I_{sc}$ responses to VIP or prostaglandin E₂ alone were the initial increase followed by a gradual decrease, in most cases, to below the base line level. During the increasing phase the $G_i$ also increased, and further increase in the $G_i$ was observed during the decreasing phase of $I_{sc}$. The decrease in $I_{sc}$ that was associated with an increase in $G_i$ was quite similar to the response to 8-bromocyclic AMP (see below).

Evidence is accumulating that VIP and prostaglandin E₂ cause an elevation of intracellular cyclic AMP (Kimberg et al., 1971, 1974; Schwartz et al., 1974). Thus, we next examined whether cyclic AMP added exogenously can similarly enhance the bethanechol-induced $I_{sc}$ change, using 8-bromocyclic AMP. As shown in Fig. 2, 8-bromocyclic AMP at the concentration of $10^{-3}$ M caused a decrease in $I_{sc}$ which was accompanied by an increase in $G_i$ in most of the tissues used in the present experiment. The bethanechol-induced increase in both $I_{sc}$ and $G_i$ was markedly potentiated by pretreatment of the tissue with 8-bromocyclic AMP. The results of nine such measurements are summarized in Table 1A. Bethanechol-induced in-

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Creases in ISO and Gt were enhanced as much as 8 and 11 fold, respectively, in the presence of 10^-3 M 8-bromocyclic AMP. The induction of the marked increase in ISO did not depend on the order of application of bethanechol and 8-bromocyclic AMP. As shown in Fig. 3, when the tissue was pretreated with bethanechol the ISO response to 8-bromocyclic AMP was altered from a decrease to a marked increase to a level essentially similar to that observed in Fig. 2. The increase in Gt was also potentiated. Addition of atropine (10^-6 M) to the serosal bathing solution promptly reduced the ISO and the Gt to the level which might have been attained when 8-bromocyclic AMP was applied alone, indicating that muscarinic receptor activation is an essential process for this potentiation phenomenon. The mean values of four such experiments are summarized in Table 1B.

2. Mucosal high-K+ condition

In the rabbit and the guinea pig colon, electrogenic K+ secretion, which is stimulated by β-adrenergic agonist (probably mediated by cyclic AMP), has been demonstrated (HALM and FRIZZELL, 1986; Plass et al., 1986; SMITH and McCabe, 1986; Ishida and Suzuki, 1987). It is likely that the decrease in ISO induced by 8-bromocyclic AMP as shown above is mainly due to enhancement of K+ secretion. In the following experiments, we bathed the mucosal side with high-K+ (105.4 mM) solution to inhibit K+ secretion so that we could show changes caused by Cl- secretion more clearly. Actually, stimulation of K+ secretion by isoproterenol is

Fig. 2. Potentiation of the responses of ISO and Gt to bethanechol (BCH) in the presence of 8-bromocyclic AMP (8-Br-cAMP). A representative recording. Bethanechol was added to the 8-Br-cAMP-treated tissue when the ISO response to 8-Br-cAMP attained stability (2–3 min).
Fig. 3. Responses of $I_{sc}$ and $G_i$ to 8-bromocyclic AMP (8-Br-cAMP) in the presence and absence of bethanechol (BCH) and the effect of atropine. A representative recording. 8-Br-cAMP was added to the BCH-treated tissue when the $I_{sc}$ response to BCH attained its peak (about 1 min). The increase in $I_{sc}$ induced by 8-Br-cAMP in the presence of BCH became apparent approximately 1 min after application.

Table 1. Changes in $I_{sc}$ and $G_i$ caused by bethanechol and 8-bromocyclic AMP.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>$\Delta I_{sc}$ ($\mu$A·cm$^{-2}$·h$^{-1}$)</th>
<th>$\Delta G_i$ (mS·cm$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1) Bethanechol ($10^{-4}$M)</td>
<td>35 ± 10</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>2) 8-Bromocyclic AMP ($10^{-3}$M)</td>
<td>$-56 \pm 6$</td>
<td>4.8 ± 0.4</td>
</tr>
<tr>
<td>+ Bethanechol ($10^{-4}$M)</td>
<td>$239 \pm 29$</td>
<td>9.1 ± 0.6</td>
</tr>
<tr>
<td>(Bethanechol-induced change)</td>
<td>$295 \pm 29^{a}$</td>
<td>$4.3 \pm 0.4^{a}$</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1) 8-Bromocyclic AMP ($10^{-3}$M)</td>
<td>$-36 \pm 15$</td>
<td>4.5 ± 0.4</td>
</tr>
<tr>
<td>2) Bethanechol ($10^{-4}$M)</td>
<td>$13 \pm 5$</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>+ 8-Bromocyclic AMP ($10^{-3}$M)</td>
<td>$176 \pm 53$</td>
<td>8.1 ± 1.6</td>
</tr>
<tr>
<td>(8-Bromocyclic AMP-induced change)</td>
<td>$163 \pm 51^{b}$</td>
<td>$7.8 \pm 1.5^{c}$</td>
</tr>
<tr>
<td>+ Atropine ($10^{-6}$M)</td>
<td>$-36 \pm 7$</td>
<td>5.7 ± 1</td>
</tr>
</tbody>
</table>

Experimental protocols for determining the values in part A and B are given in Figs. 2 and 3, respectively. $\Delta I_{sc}$ and $\Delta G_i$ are differences from the base line except those in parentheses. $^{a)}p<0.01$ compared with bethanechol alone. $^{b)}0.01<p<0.05$ compared with 8-bromocyclic AMP alone. $^{c)}$Although statistically insignificant (0.1 $<p<0.2$, paired $t$-test), the values were larger than those with 8-bromocyclic AMP alone in all tissues measured. $n=9$ for part A and $n=4$ for part B. Paired $t$-test.

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mostly abolished under the mucosal high-K⁺ condition (ISHIDA and SUZUKI, 1987). The basal $I_{sc}$ and $G_{t}$ in the presence of mucosal high-K⁺ solution were $99 \pm 26 \mu A \cdot cm^{-2}$ and $13.1 \pm 1.4 mS \cdot cm^{-2}$, respectively ($n = 14$). As is shown in Fig. 4a, under the mucosal high-K⁺ condition, $10^{-4} M$ bethanechol induced an increase in $I_{sc}$ which is similar to the response under the control condition. The addition of bumetanide ($5 \times 10^{-5} M$), an inhibitor of Cl⁻ secretion, partially inhibited this response. The increase in $I_{sc}$ caused by bethanechol was $30 \pm 8 \mu A \cdot cm^{-2}$, which was reduced to $16 \pm 8 \mu A \cdot cm^{-2}$ by bumetanide (both values are differences from the pre-stimulation level, $n = 6$). Bumetanide did not change $I_{sc}$ in the absence of stimulants. On the other hand, the 8-bromocyclic AMP-induced change in $I_{sc}$ was altered from a decrease under control condition to an increase by bathing the mucosal side with high-K⁺ solution (Fig. 4b). This increase was partially inhibited by bumetanide. The increase in $I_{sc}$ induced by $2 \times 10^{-3} M$ 8-bromocyclic AMP was $83 \pm 21 \mu A \cdot cm^{-2}$, which was reduced to $27 \pm 3 \mu A \cdot cm^{-2}$ by bumetanide ($n = 5$).

A marked potentiation of the bethanechol-induced increase in $I_{sc}$ in the presence of 8-bromocyclic AMP was elicited under the mucosal high-K⁺ conditions (Fig. 4c). This increase was mostly inhibited by bumetanide. The total change in $I_{sc}$ caused by the simultaneous presence of $2 \times 10^{-3} M$ 8-bromocyclic AMP and $10^{-4} M$
bethanechol was 438 ± 133 (the bethanechol-induced increase was 392 ± 116) µA · cm⁻², which was reduced by bumetanide to 71 ± 29 µA · cm⁻² (n = 3). These findings suggest that bethanechol and 8-bromocyclic AMP can stimulate Cl⁻ secretion independently and when these secretagogues are applied simultaneously, Cl⁻ secretion is markedly potentiated.

3. Cl⁻ and Na⁺ fluxes
   In order to confirm the transport processes responsible for these potentiated increases in $I_{sc}$, we measured the bidirectional fluxes of $^{36}$Cl and $^{22}$Na. Figure 5A illustrates that the addition of 8-bromocyclic AMP (5 x 10⁻⁴ M) plus bethanechol (10⁻⁴ M) caused an increase in the unidirectional flux of Cl⁻ from serosa to mucosa while that from mucosa to serosa was not affected significantly. The change in serosal-to-mucosal flux agreed well with the concomitant increase in $I_{sc}$ (Fig. 5B). On the other hand, similar stimulation (10⁻³ M 8-bromocyclic AMP plus 10⁻⁴ M bethanechol) failed to alter the net absorptive flux of Na⁺ (mucosal-to-serosal flux minus serosal-to-mucosal flux) significantly (7.35 ± 3.05 µmol·cm⁻²·h⁻¹, mean value of two flux periods before stimulation, vs. 6.43 ± 3.72 µmol·cm⁻²·h⁻¹, mean...
value of three flux periods after stimulation, \( p > 0.5, n = 7 \), paired \( t \)-test). These results substantiate that the marked increase in \( I_{\text{sc}} \) elicited by the simultaneous application of bethanechol and 8-bromocyclic AMP results primarily from the activation of \( Cl^- \) secretion.

4. Dose-dependency

Bethanechol-induced changes in \( I_{\text{sc}} \) at various concentrations of bethanechol were determined in the absence and the presence of \( 10^{-3} \) M 8-bromocyclic AMP, and the result is summarized in Fig. 6. Potentiation by 8-bromocyclic AMP was elicited at most of the concentrations of bethanechol examined. The half-maximum dose of bethanechol was not noticeably affected by the 8-bromocyclic AMP treatment. LUNDBERG et al. (1982) suggested that the potentiating effect of VIP on acetylcholine-induced saliva secretion involves an increase in the affinity of muscarinic receptors. This possibility may not be the main mechanism of the potentiation in the colon.

Figure 7 shows the changes in \( I_{\text{sc}} \) induced by 8-bromocyclic AMP and the
potentiation of bethanechol-induced changes in $I_{sc}$ by 8-bromocyclic AMP at various concentrations of 8-bromocyclic AMP. 8-Bromocyclic AMP alone caused a decrease in $I_{sc}$ at concentrations of $10^{-5}$ to $10^{-4}$ M. At higher concentrations than these, however, the $I_{sc}$-response to 8-bromocyclic AMP exhibited a rather smaller decrease or in some cases an increase (e.g. Fig. 9). 8-Bromocyclic AMP failed to potentiate the $I_{sc}$ increase caused by bethanechol at the concentration of $10^{-5}$ to $10^{-4}$ M. However, at $5 \times 10^{-4}$ M or higher concentrations, it markedly potentiated the $I_{sc}$ response to bethanechol. Figure 7 shows, in addition, the result of experiments on dose-dependency of the effect of 8-bromocyclic AMP under the mucosal high-$K^+$ condition. In the presence of high concentration of $K^+$ in the mucosal bathing solution, 8-bromocyclic AMP increased $I_{sc}$ at $5 \times 10^{-4}$ M or higher concentrations. On the other hand, the potentiating effect of 8-bromocyclic AMP on bethanechol-induced increase in $I_{sc}$ were indistinguishable between the control and the mucosal high-$K^+$ condition.
Interaction between A23187 and 8-bromocyclic AMP

An increase in cytosolic free Ca²⁺ is believed to play an important part in the intermediate processes from the muscarinic receptor activation to Cl⁻ secretion in the intestinal epithelium (Bolton and Field, 1977; Donowitz, 1983; Dharmsathaphorn and Pandol, 1986). Thus, we examined whether the application of A23187, a Ca ionophore, can mimic the effect of bethanechol on 8-bromocyclic AMP-induced Iₑc changes. Figure 8 shows that when the tissue was pretreated with A23187 the 8-bromocyclic AMP-induced change in Iₑc was altered from a decrease to a marked increase, similar to the effect of bethanechol. The Gᵢ increment by 8-bromocyclic AMP was also augmented in the presence of A23187. The mean values of five such measurements are summarized in Table 2.

### Table 2. Effect of A23187 on 8-bromocyclic AMP-induced change in Iₑc and Gᵢ

<table>
<thead>
<tr>
<th>Conditions</th>
<th>ΔIₑc (µA·cm⁻²·h⁻¹)</th>
<th>ΔGᵢ (mS·cm⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) 8-Bromocyclic AMP (10⁻³ M)</td>
<td>-25 ± 15</td>
<td>4.5 ± 0.6</td>
</tr>
<tr>
<td>2) A23187 (5 × 10⁻⁶ M)</td>
<td>9 ± 2</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>+ 8-Bromocyclic AMP (10⁻³ M)</td>
<td>256 ± 26</td>
<td>9.2 ± 0.6</td>
</tr>
<tr>
<td>(8-Bromocyclic AMP-induced change)</td>
<td>247 ± 27ᵃ</td>
<td>8.6 ± 0.5ᵃ</td>
</tr>
</tbody>
</table>

Experimental protocol for determining the values is given in Fig. 8. A23187 was applied 10 min before the addition of 8-bromocyclic AMP. ΔIₑc and ΔGᵢ are differences from the base line except those in parentheses.ᵃ p < 0.01 compared with the absence of A23187. n = 5. Paired t-test.

5. **Interaction between A23187 and 8-bromocyclic AMP**

An increase in cytosolic free Ca²⁺ is believed to play an important part in the intermediate processes from the muscarinic receptor activation to Cl⁻ secretion in the intestinal epithelium (Bolton and Field, 1977; Donowitz, 1983; Dharmsathaphorn and Pandol, 1986). Thus, we examined whether the application of A23187, a Ca ionophore, can mimic the effect of bethanechol on 8-bromocyclic AMP-induced Iₑc changes. Figure 8 shows that when the tissue was pretreated with A23187 the 8-bromocyclic AMP-induced change in Iₑc was altered from a decrease to a marked increase, similar to the effect of bethanechol. The Gᵢ increment by 8-bromocyclic AMP was also augmented in the presence of A23187. The mean values of five such measurements are summarized in Table 2.

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The above results suggest that the combination of two groups of secretagogues, one presumably increasing cyclic AMP and the other presumably increasing Ca²⁺, results in the potentiation of Cl⁻ secretion. We finally examined whether or not such synergistic interaction occurs among the secretagogues of the same groups. 8-Bromocyclic AMP at the concentration of 10⁻³ M did not cause noticeable potentiation, if any, either in prostaglandin E₂-induced ISO response or VIP-induced ISO response (Fig. 9A and B, n = 3 for each drug). The effect of A23187 (5 x 10⁻⁶ M) on bethanechol-induced response was also scarcely detectable (Fig. 9C, n = 4).

DISCUSSION

The results of this study demonstrate the synergistic interaction between Ca²⁺ and cyclic AMP messenger systems in activating Cl⁻ secretion in the isolated colonic mucosa from the guinea pig. We applied 8-bromocyclic AMP and bethanechol to activate cyclic AMP-mediated and Ca²⁺-mediated processes, respectively (BOLTON and FIELD, 1977; DONOWITZ, 1983; DHARMSATHAPHORN and PANDOL, 1986). When both stimulants are present, increases in ISO and GI induced by bethanechol were remarkably potentiated (Table 1). The potentiated increase in ISO was essentially due to the increase in Cl⁻ flux from serosa to mucosa (Fig. 5). In addition, the bethanechol-induced increase in ISO was markedly enhanced in the

Fig. 9. Interaction between prostaglandin E₂ (PGE₂) and 8-bromocyclic AMP (8-Br-cAMP) (A), between VIP and 8-Br-cAMP (B), and between A23187 and bethanechol (BCH) (C). Representative recordings.
presence of VIP or prostaglandin E$_2$ (Fig. 1), both being known to increase Cl$^-$ secretion through the generation of cyclic AMP (Kimberg et al., 1971, 1974; Schwartz et al., 1974; Laburthe et al., 1979). Furthermore, the potentiation of $I_{sc}$ increase could also be elicited by the combination of 8-bromocyclic AMP and Ca$^{2+}$ ionophore A23187 (Table 2). These findings are consistent with the idea that synergistic interaction does occur between cyclic AMP-mediated and Ca$^{2+}$-mediated Cl$^-$ secretion.

Although the cellular and molecular processes underlying the potentiation of Cl$^-$ secretion observed in the present preparation are yet to be explored, the augmentation of $I_{sc}$ increase by the simultaneous addition of 8-bromocyclic AMP and A23187 suggests that at least synergistic interaction takes place in the process after the increases in cyclic AMP and Ca$^{2+}$.

It seems most likely that the synergistic interaction demonstrated in the present study occurs within the Cl$^-$-secreting epithelial cells. Secretagogues used in the present study, i.e. VIP, prostaglandin E$_2$, and bethanechol, have all been shown to interact with specific receptors on the enterocyte (Prieto et al., 1979; Laburthe et al., 1979; Rimele et al., 1981; Smith et al., 1987). However, the mucosal preparation used in the present study consists of not only Cl$^-$-secreting epithelial cells but also other cell types, e.g. fibroblasts, immune cells, neurons, enteroendocrine cells, etc. These latter cell types might also be activated by the substances used in this study leading to alteration of the function of the epithelial cells. We can eliminate the involvement of nerve excitation and prostaglandin synthesis, since, in the present experiments, tetrodotoxin and indomethacin were added to the bathing solution. However, we cannot entirely exclude the possibility that substances from enteroendocrine cells, immune cells, and nerve terminals are released and have some influence on the potentiation of Cl$^-$ secretion observed in the present study.

Synergistic interaction of two messenger systems in the activation of intestinal secretion has been reported. In guinea pig ileal mucosa, VIP enhanced the increase in short-circuit current evoked by bethanechol. In addition, VIP and other substances that stimulate secretion by increasing cyclic AMP were shown to potentiate the cholinergic phase in the chloride secretory responses evoked by neural stimulation (Cooke et al., 1987). Dharmsathaphorn and his colleagues have clearly shown in the colonic cancer cell line T-84 that potentiation of Cl$^-$ secretion occurs in the presence of both Ca$^{2+}$-mediated secretagogues (carbachol and A23187) and cyclic AMP-mediated secretagogues (prostaglandin E$_2$ and VIP) (Cartwright et al., 1985; Dharmsathaphorn and Pandol, 1986). They demonstrated that the increase in free cytosolic Ca$^{2+}$ caused by carbachol was not influenced by the addition of prostaglandin E$_2$ or VIP and that the increase in cyclic AMP by prostaglandin E$_2$ and VIP was not influenced by the addition of carbachol, indicating that the potentiation cannot be explained by changes in either Ca$^{2+}$ or cyclic AMP alone (Dharmsathaphorn and Pandol, 1986). Furthermore, they showed that there are two kinds of K$^+$ channels in the basolateral membrane, one activated through the generation of cyclic AMP and the other activated by the
elevation of cytosolic Ca\(^{2+}\). Since the K\(^+\) channels in the basolateral membrane play a supportive role in Cl\(^-\) secretion, they suggest that the activation of both channels concomitantly would markedly enhance the Cl\(^-\) secretion (McROBERTS et al., 1985; CARTWRIGHT et al., 1985; DHARMSATHAPHORN and PANDOL, 1986). To determine whether a similar mechanism is responsible for the potentiation of Cl\(^-\) secretion in the guinea pig colon would be interesting and remains for future study.

The addition of 8-bromocyclic AMP caused a decrease in \(I_{sc}\) which was associated with a marked increase in \(G_t\) (Fig. 2). This decrease in \(I_{sc}\) is mostly due to activation of electrogenic K\(^+\) secretion, since the mucosal high-K\(^+\) condition abolished the decrease in \(I_{sc}\). In rabbit and guinea pig colons \(\beta\)-adrenergic agonist, which elevates intracellular cyclic AMP concentration, has been shown to elicit a decrease in \(I_{sc}\) associated with an increase in \(G_t\), both of which are due to stimulation of K\(^+\) secretion (SMITH and MCCABE, 1986; Plass et al., 1986; Halm and Frizzell, 1986; Ishida and Suzuki, 1987). The decreases in \(I_{sc}\) together with the \(G_t\) increase after a peak configuration in response to VIP or prostaglandin E\(_2\) (Fig. 1) are also likely, at least in part, a consequence of the activation of K\(^+\) secretion. In rabbit colon, A23187, 8-bromocyclic AMP, and PGE\(_1\) (or PGE\(_2\)) have been shown to stimulate both K\(^+\) and Cl\(^-\) secretions (Mccabe and Smith, 1985). It is interesting that at higher concentrations of 8-bromocyclic AMP the decrease in \(I_{sc}\) was smaller when compared with low concentrations (Fig. 7). A similar dose-I\(_{sc}\) relation was observed in rabbit colon obtained during the night portion of the light:dark cycle (Halm and Frizzell, 1986). The interpretation of this finding could be that K\(^+\) secretion is activated at a relatively low concentration of cyclic AMP, while at a higher concentration Cl\(^-\) secretion is gradually activated resulting in cancellation of part of the \(I_{sc}\) decrease caused by K\(^+\) secretion. In the presence of mucosal high-K\(^+\) solution to suppress K\(^+\) secretion, the dose-dependent increase in \(I_{sc}\) produced by 8-bromocyclic AMP (Fig. 7) is consistent with this interpretation. It is not clear whether there are some interactions between K\(^+\) and Cl\(^-\) secretions under some conditions. However, the enhancement of K\(^+\) secretion is not involved in the synergistic interaction of Cl\(^-\) secretion induced by cyclic AMP and Ca\(^{2+}\), since the potentiation of \(I_{sc}\) response to bethanechol by 8-bromocyclic AMP was not influenced by mucosal high-K\(^+\) condition.

The synergic interaction is likely to play an important physiological function, since under most physiological conditions multiple secretagogues are assumed to participate simultaneously in stimulation of Cl\(^-\) secretion. For instance, many kinds of transmitter substances which are known to activate Cl\(^-\) secretion through Ca\(^{2+}\) or cyclic AMP have been demonstrated to exist in the nerve terminals in the mucosa (Schultzberg et al., 1980; Keast et al., 1985; Costa et al., 1987). Stimulation of the enteric nerve experimentally, e.g., by means of electrical field stimulation, was shown to activate Cl\(^-\) secretion by releasing at least two kinds of transmitters (acetylcholine plus others), since atropine only partially blocked this response (Hubel, 1985; Cooke et al., 1987; Kuwahara et al., 1987). Therefore, the potentiation might well occur when enteric nerves are activated physiologically.
Potentiation might also be important under some pathological conditions. For example, under inflammatory conditions, the production of eicosanoids (cyclic AMP-mediated), and the release of histamine (Ca2+-mediated), 5-hydroxytryptamine (Ca2+-mediated), and kinins (Ca2+- and cyclic AMP-mediated) may all be enhanced to some extent (CASTRO, 1982; LAWSON and POWELL, 1987; WHittle and VANE, 1987).

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