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Regulation of Guinea Pig Ileal Electrolyte Transport by M_3-Muscarinic Acetylcholine Receptors *In Vitro*

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SUMMARY

To determine the muscarinic receptor subtype mediating guinea pig ileal mucosal electrolyte secretion, we compared the potencies (K_b) of selective M_1 (pirenzepine) (PZ), M_2 (AF-DX 116, methoctramine), and M_3 [4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP), hexahydrosiladifenidol (HHSiD)] antagonists as inhibitors of carbachol-induced reductions in guinea pig atrial heart rate and ileal longitudinal muscle contractions, responses mediated by M_2 and M_3 receptors, respectively. Pretreatment with all five muscarinic antagonists shifted the carbachol concentration-response curve to the right, in a manner suggesting competitive antagonism. The following affinity profiles (K_b, nM) were obtained for: 1) ileal mucosa: 4-DAMP (2.7) > HHSiD (23.0) > PZ (110) ≥ methoctramine (395) > AF-DX 116 (784); 2) atrial heart rate: 4-DAMP (9.5) ≃ methoctramine (11) > AF-DX 116 (63) > HHSiD (222) > PZ (256); and 3) ileal longitudinal muscle: 4-DAMP (3.1) > HHSiD (21) > PZ (143) > methoctramine (388) ≥ AF-DX 116 (482). The selectivity profiles of these antagonists suggest that muscarinic receptors in the ileal mucosa more closely resemble those in the ileal muscle (M_3) than those in atrial muscle (M_2). Moreover, M_1-muscarinic receptors appear to be relatively unimportant in mediating the effects of carbachol on short circuit current (I_{SC}). Carbachol-induced increases in I_{SC} were also unaffected by pretreatment with 0.5 μM tetrodotoxin, suggesting that electrolyte transport in the guinea pig ileal mucosa may be mediated, in part, by postsynaptic M_3-muscarinic receptors on the enterocytes.

Activation of muscarinic receptors in the guinea pig ileum evokes increases in I_{SC} and stimulates Cl^- secretion by mucosal enterocytes (1–4). It is believed that acetylcholine released from enteric cholinergic neurons acts upon epithelial crypt cells, inducing the secretion of chloride ions into the lumen. Cholinergic neurons innervate the colonic mucosa of the rat (5) and small intestinal mucosa of the guinea pig (6), and muscarinic receptors have been identified on plasma membranes of both colonic and small intestinal epithelial cells (7).

From molecular cloning studies, five unique gene sequences encoding muscarinic receptors have been identified (8). Pharmacologically, however, muscarinic receptors have been classified into three subtypes (M_1, M_2, and M_3), defined on the basis of selective muscarinic receptor antagonists (9, 10). The M_1 "neuronal" subtype has a high affinity for PZ and (+)-telenzepine and is localized primarily in areas of the autonomic ganglia and brain. M_2 (or $M_{2\alpha}$) receptors, found in the heart, bind PZ with a low affinity but exhibit a high affinity for the cardioselective antagonists AF-DX 116 and methoctramine (11, 12). M_3 (or $M_{2\beta}$) receptors are defined by their high affinity for HHSiD, *p*-fluoro-HHSiD, and 4-DAMP.

In order to determine the muscarinic receptor subtype mediating guinea pig ileal mucosal electrolyte secretion, comparisons of the relative potencies (K_b) of selective M_1 (PZ), M_2 (AF-DX 116, methoctramine), and M_3 (4-DAMP, HHSiD) muscarinic antagonists were made in three functional assay systems, as inhibitors of carbachol-induced increases in I_{SC} in ileal mucosa and as inhibitors of both carbachol-induced reductions in atrial heart rate and contractions of ileal longitudinal muscle.

Materials and Methods

Inhibition of carbachol-induced contraction of guinea pig ileum longitudinal muscle. Ileal contractions were measured as previously described (13). Distal ileum (5 cm above the ileocecal junction) excised from male Hartley guinea pigs (300–500 g) was placed immediately in oxygenated Tyrode's solution of the following composition (in mM): NaCl, 137; KCl, 2.7; $NaHCO_3$, 11.9; NaH_2PO_4, 0.36; $MgCl_2 \cdot 6\,H_2O$, 1.0; $CaCl_2$, 1.8; dextrose, 5.6. Longitudinal muscle strips (3–4 cm) were suspended in 10-ml organ baths at 37° and gassed continuously (5% CO_2 in O_2). Eight tissue segments were used in parallel from each animal and stabilized for 60 min before drug addition. Initial tension was set at 0.5 g. Isometric contractions were recorded by means of an electromechanical transducer on a potentiometric recorder.

Carbachol concentration-response curves were constructed by cumulative additions after each contraction elicited by the preceding

ABBREVIATIONS: I_{SC}, short circuit current; 4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine methiodide; HHSiD, hexahydrosiladifenidol; PZ, pirenzepine; PD, potential difference.

concentration had reached a steady value. After a control concentration-response curve was obtained, the tissue was washed several times and then additional curves in the presence of increasing concentrations of test antagonist were constructed. Responses were expressed as a percentage relative to the maximum contraction elicited by carbachol in the absence of antagonist. Anti-muscarinic potencies (K_b) were calculated as previously described (13).

Inhibition of carbachol-induced I_{sc} in guinea pig ileal mucosa. Ussing chamber studies were conducted as previously described (13). After the terminal 5 cm of ileum were discarded, a ~15-cm segment of distal ileum from male Hartley guinea pigs (200–400 g) was removed and stripped of its underlying longitudinal muscle by blunt dissection. Six adjacent tissues were then mounted as flat sheets between two Lucite half chambers (exposed area = 0.64 cm^2) and bathed on both sides by a physiological salt solution that was circulated by gas lift and maintained at 37° by water-jacketed reservoirs. The solution was gassed continuously with 5% CO_2 in O_2 (pH 7.4). The ionic composition was (in mM): Na^+, 142; K^+, 5; Cl^-, 123.7; HCO_3^-, 25; HPO_4^{2-}, 1.65; $H_2PO_4^-$, 0.3.

Electrical measurements were monitored with an automatic voltage clamp (model DVC-1000; World Precision Instruments). Two calomel electrodes with 4% agar Krebs Ringer bicarbonate bridges were used to measure the transepithelial PD across the isolated mucosa. The spontaneous PD (usually 1.5–5.0 mV) was short-circuited throughout the experiment, and the clamp current (I_{sc}) was passed with Ag/AgCl electrodes located on each side of the tissue. I_{sc}, which is defined as the current necessary to nullify the PD, is the electrical sum of all ion movement. I_{sc} measurements were recorded on a Gould strip-chart recorder. Tissue resistance was calculated (Ohm's law) from periodic measurements of I_{sc} and open-circuit PD.

Tissues were allowed to equilibrate for 45 min before addition of drugs. Transepithelial resistance at the time of drug addition was between 25 and 50 $\Omega \cdot cm^2$. Cumulative additions of carbachol (0.1–100 μM) were added simultaneously to both the mucosal and serosal sides of the tissue. Maximal response was defined when the last incremental concentration of carbachol produced no further increase in I_{sc}. In test tissues, antagonists were added to both serosal and mucosal sides 5 min before carbachol.

Inhibition of carbachol-induced negative chronotropic effects in guinea pig atria. Hearts from male albino guinea pigs were removed and immediately placed into an oxygenated Krebs-Henseleit buffer of the following composition (in mM): NaCl, 116.2; $NaHCO_3$, 22.4; KCl, 6.0; NaH_2PO_4, 0.98; $MgSO_4$, 1.0; $CaCl_2$, 2.46; dextrose, 11.1. After the ventricles were dissected away, atria were bisected to isolate the right atrium. Tissues were suspended with silk suture in 10-ml water-jacketed tissue baths maintained at 33°, containing oxygenated buffer (95% O_2/5% CO_2). Isometric contractions were measured with an electromechanical force displacement transducer coupled to a physiograph. Initial tension was set at 0.5 g. Because right atria beat spontaneously, resting tension was adjusted to the peak of the length-tension curve for each tissue following a 30-min equilibration period. Tissue baths were then emptied, refilled, and reequilibrated for an additional 30-min period.

Cumulative concentration-response curves for carbachol were obtained in the absence and presence of increasing concentrations of antagonists. K_b values were calculated as previously described (see above) and represent means ± standard errors of at least three experiments.

Materials. Tetrodotoxin and carbamylcholine chloride were purchased from Sigma (St. Louis, MO) and methoctramine·HCl from Research Biochemicals, Inc. (Wayland, MA). Pirenzepine·HCl was obtained as a gift from Dr. Karl Thomae GMBH (Biberach an der Riss, Germany); 4-DAMP and HHSiD were synthesized by Drs. Ted Adams and Vicki Audia, respectively (NOVA Pharmaceutical Corporation, Baltimore, MD).

Results

Isolated ileal mucosa. Carbachol-induced increases in I_{sc} were inhibited by all antagonists tested, with a rank order of potency of 4-DAMP > HHSiD > PZ > methoctramine > AF-DX 116. In each case, the carbachol dose-response curve was shifted competitively to the right, without depression of the maxima; representative data for HHSiD and AF-DX 116 at equivalent concentrations (1.0 μM) are shown in Fig. 1. HHSiD was about 20- to 40-fold more potent than the cardioselective agents AF-DX 116 and methoctramine in antagonizing mucosal Cl^- secretion; 4-DAMP was the most potent antagonist (Table 1). None of the muscarinic antagonists affected basal I_{sc} or transepithelial resistance at the concentrations tested. The neurotoxin tetrodotoxin (0.5 μM) did not block (but significantly enhanced) the I_{sc} response to 10 μM carbachol (57 ± 10 $\mu A/cm^2$ for control versus 93 ± 7 $\mu A/cm^2$ in the presence of tetrodotoxin; $n = 3$ animals; $p < 0.05$), suggesting that muscarinic receptors situated on the enterocyte may be activated by muscarinic agonists.

Isolated ileal longitudinal muscle and right atria. The rank order of potency of the antagonists in inhibiting carbachol-induced contractions in ileal muscle was similar to their rank order of potency as antagonists of Cl^- secretion in the mucosa, suggesting no significant difference between muscarinic receptors mediating these two responses. In the atria and in the ileal muscle, increasing concentrations of antagonist

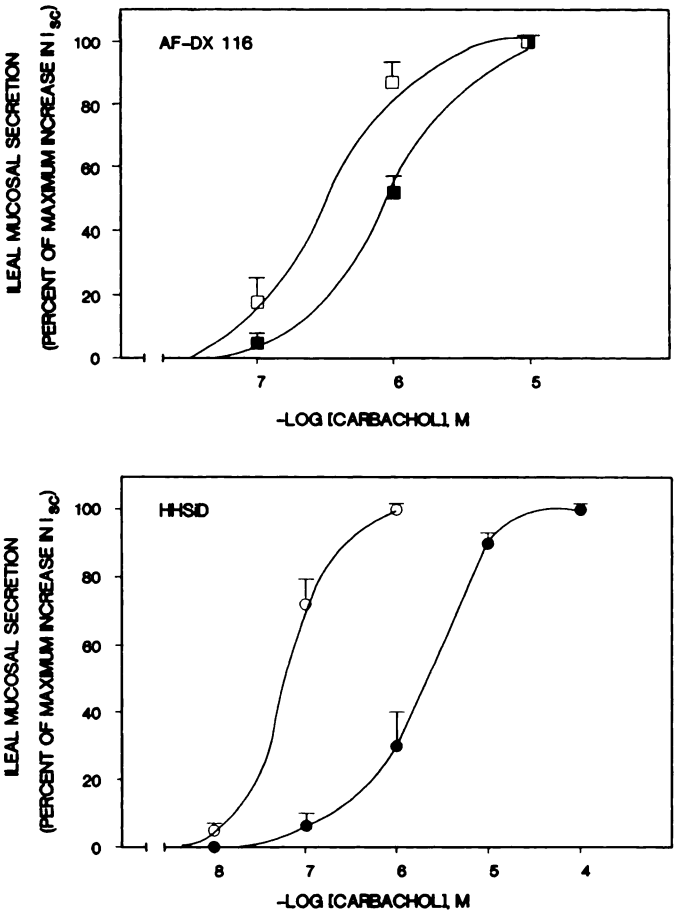

Fig. 1. Effect of 1.0 μM AF-DX 116 (■) or 1.0 μM HHSiD (●) on the carbachol dose-response curve (□, ○) in ileal mucosa preparations. *Points* are means ± standard errors.

TABLE 1

Affinity profile of selective muscarinic antagonists in guinea pig ileal mucosa, ileal muscle, and atria

See Materials and Methods for details. The numbers in parentheses are the numbers of animals used. For the mucosal studies, the K_b values are derived from the means of three to five animals. Values are mean ± standard error.

Drug (receptor subtype)	K_b values			Selectivity ratios		
	Muscle		Mucosa, ileum (?)	Ileal muscle/ atrial muscle	Ileal mucosa/ atrial muscle	Ileal mucosa/ ileal muscle
	Ileum (M_3)	Atria (M_2)				
	nM					
Methoctramine (M_2)	388 ± 64 (4)	11.0 ± 1.1 (5)	395 ± 92	35.3	35.9	1.0
AF-DX 116 (M_2)	482 ± 55 (7)[a]	63 ± 8 (9)[a]	784 ± 80	7.7	12.4	1.6
HHSiD (M_3)	21 ± 4 (4)[a]	222 ± 41 (6)[a]	23 ± 8.5	0.09	0.1	1.1
4-DAMP (M_3)	3.1 ± 0.6 (4)	9.5 ± 0.7 (5)	2.7 ± 0.2	0.3	0.3	0.9
Pirenzepine (M_1)	143 ± 22 (6)[a]	256 ± 26 (5)[a]	110 ± 22	0.6	0.4	0.8

[a] Data taken from Noronha-Blob *et al.* (23).

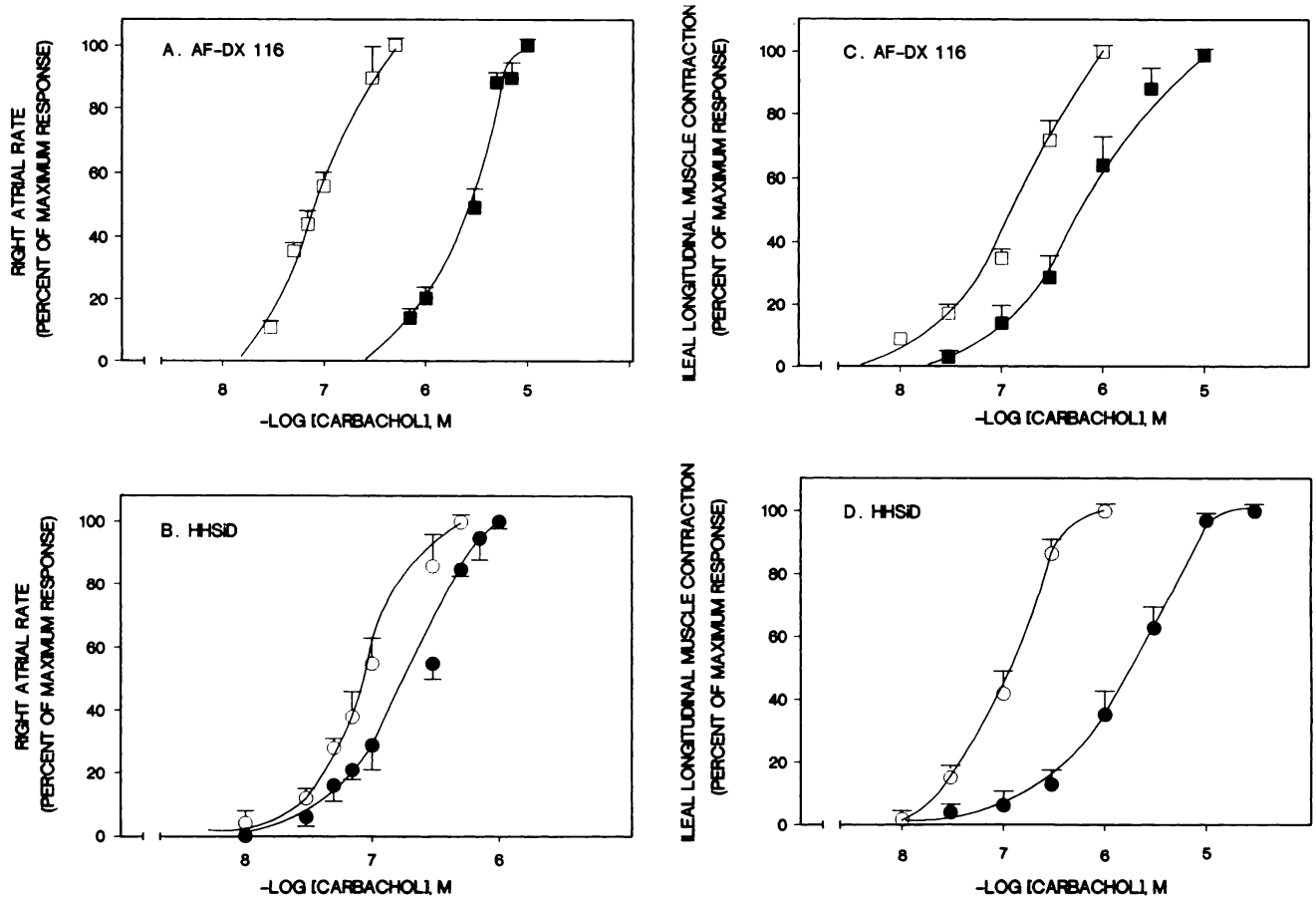

Fig. 2. Effect of 1.0 μM AF-DX 116 (■) or 1.0 μM HHSiD (●) on right atrial heart rate (A and B) and ileal longitudinal muscle contractions (C and D). □, ○, Concentration-response curve for carbachol in the absence of antagonists. *Points* are means ± standard errors.

caused parallel rightward shifts of the carbachol concentration-response curve, with the same maximum response, suggesting competitive interactions. Fig. 2 shows that, in the atria, AF-DX 116 (1.0 μM) produced a greater rightward shift than an equivalent concentration of HHSiD. In contrast, in the ileum, HHSiD was more potent than AF-DX 116 (~10-fold) (Fig. 2, Table 1), confirming the selectivity of AF-DX 116 and HHSiD for M_2 and M_3 receptors, respectively. The affinities of all the antagonists for muscarinic receptors in the ileal and atrial muscle and ileal mucosa and the selectivity ratios derived from these values are shown in Table 1.

Correlation among the potencies of muscarinic antagonists to antagonize Cl^- secretion, ileal smooth muscle contraction, and reduction of atrial heart rate depression. The similarity between the rank order of potency of muscarinic antagonists to antagonize carbachol-induced Cl^- secretion in the ileal mucosa and ileal smooth muscle contraction prompted a least squares regression analysis to test for potential correlation of these inhibitory potencies. Significant positive linear correlations were calculated for five muscarinic antagonists ($r = 1.0$, $p < 0.01$) in inhibiting mucosal Cl^- secretion and ileal longitudinal muscle contraction, with a slope approaching unity. In contrast, there was no correlation ($r = 0.2$, $p > 0.5$) among the potencies of the antagonists in inhib-

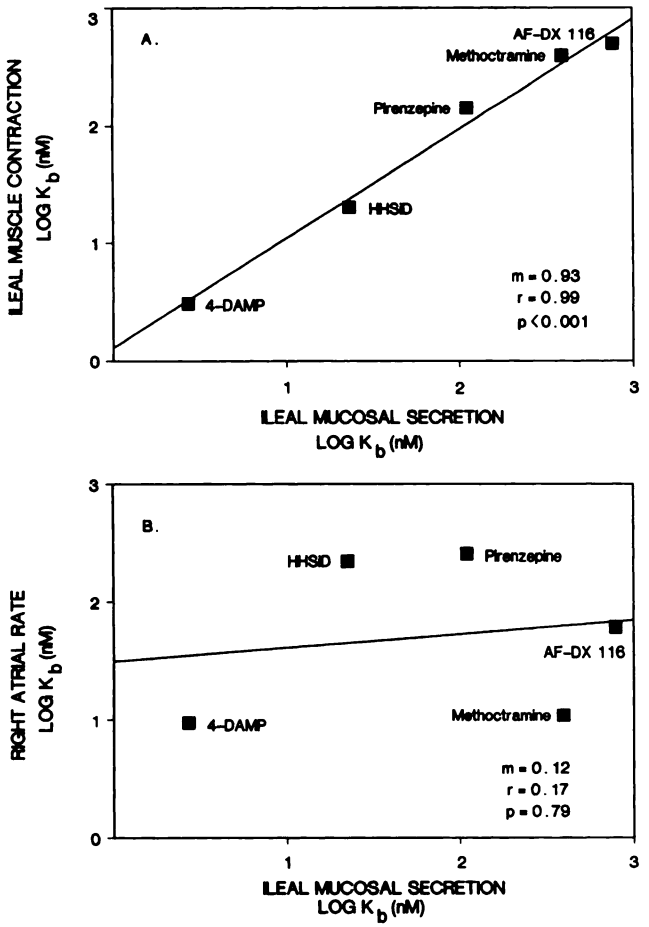

Fig. 3. Correlation between the potencies (K_b) of muscarinic antagonists to inhibit Cl^- secretion in the ileal mucosa and to inhibit ileal muscle contraction (A) or prevent reductions in heart rate (B). Data are summarized in Table 1. *Lines* were determined by linear regression analysis. $p < 0.05$ was considered significant.

iting I_{SC} increases and atrial muscle contractile responses (Fig. 3).

Discussion

Based on a comparison of the selectivity profiles of known reference muscarinic antagonists, it appears that muscarinic receptors of the M_3 subtype mediate both ileal mucosal Cl^- secretion and ileal longitudinal muscle contractility. Thus, there was a significant positive correlation among the antagonists in mediating these two functional responses (Fig. 3).

In the guinea pig ileum, muscarinic agonists stimulate electrogenic Cl^- secretion by crypt cells with little effect on other ions (14). In these ileal studies, carbachol both increased I_{SC}, indicative of Cl^- secretion, and induced ileal longitudinal muscle contractions, with a similar potency. None of the muscarinic antagonists affected basal I_{SC} at concentrations that competitively antagonized carbachol-induced Cl^- secretion, consistent with findings of Cooke (1, 15), in which atropine had little effect on basal Na^+ and Cl^- transport rates. HHSiD, methoctramine, AF-DX 116, and 4-DAMP competitively antagonized Cl^- secretion in the ileal mucosa, similar to their inhibitory effects in other peripheral tissues (11, 12, 16, 17). Methoctramine, a polymethylene tetramine with cardioselective antagonist properties, was 35-fold more selective for atrial versus ileal or mucosal muscarinic receptors (Table 1); likewise AF-DX 116, another cardioselective agent, showed about an 8–12-fold selectivity for atrial receptors. Both methoctramine and AF-DX 116 were equipotent antagonists at ileal muscle and ileal mucosal muscarinic receptors (Table 1).

Mutschler and Lambrecht and co-workers (17, 18) identified a series of ileal-selective compounds (e.g., HHSiD) related to procyclidine and difenidol, and Barlow *et al.* (16), using a series of structurally related 4-DAMP analogs, proposed differences between subtypes of muscarinic receptors in atria and ileum. Our findings with 4-DAMP and HHSiD confirm the selectivity of these agents for ileal over atrial muscle receptors. More importantly, however, the potency of these agents to block Cl^- secretion in the ileal mucosa was identical to their potency as inhibitors of ileal contractile activity. Together, the data suggest a similarity between M_3 receptors that mediate muscle contractility and epithelial Cl^- secretion. These findings differ from those of Herawi *et al.* (19), who proposed the existence of two different subtypes of M_3 ($M_{2\beta}$) muscarinic receptors on gastric smooth muscle and mucosa. Based on radioligand binding studies, they found that the selectivity of AF-DX 116 was greater between cardiac (M_2) and mucosal (M_3) receptors than between cardiac and muscle (M_3) receptors.

Electrophysiological studies in the guinea pig ileum showed that PZ potently antagonized muscarinic depolarization of submucous neurons ($K_b < 100$ nM), suggesting the involvement of M_1-muscarinic receptors (20). In our studies, however, carbachol appears to act primarily at receptors localized on the serosal surface of enterocytes, because the neural toxin tetrodotoxin has no inhibitory effect. Furthermore, PZ has weak inhibitory effects on carbachol-induced increases in I_{SC}, also suggesting that M_1-muscarinic receptors contribute little towards mediating the effects of carbachol. Carey *et al.* (14), in agreement with our findings, have also suggested a minimal involvement of M_1-muscarinic sites in the overall secretory response in the guinea pig ileum. M_1 receptors, therefore, probably mediate acetylcholine effects in the ganglia, whereas M_3 receptors mediate its effects on the enterocyte. Recently, rat gastric parietal cells (21) and human HT-29 colon carcinoma cells (22) have been shown to contain M_3-muscarinic receptors coupled to phosphoinositide breakdown.

In summary, our findings suggest that muscarinic receptors on enterocytes in ileal mucosa that mediate Cl^- secretion belong to the M_3 subtype and are similar to the muscarinic receptor subtype that mediates contraction of ileal longitudinal muscle. The effect of carbachol at M_3 receptors on both smooth muscle and enterocytes may contribute to ileal secretion in the whole animal.

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