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# Restoration by VIP of the Carbachol-Stimulated $Cl^-$ Secretion in TTX-Treated Guinea Pig Distal Colon

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**Abstract:** To determine if vasoactive intestinal peptide (VIP) restores neural activity from tetrodotoxin (TTX) blockade, we studied the effects of VIP and related agents on carbachol (Cch)-induced $Cl^-$ secretion in control-isolated guinea pig distal colon and in that treated with TTX. The short circuit current ($I_{sc}$) increased dose-dependently after serosal applications of Cch ($10^{-6}$ – $2 \times 10^{-5}$ M) and VIP ($5 \times 10^{-9}$ – $10^{-7}$ M). But no additive or synergistic increase in $I_{sc}$ was observed. Cch- and VIP-induced $I_{sc}$ was completely abolished by a serosal application of TTX ($10^{-6}$ M). However, a serosal application, not mucosal, of VIP ($10^{-7}$ M) and 8-bromo-cAMP ($10^{-3}$ M) restored the Cch-stimulated, TTX-inhibited $I_{sc}$ by 113% and 75.8%, respectively. Furthermore, mucosal and serosal applications of forskolin (adenylate cyclase activator) restored the $I_{sc}$ by 43.9% and 65.3%, respectively. The restored $I_{sc}$ was completely abolished by atropine (muscarinic receptor antagonist). These results suggest that VIP may restore the cholinergic activity by increasing the level of intracellular cAMP, and that cholinergic neuron is very likely to be responsible for the regulation of $Cl^-$ secretion at neuroepithelial junctions. The exact mechanism of VIP's effect on the TTX-inhibited epithelial $Cl^-$ secretion, and its possible usefulness in the treatment of TTX-induced pathophysiological conditions, remain to be determined. [The Japanese Journal of Physiology 55: 317–324, 2005]

*Key words*: tetrodotoxin, chloride secretion, cholinergic neuron, VIPergic neuron, forskolin, cAMP.

Under normal, non-inflammatory conditions, the intestinal transport of fluid and electrolytes is regulated by the enteric nervous system, especially the submucosal (Meissner's) plexus in combination with the myenteric plexus. The submucosal secretomotor neurons, made up by the cholinergic and vasoactive intestinal peptide (VIP)-ergic neurons, directly innervate the epithelial crypt cells [1, 2]. There is no doubt that acetylcholine (Ach) [3–9] and VIP [5, 6, 8–11] are neurotransmitters important for the regulation of intestinal secretion. However, it is still uncertain whether VIP directly acts at neuroepithelial junctions, or whether its action is mediated by the cholinergic neuron after potentiation [6]. Recently, Neunlist *et al.* [9] reported cross-potentiation between the cholinergic and VIPergic neurons in the guinea pig colonic mucosa. They found that in the presence of tetrodotoxin (TTX), VIP increased short-circuit currents ($I_{sc}$) dose-dependently, and the effects were dramatically increased by the pre-application of carbachol (Cch) and vice versa. However, this cannot rule out the possibility that VIP may reduce the inhibitory effects of TTX on Cch-stimulated $I_{sc}$.

VIP, a 28 amino acid peptide, binds with a high affinity to the G-protein-coupled receptors stimulating adenylyl cyclase [12]. There is ample evidence that the brain $Na^+$ channels are phosphorylated on the PKA and PKC sites [13, 14] and can be dephosphorylated by phosphatases, such as by calcineurin and phosphatase 2A [15, 16]. Further, VIP modulates nicotinic Ach re-

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ceptor in chick [17] and rat [18] ganglion neurons, and there is evidence that VIP shows neuro-protective action in cultured retinal ganglion cells. In these preparations, VIP-mediated increase in intracellular cAMP prevented TTX-induced cell death [19–21]. These results postulate that VIP may reduce the inhibition of TTX on the epithelial $Cl^-$ secretion through a cAMP-dependent process. It is therefore particularly important to study the exact mechanism of the observed cross-potentiation [9] and to determine the target of VIP in the colon.

In the present study, upon the mucosa-submucosa preparation mounted in Ussing-type chambers, we added VIP and Cch (or to a lesser extent Cch and VIP) sequentially to a serosal solution in the presence and absence of TTX. The object of this study was to determine whether forskolin and 8-bromo-cAMP could mimic the action of VIP on the Cch-stimulated $Cl^-$ secretion in the TTX-treated guinea pig distal colon.

## MATERIALS AND METHODS

**Animals and tissue preparation.** Experiments were performed using male Hartley strain guinea pigs (250 to 350 g body weight; Japan SLC Inc., Shizuoka) in accordance with the Kitasato University School guidelines for animal care. They were sacrificed by decapitation under ether anesthesia. Tissue preparation was exactly the same as previously described in [22]. Briefly, the distal segment of the colon (10 cm proximal to the anus) was removed, opened longitudinally along the mesenteric border, and rinsed with Tyrode solution.

**Solutions.** In all experiments, the mucosal and serosal Tyrode solutions were identical, and unless otherwise mentioned they contained (mmol·$l^{-1}$): 137 NaCl, 2.7 KCl, 1.8 $CaCl_2$, 1.1 $MgCl_2$, 11.9 $NaHCO_3$, 0.4 $NaH_2PO_4$, and 5.6 D-glucose. The solution was continuously gassed with 95% $O_2$/5% $CO_2$ and buffered at pH 7.4.

**Electrical measurement.** Submucosa-mucosa preparations were mounted in conventional Ussing chambers with a serosal surface area of 0.5 $cm^2$ and were bathed on both sides with 10 ml of Tyrode solution warmed to 37°C. Short-circuit current ($I_{sc}$) and tissue conductance ($G_t$) were measured using a voltage clamp apparatus (CEZ-9100, Nihon-Koden, Tokyo). Small, reversed rectangular pulses (2 mV, 1 s duration, 5–10 s intervals) were applied across the tissue through salt agar bridges. But no "electric field stimulation (EFS)" [3, 23] was applied to the tissue [22]. Most results are expressed as a peak of $I_{sc}$ response. However, when irregular oscillatory increases are observed in the $I_{sc}$ response, it is integrated over a 3 min period and is then averaged. The fluid resistance between the voltage-sensing electrodes (1 M KCl agar bridges) was automatically corrected. Data were digitized and stored in computer memory with McLab/4e as a multi-channel chart recorder (ADInstruments Pty Ltd., Australia).

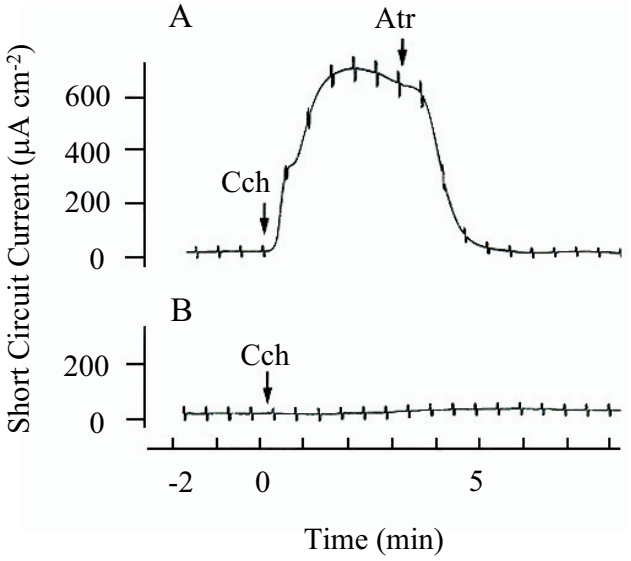


**Fig. 1. Representative traces showing changes in short-circuit current ($I_{sc}$) and tissue conductances ($G_t$) by the serosal application of carbachol (Cch). (A)** In the absence of tetrodotoxin (TTX), Cch ($2 \times 10^{-5}$ M) increased $I_{sc}$ and $G_t$, which are abolished by the serosal application of $10^{-6}$ M atropine (Atr). **(B)** In the presence of TTX ($10^{-6}$ M), Cch fails to increase $I_{sc}$ and $G_t$.

**Chemicals.** Vasoactive intestinal peptide (VIP), forskolin, 8-bromo-cAMP, and 8-bromo-cGMP were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA). Tetrodotoxin (TTX) was obtained from Seikagaku Corporation (Tokyo). The other chemicals were purchased from Wako Pure Chemical Industries (Osaka).

**Statistics.** The results are expressed as the means ± SEM. The number of experiments is indicated by "*n*." The differences were analyzed using a paired or unpaired Student's *t*-test. When more than two means were compared, an analysis of variance (ANOVA) was used. The values of $P < 0.05$ were considered statistically significant.

## RESULTS

### Carbachol-induced short-circuit current

In the absence of tetrodotoxin (TTX), isolated colonic mucosa of guinea pigs showed average resting

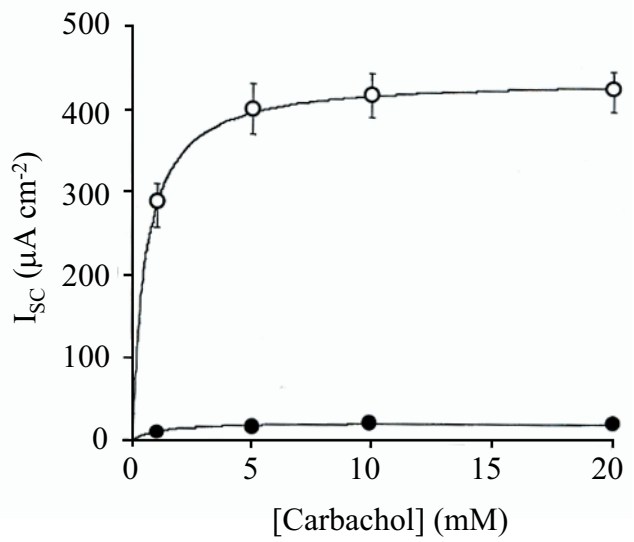


**Fig. 2. Concentration-response curves for Cch-induced $I_{sc}$ in the absence (open circles) and presence (closed circles) of $10^{-6}$ M TTX.** The regression lines were drawn by the Michaelis-Menten equation: $I_{sc} = I_{max}$ [Cch]/($K_m$ + [Cch]), where 439.5 μA cm$^{-2}$ (–TTX) and 23.1 μA cm$^{-2}$ (+TTX) are the maximum $I_{sc}$ ($I_{max}$), and 0.5 μM (–TTX) and 1.3 μM (+TTX) are the half-maximum concentrations of Cch ($K_m$).

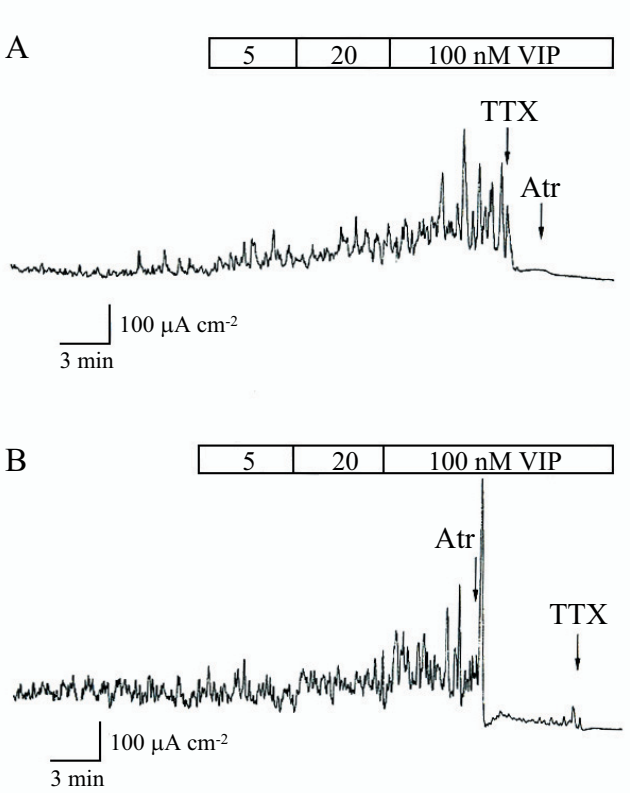


**Fig. 3. Effects of TTX and atropine on VIP-induced $I_{sc}$.** A representative trace of VIP-induced $I_{sc}$ and a following addition of TTX ($10^{-6}$ M) and atropine ($10^{-6}$ M) in this sequence **(A)** and vice versa **(B)**.

short-circuit currents ($I_{sc}$) of 30.6 ± 4.9 μA·cm$^{-2}$ ($n$ = 21) with spontaneous fluctuations in Ussing chambers containing Tyrode solutions. Tissue conductance ($G_t$), calculated by voltage deflections, was 10.2 ± 0.6 mS·cm$^{-2}$ ($n$ = 12). These values were similar to those reported previously [22, 24–26]. The addition of 2 × $10^{-5}$ M carbachol (Cch), a muscarinic and nicotinic agonist, to the serosal solution promptly caused large increases in $I_{sc}$ and $G_t$, which were sustained and slowly decreased (Fig. 1A). They were reversed to the initial levels by a serosal application of $10^{-6}$ M atropine. Thus Cch-induced $I_{sc}$ is expressed as the change in $I_{sc}$ above basal currents. The maximum values of $I_{sc}$ and $G_t$ were 423.5 ± 24.7 μA·cm$^{-2}$ ($n$ = 21) and 19.2 ± 0.6 mS·cm$^{-2}$ ($n$ = 12), respectively. On the other hand, in the presence of $10^{-6}$ M TTX, Cch failed to increase the $I_{sc}$ and $G_t$ (Fig. 1B). TTX used through the present study was 100-times higher in concentration than in the previous paper [22]. Their peak values were 24.8 ± 3.1 μA·cm$^{-2}$ ($n$ = 21) and 9.7 ± 0.9 mS·cm$^{-2}$ ($n$ = 12). Cch-induced $G_t$ (+TTX) was not significantly different from that of the resting level (10.2 ± 0.6 mS·cm$^{-2}$). Figure 2 illustrates the peak of Cch-induced $I_{sc}$ as a function of concentrations of Cch in the absence and presence of TTX, showing that Cch-induced $I_{sc}$ is almost completely sensitive to TTX.

## Effect of TTX and atropine on VIP-induced $I_{sc}$

A representative response to VIP is shown in Fig. 3. A cumulative addition of VIP (serosal) gradually increased the baseline of $I_{sc}$ and its fluctuations in a dose-dependent manner. The maximal response to VIP was obtained at $10^{-7}$ M. The averaged $I_{sc}$ over a 3 min interval was 107.5 ± 8.6 μA·cm$^{-2}$ ($n$ = 8). To determine whether VIP-induced $I_{sc}$ was neurally stimulated, or whether its action was mediated by the cholinergic neurons, TTX and atropine (or atropine and TTX) were sequentially added to the serosal solution. Either TTX ($10^{-6}$ M) or atropine ($10^{-6}$ M) completely inhibited the VIP ($10^{-7}$ M)-induced $I_{sc}$ and fluctuations (Fig. 3, A and B). The inhibitory effects of TTX and atropine on VIP-induced $I_{sc}$ were summarized in Fig. 4.

## Effects of VIP and 8-bromo-cAMP on Cch-induced $I_{sc}$ (–TTX)

The potentiation of either VIP or 8-bromo-cAMP on Cch-induced $I_{sc}$ was evaluated in the absence of TTX. Either VIP ($10^{-7}$ M) or 8-bromo-cAMP ($10^{-3}$ M) was applied to the serosal solution at least 3 min before the addition of Cch. The Cch-induced $I_{sc}$ under the pre-application of VIP ($n$ = 5) or 8-bromo-cAMP ($n$ = 4) was not significantly different from the controls ($P$ > 0.05) (Fig. 5).

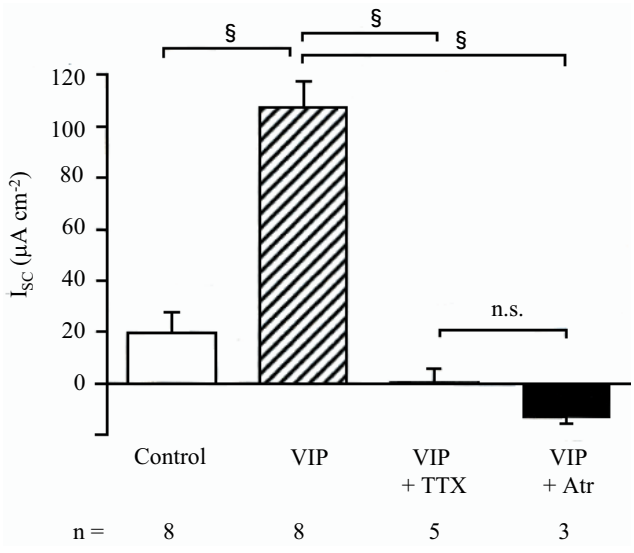


**Fig. 4. Amplitude of basal- and VIP-induced $I_{sc}$ shown in Fig. 3.** Control (open), VIP-induced $I_{sc}$ (hatched), and VIP-induced $I_{sc}$ with TTX or atropine (closed). § $P < 0.0001$, n.s: not significant.

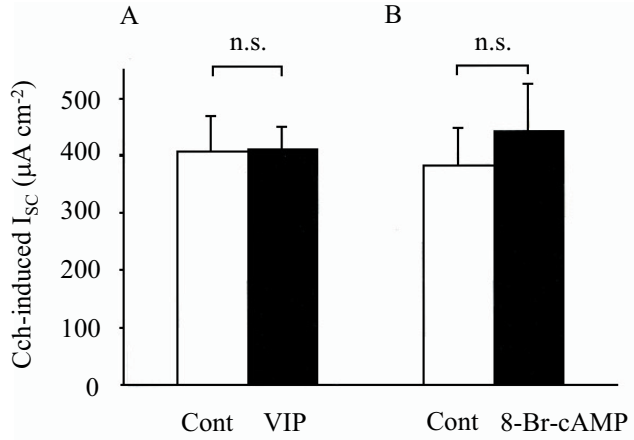


**Fig. 5. Effects of VIP and 8-bromo-cAMP on Cch-induced $I_{sc}$ in the absence of TTX. (A)** Cch-induced $I_{sc}$ (366.2 ± 60.5 μA cm$^{-2}$) is not significantly increased with a pre-application of $10^{-6}$ M VIP (399.8 ± 31.1 μA cm$^{-2}$) ($n$ = 5). **(B)** Cch-induced $I_{sc}$ (376.3 ± 65.2 μA cm$^{-2}$) is not significantly increased with a pre-application of $10^{-3}$ M 8-bromo-cAMP (436.8 ± 81.5 μA cm$^{-2}$) ($n$ = 4). $P > 0.05$.

## Effect of VIP on Cch-stimulated $I_{sc}$ (+TTX)

In the presence of TTX ($10^{-6}$ M), a serosal application of Cch could not increase $I_{sc}$. However, it could induce the $I_{sc}$ by a pre-application of $10^{-7}$ M VIP to the serosal solution (Fig. 6A). The restoration by VIP of Cch-stimulated $I_{sc}$ was promptly inhibited by serosal atropine ($10^{-6}$ M), but not by the secondary addition of TTX ($10^{-6}$ M). Moreover, when the mucosa-submucosa preparations were pretreated with Cch in the serosal side, a subsequent application of serosal VIP ($10^{-7}$ M) dramatically increased $I_{sc}$ (Fig. 6B). In contrast, a

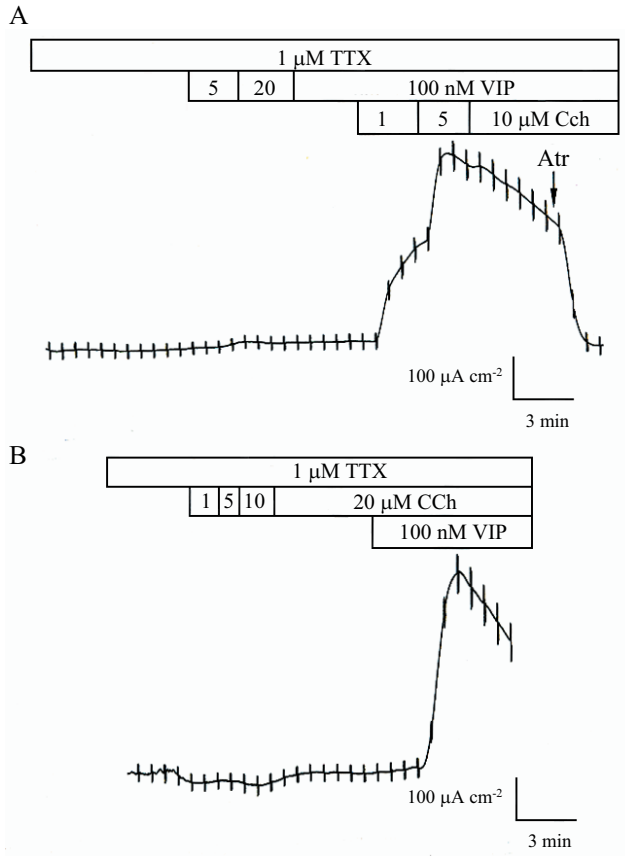


**Fig. 6. Effect of VIP on Cch-stimulated $I_{sc}$ in the presence of TTX. (A)** A representative trace of a cumulative addition of VIP and a subsequent application of Cch in the presence of TTX. **(B)** A representative trace of a cumulative addition of Cch and a subsequent application of VIP in the presence of TTX.

mucosal application of VIP ($10^{-7}$ M) failed to restore the TTX-inhibited, Cch-stimulated $I_{sc}$ ($n$ = 4). These results are summarized in Fig. 7.

## Effect of 8-bromo-cAMP on Cch-stimulated $I_{sc}$ (+TTX)

To determine whether the submucosal plexus is a target of VIP, we applied 8-bromo-cAMP ($10^{-3}$ M), membrane permeable cAMP, to either mucosal or serosal solution before addition of Cch, which dramatically increased $I_{sc}$ by the pre-application of 8-bromo-cAMP in the serosal solution. This current was completely inhibited by the subsequent application of atropine ($10^{-6}$ M). Conversely, the mucosal application of 8-bromo-cAMP could not restore the Cch-stimulated $I_{sc}$ in the presence of TTX (Fig. 8). These results postulate that a restoration by VIP of Cch-stimulated $I_{sc}$ may be the result of a cAMP-increasing process in the submucosal plexus. Further proof for this hypothesis was obtained from the effect of 8-bromo-cGMP.

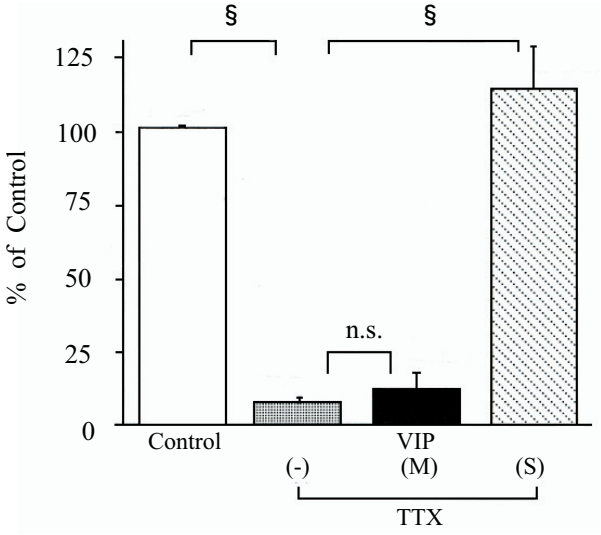


**Fig. 7. Amplitude of Cch-induced $I_{sc}$ shown in Fig. 6.** M and S indicate mucosal and serosal sides, respectively. TTX is present throughout the experiment.

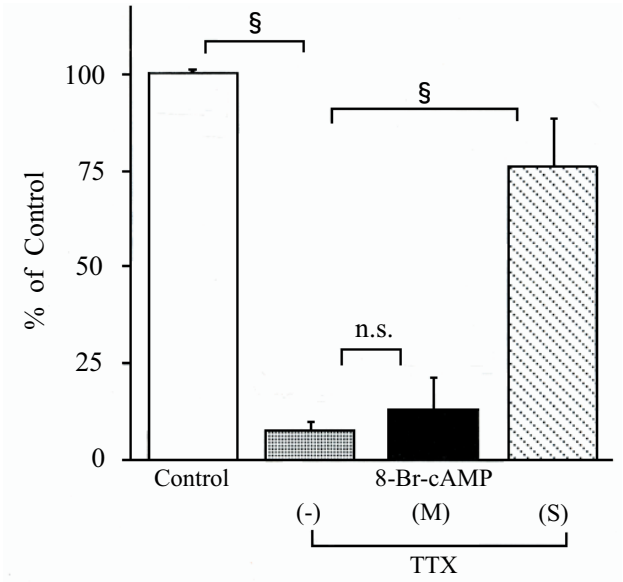


**Fig. 8. Effects of 8-bromo-cAMP on Cch-induced $I_{sc}$ in the presence of TTX.** M: mucosal, S: serosal, § $P < 0.0001$.

Cch-stimulated, but TTX-inhibited $I_{sc}$ was not significantly restored by 8-bromo-cGMP ($10^{-3}$ M). Its recovery rate was 1.7 ± 0.8% ($n$ = 5). These results suggest that this restoration by VIP of the Cch-stimulated $I_{sc}$ is specifically cAMP-dependent.

## Effect of forskolin on Cch-stimulated $I_{sc}$ (+TTX)

To further investigate the target of the cAMP-dependent process, we added forskolin, an adenylate cyclase activator, to mucosal, serosal, and both solutions of the chambers. Recovery rates by forskolin

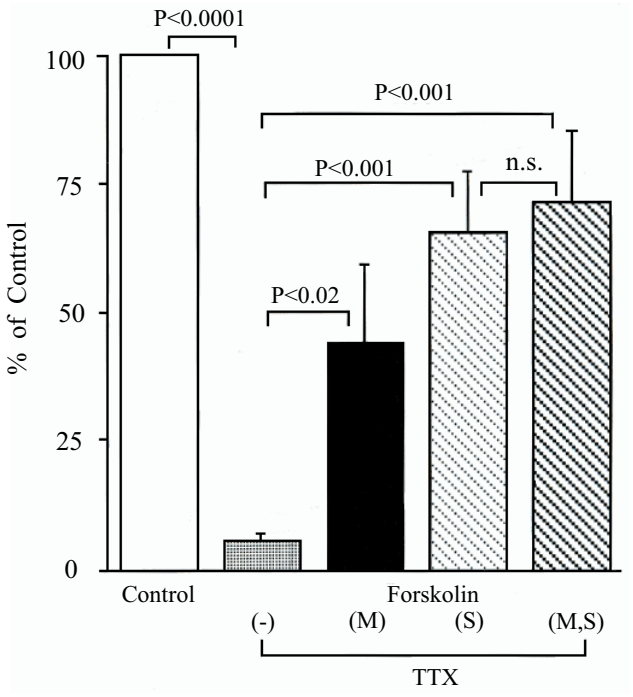


**Fig. 9. Effects of forskolin on Cch-induced $I_{sc}$ in the presence of TTX.** M: mucosal, S: serosal, M,S: both.

($10^{-6}$ M) were 43.9 ± 14.8% (mucosal), 65.3 ± 11.9% (serosal), and 71.0 ± 13.9% (both) ($n$ = 5 each) (Fig. 9). These results support a hypothesis that pretreatment with forskolin reduced the inhibitory effects of TTX on the Cch-stimulated $I_{sc}$.

## DISCUSSION

The present study has demonstrated for the first time that vasoactive intestinal peptide (VIP) and cAMP-increasing agents can restore the Cch-stimulated short-circuit current ($I_{sc}$) in the TTX-treated guinea pig distal colon. Further, our study gives new insights into cross-potentiation between the cholinergic and VIPergic neurons in the epithelial $Cl^-$ secretion.

Epithelial $Cl^-$ secretion across the mucosa-submucosa preparation isolated from the guinea pig distal colon is dose-dependently stimulated by the serosal application of Cch and VIP. But their stimulation is not synergistic. This is consistent with the previous functional and immunohistochemical studies showing that VIP as well as acetylcholine (Ach) may be present in both interneurons and secretomotor neurons and that they stimulate $Cl^-$ secretion [8, 27, 28]. On the other hand, Cch-induced $I_{sc}$ was increased by IBMX (inhibitors of phosphodiesterase)/forskolin in human colon epithelium [29]. Increased intracellular cAMP stimulated a sustained lumen-negative current in this preparation. Further, Cch- and VIP-induced $Cl^-$ secre-

tion is completely inhibited by TTX, a voltage-gated $Na^+$ channel inhibitor, and, more important, by atropine, a muscarinic receptor antagonist. This suggests that Ach mainly acts at the neuroepithelial junction to regulate the epithelial $Cl^-$ secretion. The present study supports the working model A of [6] in which VIPergic interneurons are synaptically coupled with the cholinergic secretomotor neuron. Our study does not rule out the possibility that the VIPergic neuron directly acts at neuroepithelial junctions in response to mucosal stroking [8] and intestinal distention [11].

As Neunlist *et al.* [9] reported, an apparent potentiation between the cholinergic and VIPergic neurons was observed in our preparation treated with TTX. However, no synergistic action was observed between Cch and VIP in the absence of TTX. This suggests that Ach may be a common transmitter at neuroepithelial junctions. Thus the increased $I_{sc}$ on the pre-application of Cch or VIP in the TTX-treated colon may be due to a reduction of inhibitory effects of TTX on the $Cl^-$ secretion. The restoration by VIP of Cch-stimulated $Cl^-$ secretion (+TTX) was mimicked by either serosal 8-bromo-cAMP ($10^{-3}$ M) or mucosal/serosal forskolin ($10^{-6}$ M), but not by serosal 8-bromo-cGMP or mucosal 8-bromo-cAMP. These results are consistent with the finding that TTX significantly reduced Ach-induced $I_{sc}$ in rat colonic epithelia, but TTX was without effect on the stimulation of $I_{sc}$ by cAMP-increasing secretagogues, such as prostaglandin $E_2$ and forskolin [7].

On the other hand, our results of VIP-induced $I_{sc}$ may be different from the previous study in which VIP-induced $I_{sc}$ is only partially inhibited by TTX and atropine in guinea pig distal colon [5]. One possible explanation for the different responses to TTX may arise from a difference in experimental conditions, i.e., the application or no application of EFS. In response to huge currents of EFS, various neurotransmitters and secretagogues, such as histamine and VIP [3, 30], may be released from the submucosal plexus and other cells. Although the electrical action of EFS may be transient, these chemicals released are very likely to potentiate the submucosal neurons and cells through various cellular processes including cAMP. It is reported that $Na^+$ channel activity is different at it phosphorylation state [13–16]. A mechanism of atropine-insensitive $I_{sc}$ remains unknown.

What is the target of VIP acting as increasing cAMP? The current model of $Cl^-$ secretion in the present preparation includes the colonic crypt cells, enteric nervous system, and non-neuronal cells including inflammatory cells. It is possible that VIP upregulates a luminal $Cl^-$ conductance and a basolateral $K^+$ conductance of the crypt cells by increasing intracellular cAMP [31]. Simultaneous activation of the $Ca^{2+}$- and cAMP-mediated system increased $Cl^-$ secretion in guinea pig distal colon [32]. An activation of a basolateral $P2Y_6$ receptor in rat colonic enterocytes stimulates a sustained NaCl secretion via a synergistic increase of $[Ca^{2+}]_i$ and cAMP [33]. However, a mucosal application of 8-bromo-cAMP could not restore the Cch-stimulated $I_{sc}$ in the present study. Further, it is reported that lamina propria mononuclear cells in human colon do not respond to VIP [34]. In the mucosa-submucosa preparation, the epithelial $Cl^-$ secretion can be stimulated by either neurotransmitters or secretagogues released from the submucosal cells (a subsequent activation of the epithelial crypt cells) [1, 2]. $Ca^{2+}$ entry through L-type $Ca^{2+}$ channels of the presynaptic terminals is essential for the release of neurotransmitters. The cAMP response element-binding protein and the cAMP-dependent system of phosphorylation, may be key steps for gene expression [35] and activity [36], respectively, of the L-type $Ca^{2+}$ channels. In pathophysiological conditions, $P2Y_6$ receptors are upregulated in T cells infiltrating regions of inflamed bowel [37] and involved in monocytic release of IL-8 [38]. It is thus probable that immunocytes are a source of nucleotide release [39] and cause diarrhea of inflammatory bowel disease. However, this is not so in the present study. Finally, the recovery rates in the TTX-treated colon were 113% (serosal VIP), 75.8% (serosal 8-bromo-cAMP), 43.9% (mucosal forskolin), and 63.5% (serosal forskolin). These results indicate that (i) a serosal application of VIP restores the Cch-stimulated $I_{sc}$ by increasing intracellular cAMP, (ii) the target of VIP acting may not be the submucosal immune cells, and (iii) cAMP-dependent phosphorylation of mucosal $Cl^-$ channels in the crypt cells may not be a rate-limiting step in the epithelial $Cl^-$ secretion. When these indications are taken together, we conclude that a target tissue of the serosal application of VIP is probably the submucosal cholinergic neurons.

It is questionable why a mucosal addition as well as a serosal addition of forskolin increased the $I_{sc}$ in the presence of TTX ($n = 5$). The mechanism can be speculated as follows: Although forskolin and 8-bromo-cAMP are permeable to the cell membrane, 8-bromo-cAMP, becoming impermeable after hydrolysis in the cell, cannot cross the epithelium. In contrast, forskolin added to the mucosal side can cross the mucosal and basolateral membranes of the crypt cells and could stimulate the submucosal neural activity.

In conclusion, our study has presented that VIP and cAMP-increasing agonists can restore the Cch-stimulated $I_{sc}$ in the TTX-treated guinea pig distal colon. Our data and hypothesis may provide an important framework for the functional interactions between the cholinergic and VIPergic neurons of mucosa-submucosa preparation in the future.

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