Cholinergic inhibition of electrogenic sodium absorption in the guinea pig distal colon

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Hayashi, Hisayoshi, Tomoko Suzuki, Takeshi Yamamoto, and Yuichi Suzuki. Cholinergic inhibition of electrogenic sodium absorption in the guinea pig distal colon. Am J Physiol Gastrointest Liver Physiol 284: G617–G628, 2003.—Submucosal cholinergic and noncholinergic neurons in intestines have been shown to be involved in regulating epithelial transport functions, particularly stimulating Cl− secretion. This study investigates the role of submucosal cholinergic neurons in regulating electrogenic Na+ absorption in distal colon. Amiloride-sensitive short-circuit current (Isc) and 22Na+ flux were measured in mucosal and mucosal-submucosal preparations mounted in Ussing chambers. In the mucosal preparation, carbachol (CCh) added to the serosal side inhibited amiloride-sensitive Isc and amiloride-sensitive 22Na+ absorption. The inhibitory effect of CCh was observed at ∼0.1 μM, and maximum inhibition of ∼70% was attained at ∼30 μM (IC50 = ∼1 μM). CCh-induced inhibition of amiloride-sensitive Isc was almost totally abolished by 10 μM atropine. Treatment of the tissue with ionomycin markedly reduced amiloride-sensitive Isc, but a subsequent addition of CCh further decreased it. Also, CCh still had an inhibitory effect, although significantly attenuated, after the tissue had been incubated with a low-Ca2+ solution containing ionomycin and BAPTA-AM. Applying electrical field stimulation to submucosal neurons in the mucosal-submucosal preparation resulted in inhibition of amiloride-sensitive Isc, ∼33% of this inhibition being atropine sensitive. Phystostigmine inhibited amiloride-sensitive Isc, this effect being abolished by atropine. In conclusion, submucosal cholinergic and noncholinergic neurons were involved in inhibiting electrogenic Na+ absorption in colon. This inhibition by cholinergic neurons was mediated by muscarinic receptor activation.

enteric nerve; intracellular Ca2+; acetylcholine; intestinal secretion; epithelial Na+ channel

THE COLON, THE TERMINAL PART OF THE GASTROINTESTINAL TRACT, performs functions of both absorption and secretion of a variety of electrolytes by epithelial transport systems. Regulation of this absorption and secretion of electrolytes by neurotransmitter, paracrine, and endocrine systems probably play an important role in maintaining the fluid and electrolyte homeostasis in the whole body as well as being involved in mucosal defensive functions (1, 25).

The colon, like other segments of the gastrointestinal tract, has an intrinsic nervous system, the enteric nervous system, consisting of two ganglionated plexi, namely the myenteric plexus and the submucosal plexus (also called the submucous plexus). Myenteric neurons mainly regulate contractile activity, whereas submucosal neurons are mainly involved with epithelial transport functions (5, 12). It has been shown that the stimulation of submucosal neurons by electrical, mechanical, or pharmacological means led to enhanced Cl− secretion (5, 12). In addition, the inhibition of electroneutral NaCl absorption (3, 23) and stimulation of K+ secretion (6, 15, 16, 28) are also likely to be induced by submucosal neurons. Cholinergic neurons are predominantly responsible for these epithelial prosecretory responses, although neurons containing vasoactive intestinal peptide are probably also involved (5, 12–14, 22–24, 32, 33, 38–40, 51, 55).

Electrogenic Na+ absorption, which involves an apical amiloride-sensitive Na+ channel and basolateral Na+/K+-ATPase/K+ channel, is one of the major pathways for Na+ absorption in the colon (1, 25). This transport pathway is known to be stimulated by aldosterone and thyroid hormones through genomic activation (1, 25, 31). In addition, our recent studies have shown that the β-adrenergic agonist activates and vasoressin and ATP inhibit electrogenic Na+ absorption (44, 49, 57). However, information concerning the regulation by enteric neurons is very limited for electrogenic amiloride-sensitive Na+ absorption in the colon, in contrast to that for colonic Cl− secretion: chemical stimulation of intrinsic cholinergic neurons and the application of the cholinergic agonist carbachol (CCh) have been reported to inhibit electrogenic Na+ absorption in the turtle colon (50, 53), but there is no such report for mammalian colon. The purpose of this study is, therefore, to elucidate the regulation of electrogenic Na+ absorption by enteric submucosal neurons, particularly the cholinergic type, in an isolated guinea pig distal colon mounted in an Ussing chamber. We examined the effect of CCh on the amiloride-sensitive short
circuit current ($I_{sc}$) and $^{22}$Na$^+$ flux. We also investigated the effect of stimulating submucosal cholinergic neurons on electrogenic Na$^+$ absorption by using electrical field stimulation (EFS).

MATERIALS AND METHODS

Tissue preparation. Hartley-strain, male guinea pigs weighing 250–550 g were used in the experiments. All animals were injected twice with 375 µg/kg body wt of aldosterone (1.39 mM in saline) to enhance the electrogenic sodium absorption, once by a subcutaneous injection in the evening before the experiment and then by an intramuscular injection 4 h before the start of the experiment. The animals were stunned by a blow to the head and bled to death. The distal colon was excised and then opened along the root of the mesentery. A mucosal preparation consisting of the mucosal layer and a part of the muscularis mucosal layer was obtained with glass microscopic slides (55). The mucosal-submucosal preparation, consisting of the mucosal, muscularis mucosal, and submucosal layers was obtained with fine forceps. All procedures involving the animals were approved by the Institutional Animal Care Board at the University of Shizuoka.

Solutions. The standard bathing solution contained (in mM) 119 NaCl, 21 NaHCO$_3$, 2.4 K$_2$HPO$_4$, 0.6 KH$_2$PO$_4$, 1.2 MgCl$_2$, 1.2 CaCl$_2$, and 10 glucose. A low-Ca$^{2+}$, high-Mg$^{2+}$ solution was prepared by omitting CaCl$_2$ and adding 0.2 mM EGTA and 10 mM MgCl$_2$. Each solution was gassed with 95% O$_2$ and 5% CO$_2$ (pH 7.3–7.4).

$I_{sc}$ measurements. The $I_{sc}$ and transmural tissue resistance ($G_t$) were measured in vitro in Ussing chambers as previously described (44). The mucosal or the mucosal-submucosal sheet was mounted vertically between acrylic resin chambers with an internal surface area of 0.5 cm$^2$. The temperature of the 10-ml bathing solution in each chamber was maintained at 37°C by a water-jacketed reservoir. The tissue was continuously short-circuited, with compensation for the fluid resistance between the two potential-sensing bridges, by using a voltage-clamping amplifier (CEZ9100; Nihon Kohden, Tokyo, Japan). The transepithelial potential was measured through a fluid resistance between the two potential-sensing bridges, by using a voltage-clamping amplifier (CEZ9100; Nihon Kohden, Tokyo, Japan). The transepithelial potential was measured through a fluid resistance between the two potential-sensing bridges, by using a voltage-clamping amplifier (CEZ9100; Nihon Kohden, Tokyo, Japan).

Statistical analyses. Each data value is presented as the mean ± SE of n guinea pigs. Statistical comparisons were performed by using the Student’s t-test (paired or unpaired, as appropriate) or ANOVA for repeated measures (Dunnett post hoc test). Significance was accepted at $P < 0.05$.

RESULTS

Effect of CCh on electrogenic Na$^+$ absorption. The experiments were performed in the mucosal preparation of the distal colon obtained from the aldosterone-treated animals. An initial indication that cholinergic agonists may inhibit electrogenic, amiloride-sensitive Na$^+$ absorption in the colon was observed in the first series of experiments (Fig. 1). Three measurements, each preceded by a washing procedure, were consecutively performed on the same preparation (Fig. 1A). The basal $I_{sc}$ value after 20–30 min of equilibration gradually decreased from the first to the third measurements (223 ± 46, 190 ± 53, and 118 ± 26 µA/cm$^2$, respectively). The $G_t$ similarly decreased (18.9 ± 3.3, 14.1 ± 2.6, and 12.7 ± 2.6 mS/cm$^2$, respectively; n = 5).

In the first measurements, amiloride-sensitive $I_{sc}$ and $G_t$ under control conditions were estimated by adding amiloride to the mucosal side (0.1 mM). In the second measurements, the effect of the cholinergic agonist CCh on the amiloride-sensitive $I_{sc}$ and $G_t$ values was examined. The addition CCh (1 mM) to the serosal side induced an initial transient $I_{sc}$ increase, which then decreased to below the basal level (Fig. 1, A and B). $G_t$ also transiently increased and then decreased to slightly above the basal level. Subsequent addition of amiloride reduced $I_{sc}$ and $G_t$, although the magnitude of these changes was smaller than that observed in the first measurements. The possibility that these decreases in the amiloride-induced $I_{sc}$ and $G_t$ values in the presence of CCh was mainly due to a time-dependent decrease, rather than to the presence of CCh, can be excluded, because, in the third measurements, the amiloride-sensitive $I_{sc}$ and $G_t$ values under control conditions were both larger than those observed in the second measurements, although they were smaller than those observed in the first measurements. Thus, as summarized in Fig. 1C, the amiloride-sensitive $I_{sc}$ and $G_t$ values were significantly attenuated by CCh, indicating that CCh inhibited the amiloride-sensitive, electrogenic Na$^+$ absorption. It is likely that the initial $I_{sc}$ and $G_t$ increases induced by CCh were due to the stimulation of the electrogenic Cl$^-$ secretion (6, 10, 24, 28, 34, 48, 56, 59).
The CCh-induced inhibition of amiloride-sensitive $I_{sc}$ was further investigated in the presence of serosal bumetanide (an Na$^+$/K$^+$/2Cl$^-$/H$^+$ cotransporter inhibitor, 10 μM) to substantially, if not totally, suppress the $I_{sc}$ components due to electrogenic K$^+$ and Cl$^-$ secretion. The cyclooxygenase inhibitor indomethacin (10 μM, mucosal and serosal sides) and nerve-conduction blocker TTX (300 nM, serosal side) were also included in the bathing solution. Under these conditions, the $I_{sc}$ value decreased rapidly, reached its lowest level within 5 min after CCh had been added to the serosal side, and then, in most cases, gradually increased, as shown in Fig. 2. The initial transient $I_{sc}$ increase that had been observed in the first series of experiments (Fig. 1) was scarcely apparent. The $G_t$ decrease also reached its maximum within 5 min. However, compared with $I_{sc}$, $G_t$ returned to its basal level more rapidly (within 10 min after CCh had been added). The $I_{sc}$ and $G_t$ decreases induced by CCh (1 mM) were substantially suppressed when the tissue had been pretreated on the serosal side with the muscarinic receptor antagonist atropine (10 μM), with >90% suppression. Atropine alone caused an increase or a decrease in $I_{sc}$ (by 10 μA/cm$^2$ at the most) in some tissues. This experiment was done in the presence of TTX and indomethacin, indicating that the inhibition of electrogenic Na$^+$ absorption induced by CCh was not substantially mediated by either the activation of intramural neurons that could have been present in the mucosal preparation or by the production of prostanoids.

Figure 3 shows the concentration dependence of the inhibitory effect of CCh on the amiloride-sensitive $I_{sc}$ value. The inhibitory effect was observed at a concentration of ~0.1 μM, and the maximum inhibition of ~70% was attained at a concentration of ~30 μM, the IC$_{50}$ value being ~1 μM.

To confirm the hypothesis that CCh inhibited the amiloride-sensitive electrogenic Na$^+$ absorption, the bidirectional $^{22}$Na flux was next measured (Table 1). In the distal colon preparation obtained from the aldosterone-treated animals, Na$^+$ absorption occurred mainly through the amiloride-sensitive electrogenic Na$^+$ absorption pathway, as was demonstrated in the control group. Mucosal benzamil (10 μM), a more spe-
specific inhibitor of the epithelial Na\(^+\) channel than amiloride, almost totally suppressed the net 22Na\(^+\) absorption \(J_{\text{net}}\) and \(I_{\text{sc}}\) values by a similar magnitude, the \(J_{\text{net}}\) decrease being mainly due to the decrease in \(J_{\text{ms}}\), with little change in \(J_{\text{sm}}\). In the experimental group, the addition of 10 \(\mu\)M CCh to the serosal side caused a moderate decrease in \(J_{\text{ms}}\) and \(J_{\text{net}}\), with little change in \(J_{\text{sm}}\). The CCh-induced decreases in \(J_{\text{ms}}\) and \(J_{\text{net}}\) were both significantly greater than the time-dependent decreases observed in the control group. The subsequent addition of benzamil further decreased \(J_{\text{ms}}\) and \(J_{\text{net}}\), with little change in \(J_{\text{sm}}\). The respective flux decreases for \(J_{\text{ms}}\) and \(J_{\text{net}}\) after the benzamil treatment were significantly smaller than those for \(J_{\text{ms}}\) and \(J_{\text{net}}\) in the control group after the benzamil treatment. These results confirm that serosal CCh inhibited the amiloride-sensitive electrogenic Na\(^+\) absorption. Similarly to benzamil, mucosal amiloride at a concentration of 0.1 mM almost totally suppressed 22Na absorption to a degree consistent with the change in \(I_{\text{sc}}\), as reported previously (57). Therefore, amiloride (0.1 mM) and benzamil (10 \(\mu\)M) were used interchangeably in the present study.

Role of Ca\(^{2+}\) in the CCh-induced inhibition of electrogenic Na\(^+\) absorption. We next examined whether the CCh-induced inhibition of amiloride (benzamil)-sensitive \(I_{\text{sc}}\) would be mediated by an increase in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) by using the mucosal preparation. To this end, the effect of CCh was assessed under two experimental conditions contrasting with each other, one with [Ca\(^{2+}\)]\(_i\), having been presumably depleted and the other with [Ca\(^{2+}\)]\(_i\), having been presumably elevated. In the first series of
experiments, tissues were bathed with the low-Ca²⁺ (nominally Ca²⁺ free + 0.2 mM EGTA), high-Mg²⁺ (10 mM) solution. This bathing solution also contained ionomycin (1 μM, mucosal and serosal sides) to deplete the intracellular Ca²⁺ store (35) and BAPTA-AM (50 μM, mucosal and serosal sides) to buffer [Ca²⁺]i. Indomethacin, TTX, and bumetanide were also included in this Ca²⁺-depleted condition. Under these Ca²⁺-depleted conditions, Gt gradually increased to 30–90 mS/cm² 30 min after the start of incubation, as shown in Fig. 4A. The increase in Gt is likely to have been due to the low Ca²⁺-induced increase in tight-junction permeability, and the increasing Mg²⁺ concentration in the solution apparently failed to prevent this change. Therefore, the following experimental values were obtained between 15 and 25 min after changing to the Ca²⁺-depletion solution before the Ca²⁺-depletion period I (Fig. 4A). The addition of CCh under the Ca²⁺-depleted condition decreased the basolateral (B)短路电流 (Isc) by 10.2 ± 0.3 mA/cm² under the control condition to 113 ± 26 mA/cm² under the Ca²⁺-depleted condition (Fig. 4, n = 5). The addition of CCh under the Ca²⁺-depleted condition decreased Isc and subsequent addition of benzamil almost totally abolished it (Fig. 4A). The percentage inhibition of benzamil-sensitive Isc by CCh was significantly attenuated, but not abolished, under the Ca²⁺-depleted condition compared with that under the control condition (Fig. 4B). We also examined the effect of Ca²⁺ depletion on the CCh-induced Cl⁻ and K⁺ secretions (Fig. 5). The measurements were done in the absence of bumetanide and in the

Table 1. Effect of serosal CCh on the unidirectional ²²Na⁺ flux and electrical properties in the guinea pig distal colon

<table>
<thead>
<tr>
<th></th>
<th>Jmax</th>
<th>Jmin</th>
<th>Jnet</th>
<th>Isc</th>
<th>Gt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12.4 ± 0.5</td>
<td>2.5 ± 0.5</td>
<td>9.9 ± 0.5</td>
<td>9.2 ± 0.5</td>
<td>11.0 ± 0.9</td>
</tr>
<tr>
<td>Period I</td>
<td>11.6 ± 0.3</td>
<td>2.6 ± 0.6</td>
<td>8.9 ± 0.5</td>
<td>8.2 ± 0.5</td>
<td>10.9 ± 0.9</td>
</tr>
<tr>
<td>Period II</td>
<td>3.7 ± 0.3</td>
<td>3.0 ± 0.9</td>
<td>0.8 ± 0.9</td>
<td>0.4 ± 0.1</td>
<td>7.2 ± 0.4</td>
</tr>
<tr>
<td>Period III (+benzamil)</td>
<td>−0.8 ± 0.5</td>
<td>0.2 ± 0.4</td>
<td>−0.9 ± 0.3</td>
<td>−0.9 ± 0.1</td>
<td>−0.1 ± 0.1</td>
</tr>
<tr>
<td>Δ Period I vs. II</td>
<td>−7.9 ± 0.4</td>
<td>0.3 ± 0.9</td>
<td>−8.2 ± 1.0</td>
<td>−8.6 ± 0.6</td>
<td>−3.7 ± 0.6</td>
</tr>
<tr>
<td>Experiment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Period I</td>
<td>13.4 ± 1.5</td>
<td>1.9 ± 0.4</td>
<td>11.5 ± 1.4</td>
<td>9.4 ± 0.6</td>
<td>11.0 ± 0.1</td>
</tr>
<tr>
<td>Period II (+CCh)</td>
<td>6.6 ± 0.4**</td>
<td>2.2 ± 0.4</td>
<td>4.4 ± 0.5**</td>
<td>2.2 ± 0.4**</td>
<td>10.0 ± 0.2</td>
</tr>
<tr>
<td>Period III (+benzamil)</td>
<td>3.8 ± 0.3</td>
<td>2.3 ± 0.7</td>
<td>1.5 ± 0.6</td>
<td>0.6 ± 0.1</td>
<td>7.5 ± 0.4</td>
</tr>
<tr>
<td>Δ CCh</td>
<td>−6.8 ± 1.6*</td>
<td>0.2 ± 0.2</td>
<td>−7.1 ± 1.7*</td>
<td>−7.1 ± 0.4**</td>
<td>−0.1 ± 0.2*</td>
</tr>
<tr>
<td>Δ benzamil</td>
<td>−2.8 ± 0.6**</td>
<td>0.1 ± 0.5</td>
<td>−2.9 ± 0.5**</td>
<td>−2.8 ± 0.5**</td>
<td>−2.5 ± 0.3</td>
</tr>
</tbody>
</table>

Values are means ± SE. Flux and current measurements are in μeq·cm⁻²·h⁻¹; transmural tissue resistance (Gt) is in mS/cm². The mucosal preparation was used. The mucosal-to-serosal (Jmuel) and serosal-to-mucosal (Jmen) fluxes of ²²Na⁺ were measured by using adjacent tissues, and the net flux (Jnet) was calculated as Jnet = Jmun − Jmuel. After the basal flux had been measured for three 10-min periods (mean value given as period I), 100 μM carbamol (CCh) was added to the serosal side (only for experimental group), and the flux was again measured for three 10-min periods (mean value for last 2 periods given as period II). Finally, benzamil (10 μM) was added to the mucosal side, and the flux was measured again for three 10-min periods (mean value for last 2 periods given as period III). The experiments were done in the presence of 10 μM indomethacin, 300 nM TTX, and 0.1 mM bumetanide all added to the serosal side. Isc, short-circuit current; Δ, change. Values for the control group and experimental group are shown; n = 4 for the control group, and n = 6 for the experimental group; significant differences between these groups are shown as *P < 0.05 and **P < 0.01 (unpaired t-test).
presence of benzamil. Under these conditions, a submaximal concentration of CCh (10 μM) caused a biphasic $I_{sc}$ response (Fig. 5A). The initial $I_{sc}$ increase, which was probably due to the stimulation of electrogenic Cl⁻ secretion, was followed by an $I_{sc}$ decrease to below the basal level, probably due to the stimulation of electrogenic K⁺ secretion. Both the increase and decrease in $I_{sc}$ induced by CCh were almost totally abolished under the Ca²⁺-depleted condition (Fig. 5, B and C). Thus, in contrast to the CCh-induced inhibition of amiloride-sensitive $I_{sc}$, the CCh-induced stimulation of Cl⁻ and K⁺ secretions, which are putatively mediated by a Ca²⁺-signaling pathway, was, in fact, strongly suppressed under the Ca²⁺-depleted condition. We also examined the effect of the same Ca²⁺-depleted condition on the 8Br-cAMP-induced stimulation of electrogenic K⁺ secretion (Fig. 5, D–E). The 8Br-cAMP-induced $I_{sc}$ decrease, which was probably due to K⁺ secretion, was significantly attenuated (by 51 ± 10%, $n = 4$) but still substantially preserved under the Ca²⁺-depleted condition, indicating that general tissue damage had not occurred under this Ca²⁺-depleted condition. On the other hand, the large stimulation of Cl⁻ secretion induced by CCh added to the 8Br-cAMP-treated tissue (28, 56) was almost totally abolished under the Ca²⁺-depleted condition, consistent with the above finding (Fig. 5, D–E).

In the second series of experiments, the effect of CCh was examined on the tissue pretreated with ionomycin (1 μM) added to both mucosal and serosal solutions to elevate the [Ca²⁺] (Fig. 6). As shown in Fig. 6, A and B, ionomycin alone inhibited the $I_{sc}$ value from 271 ± 42 μA/cm² under basal conditions to 69 ± 23 μA/cm² (73 ± 4% inhibition, $n = 4$) as shown in Fig. 6A, in agreement with our previous report (57). The addition of CCh in the presence of ionomycin caused a decrease in $I_{sc}$, and the subsequent addition of benzamil further reduced $I_{sc}$ to the level of −13 ± 5 μA/cm². This value is not significantly different from the one when benzamil was applied to an adjacent tissue under the control condition (−14 ± 3 μA/cm², $n = 5$), indicating that the $I_{sc}$ decrease induced by ionomycin and that induced by subsequently added CCh were both mainly due to the inhibition of benzamil-sensitive $I_{sc}$. The percentage

Fig. 5. Effect of intracellular Ca²⁺ depletion on electrogenic Cl⁻ and K⁺ secretion. Experimental protocol and solution conditions were the same as those described in Fig. 4, except that bumetanide was excluded and benzamil (10 μM) was added to the mucosal side during the second incubation period. A–C: effect of CCh (10 μM) added to the serosal side. A: typical trace under normal conditions. This concentration of CCh caused an initial increase and subsequent decrease in $I_{sc}$. B: typical trace under Ca²⁺-depleted conditions. *0.01 < $P$ < 0.05 vs. control; $n = 4$. D–E: effect of 8-bromo-cAMP (8Br-cAMP; 0.5 mM, mucosal and serosal sides) followed by CCh (0.1 mM). F: $I_{sc}$ decrease induced by 8Br-cAMP and $I_{sc}$ increase induced by CCh from basal levels are compared under control and Ca²⁺-depleted conditions. *0.01 < $P$ < 0.05 vs. control; $n = 4$. 

Fig. 4, except that bumetanide was excluded and benzamil (10 μM) was added to the serosal side.
inhibition of benzamil-sensitive $I_{sc}$ induced by CCh was not significantly attenuated in the tissue that had been pretreated with ionomycin compared with control tissue, despite the fact that the $[Ca^{2+}]_i$ in the ionomycin-treated tissue had been presumably highly increased (Fig. 6C). The results of these two series of experiments suggest that, at least under certain conditions, CCh can suppress electrogenic Na$^+$/H$^+$ absorption mediated by a Ca$^{2+}$-independent signaling pathway.

**Role of intramural cholinergic neurons.** We next investigated the role of submucosal cholinergic neurons in regulating electrogenic Na$^+$ absorption. The mucosal-submucosal preparation, which contained the submucosal plexus neurons, was used. As shown in Fig. 7A, the activation of the intramural neurons by an EFS protocol evoked an initial $I_{sc}$ peak that was followed by an $I_{sc}$ decrease to below the basal level. When EFS was terminated, the $I_{sc}$ value further decreased slightly and then gradually increased toward the basal level. The $G_{t}$ value (Fig. 7A) increased transiently but returned to the basal level during EFS, and, after terminating EFS, it fell to below the basal level and then gradually increased. To estimate the changes in $I_{sc}$ and $G_{t}$ derived from electrogenic Na$^+$ absorption, EFS was also applied in the presence of amiloride (0.1 mM, mucosal; Fig. 7B). The initial $I_{sc}$ peak induced by EFS in the presence of amiloride had a similar value to that in its absence but was followed by a sustained $I_{sc}$ increase instead of the decrease. The $G_{t}$ value increased during EFS. Both the increased $I_{sc}$ and $G_{t}$ levels rapidly returned to the basal level after terminating EFS. It is clear from the difference in electrical response in the presence and absence of amiloride that the amiloride-sensitive $I_{sc}$ and $G_{t}$ values were attenuated by EFS (Table 2). In particular, the lowest levels of $I_{sc}$ and $G_{t}$ observed soon after terminating EFS in the absence of amiloride would provide a good estimate for the inhibition of amiloride-sensitive $I_{sc}$ and $G_{t}$, since, at that point, the effect of EFS on the amiloride-insensitive components of electrical parameters had almost disappeared (Fig. 7B). The electrical response induced by EFS was likely to have been mediated by the submucosal neurons, because it was almost totally abolished in the presence of TTX (300 nM, serosal side; data not shown).
shown). We next examined the involvement of cholinergic neurons in the EFS-induced inhibition of amiloride-sensitive \( I_{sc} \) by applying EFS in the presence of atropine (10 \( \mu M \), serosal; Fig. 7). The \( I_{sc} \) decrease during EFS and the lowest level of \( I_{sc} \) soon after terminating EFS were both found to be significantly attenuated in the presence of atropine, in addition to the significant inhibition of the initial \( I_{sc} \) peak (Table 2). In the amiloride-treated tissue, the secondary, sustained \( I_{sc} \) increase and the \( G_t \) increase were suppressed by atropine, although the effect was not significant. Thus atropine presumably attenuated the EFS-induced inhibition of amiloride-sensitive \( I_{sc} \) by approximately one-third (Table 2), suggesting that submucosal cholinergic neurons were involved via muscarinic receptor activation in the inhibition of electrogenic \( Na^+ \) absorption. The EFS-induced increase in \( I_{sc} \) that was observed in the presence of amiloride might have been due to electrogenic anion secretion that had not been completely suppressed by serosal bumetanide.

**Effect of cholinesterase inhibitor.** To further investigate the role of intramural ACh, we examined the effect of applying the cholinesterase inhibitor physostigmine (Fig. 8). Physostigmine added to the serosal side of the mucosal-submucosal preparation (10 \( \mu M \)) induced a slow decrease in \( I_{sc} \), which reached its lowest level \( \sim 15 \) min after the physostigmine treatment, with slight recovery thereafter (maximum \( I_{sc} \) change, \( -123 \pm 40 \mu A \cdot cm^{-2} \cdot h^{-1} \); \( n = 4 \)). The \( G_t \) value was hardly affected by the physostigmine treatment \( (G_t \) change, \( -1.0 \pm 0.7 \) mS/cm\(^2) \). When atropine (10 \( \mu M \), serosal) was administered after 20–25 min of the physostigmine treatment, atropine

### Table 2. Inhibition of amiloride-sensitive \( I_{sc} \) induced by EFS and the effect of atropine on this inhibition

<table>
<thead>
<tr>
<th></th>
<th>Initial Peak, ( \Delta I_{sc} ), ( \mu A/cm^2 )</th>
<th>End of EFS</th>
<th>Lowest Level after EFS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \Delta I_{sc} ), ( \mu A/cm^2 )</td>
<td>( \Delta G_t ), mS/cm(^2 )</td>
<td>( \Delta I_{sc} ), ( \mu A/cm^2 )</td>
</tr>
<tr>
<td>Control</td>
<td>139 ± 26</td>
<td>-76 ± 22</td>
<td>-0.7 ± 0.7</td>
</tr>
<tr>
<td>+ Amiloride</td>
<td>153 ± 56</td>
<td>23 ± 7††</td>
<td>2.8 ± 0.9†</td>
</tr>
<tr>
<td>Atropine</td>
<td>48 ± 5*</td>
<td>-50 ± 19*</td>
<td>-0.1 ± 0.5</td>
</tr>
<tr>
<td>+ Amiloride</td>
<td>76 ± 22</td>
<td>12 ± 3†</td>
<td>1.6 ± 0.5†</td>
</tr>
</tbody>
</table>

Values are means ± SE. Summary of experiments shown in Fig. 6 for the mucosal-submucosal preparation. Changes in \( I_{sc} \) and \( G_t \) from basal were determined at initial peak \( I_{sc} \) level, at the end of electrical field stimulation (EFS) and at the lowest \( I_{sc} \) level after the cessation of EFS, respective values given as \( \Delta I_{sc} \) and \( \Delta G_t \). Measurements in the presence and absence of atropine with or without amiloride were performed on adjacent tissues obtained from the same animal; \( n = 7 \) for the amiloride-free experiments, and \( n = 4 \) for the amiloride experiments. Basal \( I_{sc} \) in each group was similar (in \( \mu A/cm^2)\): control, 299 ± 35; control + amiloride, 270 ± 71; atropine, 272 ± 47; atropine + amiloride, 254 ± 57. * \( P < 0.05 \) vs. control. † \( P < 0.05 \) and †† \( P < 0.01 \) vs. absence of amiloride.
the $I_{sc}$ value rapidly returned to its basal level (Fig. 8A). In addition, the atropine pretreatment completely abolished the physostigmine-induced $I_{sc}$ response (data not shown, $n = 4$). The $I_{sc}$ response to physostigmine was almost totally abolished when the tissue had been pretreated with amiloride (0.1 mM, mucosal; Fig. 8B, $n = 4$). Therefore, after its degradation had been inhibited, the tissue ACh concentration could increase enough to inhibit, via muscarinic receptor activation, the electrogenic Na$^+$ absorption. The $I_{sc}$ decrease induced by physostigmine was not affected when the tissue had been pretreated with tetrodotoxin (300 nM, serosal; maximum $I_{sc}$ decrease: control, $-79 \pm 26$; +tetrodotoxin, $-89 \pm 40 \mu$A cm$^{-2}$ h$^{-1}$; $n = 3$). Therefore, ACh released by ongoing submucosal cholinergic nerve activity is unlikely to have been mainly responsible for the physostigmine-induced $I_{sc}$ response.

**DISCUSSION**

It is well established that submucosal neurons are mainly involved in the activation of Cl$^-$ secretion in the colon. The results of this study show for the first time that submucosal neurons also inhibited electrogenic, amiloride-sensitive Na$^+$ absorption and that the cholinergic pathway is one of the major components of this mechanism. The amiloride-sensitive $I_{sc}$ value was decreased by the electrical stimulation of submucosal neurons, the response being partially blocked by atropine. In addition, externally applied CCh caused a reduction of both amiloride-sensitive $I_{sc}$ and $^{22}$Na$^+$ absorption by a similar magnitude. Inhibition of electrogenic Na$^+$ absorption during the stimulation of intrinsic cholinergic neurons has previously been reported in the turtle colon (50) but not in the mammalian colon.

The inhibitory effect of CCh on the amiloride-sensitive $I_{sc}$ value was mainly mediated by the muscarinic receptor, because the CCh-induced inhibition of amiloride-sensitive $I_{sc}$ was almost totally suppressed by atropine. The CCh-induced inhibition of $I_{sc}$ was preserved in the presence of TTX, suggesting that CCh may have activated the muscarinic receptor on the epithelial cells. The muscarinic receptor that was coupled to the inhibition of electrogenic Na$^+$ absorption in the colon might exist on the surface epithelial cells, since electrogenic Na$^+$ absorption has been suggested to occur mainly in surface epithelial cells (11, 20, 27). Muscarinic receptors have been shown to be present in the colonic mucosa or epithelial cells from the specific binding of the muscarinic antagonist [3H]quinuclidinyl benzilate (41, 42, 58). In addition, it has been demonstrated that functional cholinergic receptors are present in colonic crypt cells and colonic carcinoma cell lines (2, 8, 9, 16, 21, 26). However, it remains to be determined whether or not surface epithelial cells in the colon actually express a muscarinic receptor(s). The inhibition of electrogenic Na$^+$ absorption by CCh has previously been observed only in the turtle colon (50). In addition to the inhibition of electrogenic Na$^+$ absorption, muscarinic agonists have previously been...
reported to affect a variety of transport pathways in the colon, including the stimulation of electrogenic Cl− and K+ secretions and the inhibition of electroneutral NaCl absorption (6, 15, 16, 24, 28, 34, 48, 56, 59).

The inhibition of amiloride-sensitive electrogenic Na+ absorption by cholinergic receptor agonists has been observed not only in the turtle colon (50, 53) but also in the toad bladder (43, 52) and in frog skin (7). Electrogenic Na+ absorption involves the apical amiloride-sensitive Na channel and basolateral Na+/K+-ATPase/K+ channel (1, 25). The decrease in GI value in association with the inhibition of amiloride-sensitive I_sc induced by CCh that has been demonstrated in this study (Fig. 2) is at least consistent with the involvement of the inhibition of apical Na+ channel activity. The inhibition of both the apical Na+ channel and basolateral K+ channel have been suggested to be involved in the CCh-induced inhibition of Na+ absorption in the turtle colon (50, 53). Clearly, regulation of the transport processes responsible for the CCh-induced inhibition of electrogenic Na+ transport in the guinea pig distal colon remain to be determined.

We investigated in this study the role of the Ca2+-signaling pathway in the CCh-induced inhibition of electrogenic Na+ absorption. It was found that the CCh-induced inhibition of Na+ absorption was not markedly attenuated when the tissue was incubated with a low-Ca2+, high-Mg2+ solution containing ionomycin and BAPTA-AM, a condition whereby an agonist-induced increase in [Ca2+]i was presumably largely attenuated (35). This condition, however, almost completely suppressed the CCh-induced stimulation of Cl− and K+ secretion, consistent with the notion that these responses were mediated by an increase in [Ca2+]i. In the additional experiments, we have shown that ionomycin markedly decreased the amiloride-sensitive I_sc value that was probably mediated by the [Ca2+]i increase and that, in the presence of ionomycin, CCh still induced the inhibition of residual amiloride-sensitive I_sc. These findings suggest that a [Ca2+]i-independent signaling pathway leading to the inhibition of electrogenic Na+ absorption was activated by CCh at least under certain conditions. The Ca2+-independent signaling pathway as well as the Ca2+-dependent one is known for muscarinic receptors. Five different G protein-linked muscarinic receptor subtypes, M1–M5, have been identified (4). Activation of the M1, M3, and M5 receptors is known to be coupled to the inositol phospholipid/Ca2+-signaling pathway, whereas that of M2 and M4 is coupled to the inhibition of cAMP production. A direct action of G protein or the activation of adenyl cyclase has also been reported to mediate the response to M2, M4, and M5 activation in certain tissues. Clearly, it remains to be determined whether the Ca2+-signaling pathway is responsible and whether this putative Ca2+-independent pathway plays a role in the inhibition of electrogenic Na+ absorption induced by CCh under normal conditions.

The inhibition of amiloride-sensitive I_sc induced by EFS was partly, but not totally, suppressed by atropine, indicating that both cholinergic and noncholinergic submucosal neurons were involved in inhibiting electrogenic Na+ absorption. It has previously been shown that stimulation of the submucosal neurons activated anion secretion by activating both the cholinergic and noncholinergic pathways in the colon (12, 18, 22, 23, 32, 33, 51). Approximately half of the submucosal plexus neurons in the colon have been reported to contain ACh from choline acetyltransferase immunoreactivity and other morphological studies (13, 14, 30, 32, 33, 38, 39). Further evidence for the presence of cholinergic submucosal neurons has been the [3H]ACh release induced by nerve stimulation (19, 29, 54, 58). However, a morphological investigation of choline acetyltransferase immunoreactivity has failed to clearly demonstrate cholinergic nerve fibers in the lamina propria of colonic mucosa (38, 39), although the concentration of choline acetyltransferase in the mucosal nerve fiber may not be high enough to be detected. Vasoactive intestinal peptide and substance P have been suggested to be released and to be responsible for the noncholinergic component of Cl− secretion (17, 32, 40, 51). Whether these substances can mediate the noncholinergic component of the inhibition of electrogenic Na+ absorption remains to be determined.

The cholinesterase inhibitor physostigmine inhibited the amiloride-sensitive I_sc, the effect being abolished by atropine. Cholinesterase is known to be present in the intestinal mucosa (45, 46). This finding suggests that tissue ACh can reach a sufficiently high concentration to activate the epithelial muscarinic receptor, leading to the regulation of electrogenic Na+ absorption at least under certain conditions. The cell types from which tissue ACh was released are not clear at present. ACh release from the nerve terminal of ongoing submucosal cholinergic neurons is unlikely, because TTX failed to inhibit the physostigmine-induced response. The possibility cannot be excluded, however, that a small amount of ACh was constitutively released without action potential from the nerve terminals. Alternatively, epithelial and nonepithelial cells exhibiting immunoreactivity to choline acetyltransferase might have been the source of tissue ACh (30, 38, 39). A role of tissue ACh that was independent of the cholinergic nerve activity has been previously suggested from the finding that TTX inhibited the luminal propionate-induced Cl− secretion in the colon by 40%, whereas atropine inhibited it by 90% (55).

In summary, the submucosal neurons not only activated Cl− secretion but also inhibited electrogenic Na+ absorption, thereby leading to a prosecretory state in the colon. In support of this, the enteric neuron has previously been suggested to inhibit NaCl absorption and to stimulate mucus secretion (36, 37, 47). In addition, K+ secretion may be enhanced by cholinergic neurons, as suggested from the results of this and previous works. Collectively, the submucosal neurons may function to facilitate the movement of fecal pellets and, when excessively activated, to protect the mucosa and to flush the colonic lumen of noxious agents. Cholinergic neurons may play an important role in these activities.
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REFERENCES


