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Cholinergic Modulation of Electrogenic Ion Transport in Different Regions of the Rat Small Intestine

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Abstract

Acetylcholine acting via muscarinic receptors located in the intestinal mucosa controls ion and fluid transport. This study examined the pathway(s) by which cholinergic receptors mediate secretion in rat isolated duodenum, jejunum and ileum using the short-circuit current (Isc) as an index of electrogenic Cl^- secretion.

Carbachol and bethanechol induced electrogenic Cl^- transport which was insensitive to the neural blocker tetrodotoxin, indicating their direct action on the enterocytes. Functional characterization of electrogenic secretion activated via muscarinic receptors on jejunal and ileal enterocytes was achieved by use of selective muscarinic antagonists in the presence of tetrodotoxin. In both regions the rank order of potency of these compounds (atropine > 4-diphenylacetoxy-*N*-piperidine methiodide (4-DAMP) > hexahydro-sila-difenidol (HHSiD) > pirenzepine > methoctramine) indicated the M_3 receptor subtype. Secretion activated by the muscarinic agonist 4-[[(3-chlorophenyl)amino]carbonyl]-*N,N,N*-trimethyl-2-butyn-1-ammonium chloride (McN-A-343) was sensitive to tetrodotoxin and pirenzepine but not to the ganglionic blocker, hexamethonium, indicating the M_1 receptor subtype on post ganglionic neurons. Regional differences for bethanechol-activated secretion showed an increasing gradient in secretory capacity (Isc max) in a proximal-to-distal direction along the small intestine. Responses to McN-A-343 also showed regional differences but these were unlike those of bethanechol.

These results show that cholinomimetic-induced electrogenic Cl^- secretion in rat isolated small intestine appears to be mediated by two dissimilar populations of muscarinic receptor: M_3 muscarinic receptors positioned on enterocytes and M_1 muscarinic receptors sited on submucosal neurons.

The regulation of electrogenic Cl^- secretion in the rat small intestine appears to be mediated by two dissimilar populations of muscarinic receptors differentially localized within the intestinal mucosa. The variation in muscarinic–cholinergic-mediated ion transport in different regions of the rat small intestine is a property of these two pathways.

The cholinergic neurotransmitter acetylcholine is thought to play a major role in the management of intestinal fluid and ion transport through its interactions with muscarinic and nicotinic receptors located at neuronal or neuroepithelial junctions or both. Indeed, we and others have demonstrated the ability of cholinomimetic substances to induce intestinal electrolyte secretion (Kuwahara et al 1987; Przyborski et al 1990, 1991a,b,c, 1992; Chandan et al 1991; O'Malley et al 1995). Cholinergic nerve endings have been detected in the intestinal mucosa (Bornstein & Furness 1988) and it has been shown that the application of exogenous acetylcholine partly modulates epithelial function by its action on enteric neural pathways (Ren & Harty 1994; O'Malley et al 1995). Furthermore, stimulation of submucosal neurons in guinea-pig distal colon evoked changes in ion secretion which appeared to be mediated by neural pathways involving acetylcholine (Cooke 1984; Carey et al 1987; Kuwahara et al 1987; Chandan et al 1991). In addition, the direct action of acetylcholine on epithelial cells has been implicated by the ability of cholinergic agonists to stimulate ion secretion across epithelial monolayers in-vitro (Dharmsathaphorn & Pandol 1986; Dickinson et al 1992). Ligand-binding studies using rat colon (Rimele et al 1981; O'Malley et al 1995) and human HT29 (Kopp et al 1989) and T84 (Dickinson et al 1992) colonic carcinoma cells have provided supporting data for the presence of muscarinic receptors on gastrointestinal epithelial cells. Moreover, we have previously characterized the localization of muscarinic receptor mRNA in rat jejunal enterocytes (Przyborski & Levin 1993).

In numerous biological systems, the muscarinic action of cholinergic compounds are mediated by multiple subtypes of muscarinic receptor (see reviews by Caulfield (1993) and by Eglen & Watson (1996)). The recognition of distinct receptor subtypes and their differential distribution within the intestinal mucosa raises the question of the role of these subtypes in the regulation of intestinal ion transport. Indeed, secretory responses induced by 5-hydroxytryptamine (5-HT) in the rat intestine are thought to involve more than one 5-HT receptor subtype (Hardcastle & Hardcastle 1995) and evidence has indicated that pathways which mediate the secretory action of 5-HT vary in different regions of the gut (Franks et al 1996).

To further our understanding of the control of ion transport in the rat intestine, we examined the locus of action of various cholinergic substances which mediate electrogenic secretion and assessed whether such pathways are conserved in different regions of the rat small intestine. Preliminary reports of some of these findings have been presented (Przyborski et al 1990, 1991a, 1992).

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Materials and Methods

Chemicals

4-[[(3-Chlorophenyl)amino]carbonyl]-*N,N,N*-trimethyl-2-butyn-1-ammonium chloride (McN-A-343), 4-diphenylacetoxy-*N*-methylpiperidine (4-DAMP), methoctramine HCl and

hexahydro-sila-difenidol HCl (HHSiD) were obtained from Research Biochemicals (Natick, MA). All other chemicals and reagents were purchased from Sigma (UK).

Ion transport

Experiments were performed on pieces of proximal and distal duodenum, mid-jejunum and proximal ileum isolated from anaesthetized (pentobarbitone; 60 mg kg^{-1}, i.p.) male Wistar rats (Sheffield strain, 220–240 g). The intestinal segment was cut along its anti-mesenteric border to produce a flat sheet of submucosa-mucosa which was subsequently clamped between Using-type half-chambers (Przyborski et al 1991b). In some experiments, the preparation was stripped of its external muscle layers. The exposed tissue area (1·92 cm^2) was continuously bathed with warm (37°C) Krebs bicarbonate saline (8 mL) (Krebs & Henseleit 1932) which was oxygenated and stirred with humidified 95% O_2 5% CO_2 by a gas-lift mechanism built into the tissue-mounting plates (Przyborski et al 1991b). The serosal solution also contained 10 mM glucose for the metabolic requirements of the tissue and the mucosal solution contained 10 mM mannitol to act as an osmotic balance. Potential difference (Pd) across the tissue was measured with a high-impedance voltage clamp (DVC 1000, World Precision Instruments, New Haven, CT). All studies were performed under short-circuit conditions whereby an internal feedback circuit of the voltage clamp continuously maintained the Pd at zero by applying a current (short-circuit current, Isc) that directly opposed the tissue current. Changes in Isc were obtained on a chart recorder directly from the continuous output. In addition, transepithelial resistance (R) was calculated by changing the membrane potential and applying Ohm's law. Intestinal preparations were equilibrated under short-circuit conditions for 15 min by which time bioelectric measures had stabilized. Compounds were added to the serosal solution in volumes of 20 μL. For some experiments cumulative or non-cumulative concentration–response curves were constructed as previously described (Przyborski et al 1991c). For receptor-characterization studies, cholinergically evoked changes in secretory activity were recorded in the absence (control) and presence of a variety of muscarinic antagonists with different affinities. The EC50, calculated by non-linear regression, and maximum values were obtained from each concentration–response curve. When appropriate, such parameters were used for subsequent determination of the strength of competitive antagonism ($-\log A_2$; pA_2) for each of the inhibitory compounds used (Arunlakshana & Schild 1959). To enable monitoring of the direct action of the cholinergic compound on the intestinal epithelium, the neural blocker tetrodotoxin ($1{\cdot}25 \times 10^{-6}$ M) was added to the serosal solution to exclude involvement of enteric neurons.

Data analysis

For ion transport studies, results are expressed as the maximum change in short-circuit current response (ΔIscmax, μA cm^{-2}). All Isc values represent mean ± s.e.m. from n independent experiments unless stated otherwise. Because data complied with a normal distribution, Student's *t*-test was used to compare control and experimental values. Comparison of full concentration–response curves was achieved by Kruskal-Wallis analysis of variance followed by Conover's multiple *t*-test.

Results

Secretory responses to cholinergic agonists

Measurement of transepithelial Pd, R and Isc reflected the electrogenic secretory status of the specimen. Under basal conditions, Pd, R and Isc values of unstripped tissues were 3·3 ± 0·1 mV, 23 ± 1 Ω cm^{-2} and 40 ± 1 μA cm^2, respectively, for proximal duodenum (n = 50); 3·4 ± 0·1 mV, 14 ± 1 Ω cm^{-2} and 66 ± 2 μA cm^2, respectively, for distal duodenum (n = 50); 3·9 ± 0·2 mV, 13 ± 1 Ω cm^{-2} and 85 ± 4 μA cm^{-2}, respectively, for mid-jejunum (n = 50); 2·7 ± 0·2 mV, 16 ± 1 Ω cm^{-2} and 45 ± 2 μA cm^{-2}, respectively, for proximal ileum (n = 50), with serosa-positive. The serosal addition of either bethanechol (10^{-3} M), McN-A-343 (10^{-3} M) or dimethylphenylpiperazimium (DMPP; 10^{-4} M) induced rapid elevations in Isc values of 30 ± 3, 15 ± 2 and 18 ± 3 μA cm^{-2}, respectively, for proximal duodenum (n = 6); 58 ± 14, 48 ± 4 and 33 ± 9 μA cm^{-2}, respectively, for distal duodenum (n = 6); 83 ± 7, 34 ± 7 and 55 ± 13 μA cm^{-2}, respectively, for mid-jejunum (n = 6); 113 ± 12, 39 ± 10 and 77 ± 16 μA cm^{-2}, respectively, for proximal ileum (n = 6). Maximum responses to McN-A-343 and DMPP were achieved 1 min after addition, whereas responses to bethanechol peaked approximately 2 min later. Thereafter, Isc values declined to basal levels within approximately 5 min for McN-A-343 and DMPP, and within 20 min for bethanechol-induced responses. The serosal addition of the agonists also caused a transient rise and fall of Pd and R values, however, no changes to any electrical parameter were noted during the mucosal addition of either agonist (data not shown).

Non-cumulative concentration–response curves were constructed for bethanechol (10^{-6} to 10^{-2} M) and McN-A-343 (10^{-7} to 10^{-2} M) (Figs 1a and 1b, respectively). For these curves the maximum ΔIsc and EC50 were determined by the Eadie–Hofstee transformation (Meddings et al 1989). In each region, the maximum secretory response was always greater for bethanechol (Fig. 1a) than for McN-A-343 (Fig. 1b). Maximum bethanechol-induced ion transport was found to increase in a proximal-to-distal direction along the length of the small intestine (Fig. 1a). However, this was not observed

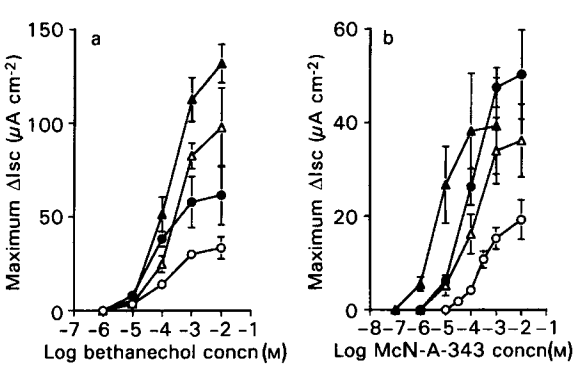

FIG. 1. Non-cumulative concentration–response curves of (a) bethanechol- and (b) McN-A-343-induced secretory responses (Isc, μA cm^{-2}) in unstripped proximal (○) and distal duodenum (●), mid-jejunum (△) and proximal ileum (▲). Each point represents mean ± s.e.m. (n = 6).

for McN-A-343 where only the proximal duodenum had significantly less secretory ability (Fig. 1b). EC50 data for McN-A-343 in the proximal (339 μM) and distal (75 μM) duodenum, mid-jejunum (75 μM) and proximal ileum (6 μM) revealed an increase in agonist affinity in a proximal-to-distal direction whereas values for bethanechol (104, 66, 292 and 200 μM, respectively) showed fewer differences and did not follow a similar trend.

Nature of the charge-carrying ion

Replacement of the chloride salts with their equivalent gluconate salts produced a Cl^- free bathing buffer. Incubation of unstripped jejunal tissues in this buffer reduced basal Isc values by $71 \pm 5\%$ ($P < 0.001$, $n = 12$) and reduced maximum responses induced by either bethanechol (10^{-3} M; $84 \pm 21\%$; $P < 0.001$, $n = 6$) or McN-A-343 (10^{-3} M; $82 \pm 14\%$; $P < 0.001$, $n = 6$). In addition, the inhibition of a specific ion co-transporter ($Na^+/K^+/2Cl^-$) by the serosal addition of the loop diuretic, bumetanide (10^{-5} M), lowered basal Isc levels by $67 \pm 8\%$ ($P < 0.001$, $n = 12$) and reduced maximum responses to bethanechol (10^{-3} M) and McN-A-343 (10^{-3} M) by $51 \pm 8\%$ ($P < 0.001$, $n = 6$) and $53 \pm 9\%$ ($P < 0.001$, $n = 6$), respectively. To confirm normal tissue viability under either of these conditions, the activity of the electrogenic glucose/sodium co-transporter was monitored by the mucosal addition of 28 mM glucose. Identical experiments performed in proximal and distal duodenum and proximal ileum gave similar results (data not shown).

Characterization of cholinergically-induced small intestinal secretion

The ability of bethanechol, McN-A-343 and DMPP to stimulate electrogenic Cl^- secretion in unstripped tissues was tested in the presence and absence of various inhibitors. The following data were recorded from mid-jejunum, however, values for proximal and distal duodenum and proximal ileum followed a similar trend and are summarized in Table 1. Responses to bethanechol (10^{-3} M; 83 ± 7 μA cm^{-2}) were not significantly affected ($P > 0.05$) by either hexamethonium (10^{-4} M; 67 ± 6 μA cm^{-2}; $n = 6$) or tetrodotoxin (1.25×10^{-6} M; 58 ± 11 μA cm^{-2}); however, they were completely abolished by atropine (10^{-6} M). Similarly, McN-A-343-induced responses (10^{-3} M; 34 ± 7 μA cm^{-2}) were not influenced by hexamethonium (10^{-4} M; 43 ± 11 μA cm^{-2}; $P > 0.05$, $n = 6$) but were entirely inhibited by tetrodotoxin (1.25×10^{-6} M; $P < 0.001$, $n = 6$) and atropine (10^{-6} M; $P < 0.001$, $n = 6$). Increased Isc stimulated by DMPP (10^{-4} M; 55 ± 13 μA cm^{-2}) was significantly reduced in the presence of hexamethonium by $89 \pm 5\%$ (10^{-4} M; 6 ± 3 μA cm^{-2}, $P < 0.001$, $n = 6$) and completely blocked by tetrodotoxin (1.25×10^{-6} M, $P < 0.001$, $n = 6$) and atropine (10^{-6} M, $P < 0.001$, $n = 6$). In addition, the selective M_1 antagonist, pirenzepine (10^{-6} M), significantly reduced McN-A-343-induced (10^{-3} M) maximum responses by $88 \pm 6\%$ ($P < 0.001$, $n = 6$) but had no significant effect ($P > 0.05$) on bethanechol (10^{-3} M)-stimulated secretion. None of the antagonists used was seen to have a significant effect on baseline values ($P > 0.05$).

Cumulative concentration–response curves for carbachol (10^{-7} to 3×10^{-3} M) were constructed in the presence and absence of various concentrations of either atropine, pirenzepine, methoctramine, HHSiD or 4-DAMP (Table 2). Such analysis was performed on stripped mid-jejunal and proximal ileal tissues. All responses were obtained in the presence of tetrodotoxin (1.25×10^{-6} M). For each antagonist concentration used the concentration–response relationship remained parallel to the control and no significant differences ($P > 0.05$) in maximum values were recorded. Subsequently, Schild plots were constructed and pA_2 values were estimated for each antagonist (Table 2). No significant differences ($P > 0.05$) were found between mid-jejunum and proximal ileum. As an example, Fig. 2 shows the right-ward shift of the carbachol concentration–response curve in the presence of increasing concentrations of HHSiD and its corresponding Schild analysis. Comparable experiments were also performed using bethanechol as the agonist and resulted in almost identical values (data not shown).

Effect of McN-A-343 on bethanchol-induced secretion

The effect of McN-A-343 on the ability of bethanechol to stimulate electrogenic secretion in stripped mid-jejunum was also examined (Fig. 3). Cumulative concentration–response curves for bethanechol (10^{-6} to 5×10^{-2} M) were constructed in the presence and absence of various concentrations

Table 1. Characterization of cholinergically-induced ion transport.

	Proximal duodenum	Distal duodenum	Mid-jejunum	Proximal ileum
10^{-4} M Dimethylphenylpiperazimium (control)	18 ± 3	33 ± 9	55 ± 13	77 ± 16
$+ 10^{-4}$ M Hexamethonium	2 ± 1†	5 ± 2†	6 ± 3†	7 ± 4†
$+ 10^{-6}$ M Atropine	0†	0†	0†	0†
$+ 1.25 \times 10^{-6}$ M Tetrodotoxin	0†	0†	0†	0†
10^{-3} M McN-A-343 (control)	15 ± 2	48 ± 4	34 ± 7	39 ± 10
$+ 10^{-4}$ M Hexamethonium	11 ± 2	47 ± 9	43 ± 11	47 ± 10
$+ 10^{-6}$ M Atropine	0†	0†	0†	0†
$+ 1.25 \times 10^{-6}$ M Tetrodotoxin	0†	0†	0†	0†
10^{-3} M Bethanechol (control)	30 ± 3	58 ± 14	83 ± 7	113 ± 12
$+ 10^{-4}$ M Hexamethonium	22 ± 2	58 ± 5	67 ± 6	85 ± 9
$+ 10^{-6}$ M Atropine	0†	0†	0†	0†
$+ 1.25 \times 10^{-6}$ M Tetrodotoxin	20 ± 4	39 ± 6	58 ± 11	83 ± 12

Secretory responses (Isc, μA cm^{-2}) to dimethylphenylpiperazimium (10^{-4} M), McN-A-343 (10^{-3} M) and bethanechol (10^{-3} M) were recorded in the absence (control) and presence of either serosal hexamethonium (10^{-4} M), atropine (10^{-6} M) or tetrodotoxin (1.25×10^{-6} M) in rat proximal and distal duodenum, mid-jejunum and proximal ileum. Results are expressed as mean ± s.e.m. ($n = 6$). Statistical comparisons with control values were performed using Student's *t*test; *$P < 0.05$, ‡$P < 0.01$, † $P < 0.001$.

Table 2. pA_2 values for various antagonists during cholinergically-induced intestinal ion transport.

Antagonist	Concentration (M)	Mid-jejunum		Proximal ileum	
		pA_2	Slope	pA_2	Slope
Atropine	3×10^{-9} to 1×10^{-7}	8·7	1·00 ± 0·09	8·8	1·05 ± 0·16
4-Diphenylacetoxy-*N*-piperidine methiodide	1×10^{-8} to 1×10^{-6}	8·0	0·93 ± 0·07	7·9	0·96 ± 0·10
Hexahydro-sila-difenidol	1×10^{-5} to 1×10^{-7}	6·9	0·86 ± 0·04	7·1	0·92 ± 0·04
Pirenzepine	1×10^{-4} to 1×10^{-5}	5·2	1·03 ± 0·08	5·1	0·92 ± 0·24
Methoctramine	3×10^{-4} to 3×10^{-5}	4·6	0·88 ± 0·05	4·7	0·85 ± 0·12

Secretory responses to carbachol were analysed in the presence of three different concentrations of different muscarinic antagonists in both mid-jejunum and proximal ileum. pA_2 values were estimated as a measure of antagonist affinity from Schild-plot regression analysis, the slope of the regression lines are given (mean ± s.e.m.; n = 3). All responses were recorded in the presence of tetrodotoxin ($1{\cdot}25 \times 10^{-6}$ M) to eliminate any contribution from enteric neural tissues.

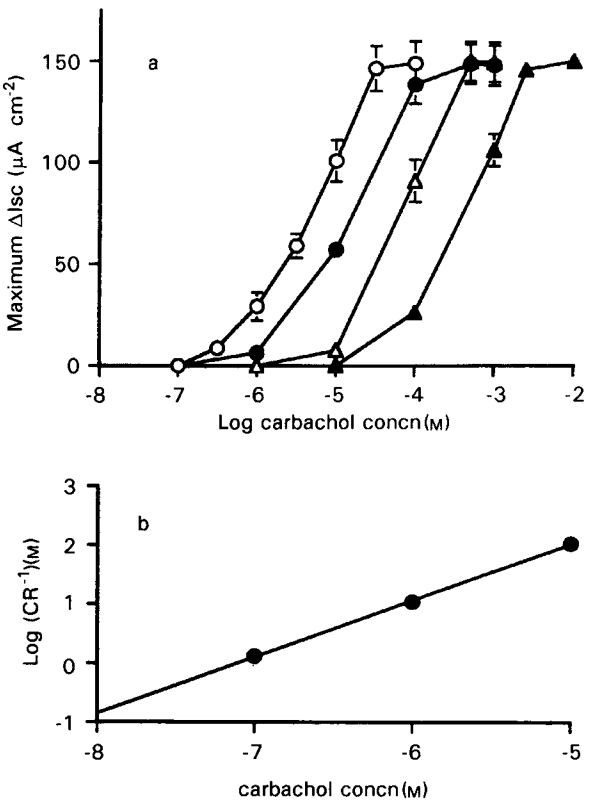

FIG. 2. Effect of HHSiD on secretory responses (Isc, $\mu A\ cm^{-2}$) stimulated by carbachol in rat proximal ileum. a. Cumulative concentration–response of carbachol-induced secretion in the absence (control, (○)) and presence of serosal HHSiD (10^{-5} M (▲); 10^{-6} M (△); 10^{-7} M (●)). All data were recorded in the presence of serosal tetrodotoxin ($1{\cdot}25 \times 10^{-6}$ M). b. Schild-plot regression employing values from the concentration–response (CR) curve seen in a. The intersection of the abscissa at zero by the regression line (slope 0·92 ± 0·04) gave a pA_2 of 7·1 for HHSiD. Results are given as mean ± s.e.m. (n = 9–12).

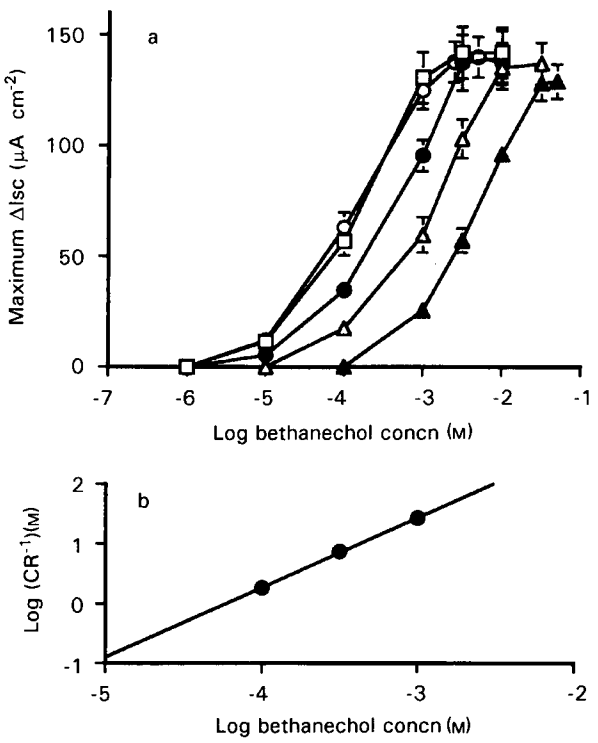

FIG. 3. Effect of McN-A-343 on secretory responses (Isc, $\mu A\ cm^{-2}$) stimulated by bethanechol. a. Cumulative concentration–response of bethanechol-induced secretion in the absence (control, (○)) and presence of serosal McN-A-343 (10^{-3} M (▲); 10^{-4} M (△); 3×10^{-4} M (●); 10^{-5} M (□)). All data were recorded in the presence of serosal tetrodotoxin ($1{\cdot}25 \times 10^{-6}$ M). b. Schild plot regression employing values from the concentration–response (CR) curve seen in a. The intersection of the abscissa at zero by the regression line (slope 1·15 ± 0·04) gave a pA_2 of 4·3 for McN-A-343. Results are given as mean ± s.e.m. (n = 8–18).

of McN-A-343 (10^{-5} to 10^{-3} M). All responses were obtained in the presence of tetrodotoxin ($1{\cdot}25 \times 10^{-6}$ M). Tetrodotoxin and McN-A-343 were added serosally 15 and 10 min, respectively, before the addition of serosal bethanechol. McN-A-343 was found to antagonize bethanechol-induced secretion competitively with an estimated pA_2 value of 4·3 (Fig. 3). Identical experiments were performed in the proximal ileum and resulted in similar values (data not shown).

Discussion

The results of this study enable characterization of muscarinic receptors which modulate the secretory status of rat isolated small intestinal preparations in response to various cholinomimetic compounds. Together with our previous observations (Przyborski et al 1990, 1991a, 1992; Przyborski & Levin 1993), these data have led to the formulation of a model consisting of two pathways through which cholinergic agonists modulate electrogenic secretion by the activation of muscarinic receptor subtypes localized to enteric neurons and enterocytes (Fig. 4). Moreover, variation of cholinergically-induced

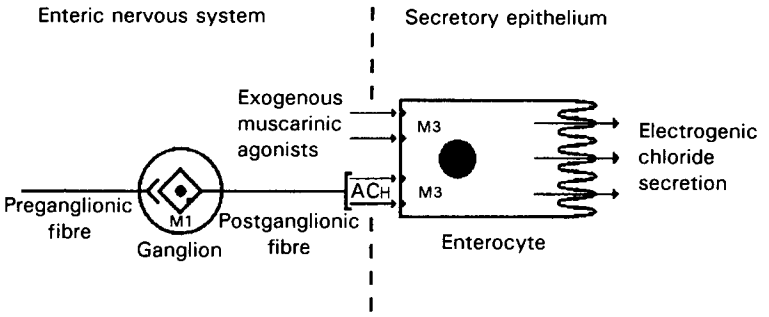

FIG. 4. Schematic diagram of the proposed location of muscarinic receptors of the M_1 and M_3 subtypes in the rat small intestinal mucosa. Activation of electrogenic chloride secretion may be mediated by the direct action of a cholinergic agonist at the enterocyte M_3 muscarinic receptor or indirectly via the M_1 subtype located on postganglionic enteric neurons.

secretion in different regions of the small intestine might be explained by varied involvement of either of these pathways.

The reduction in cholinergically-induced secretory responses by ion substitution or inhibition of the $Na^+/K^+/2Cl^-$ co-transporter indicates that Cl^- ions are the predominant charge-carrying species responsible for the electrogenic response. In agreement, intestinal studies involving ion-flux analysis (Kuwahara et al 1987), ion substitution (Chandan et al 1991) or serosal bumetanide (O'Malley et al 1995) have led to similar conclusions for cholinergically stimulated secretion. Because we did not achieve full abolition of the electrogenic response, it is arguable that a further anion is involved. Indeed, Chandan et al (1991) noted a similar 83% decrease in cholinergically evoked secretion using Cl^-- substitution in porcine jejunum; however, this inhibition was increased to 97% on additional substitution of HCO_3^- by HEPES, hence suggesting that the HCO_3^- ion carries the remainder of the secretory response.

The characterization of muscarinic-receptor subtypes mediating electrogenic Cl^- secretion can be pharmacologically defined by measuring the affinities of several key compounds. Cholinergic receptor agonists are not useful in this regard because none discriminates between individual receptor subtypes on an affinity basis by more than an order of magnitude. However, their general agonistic action can be of benefit. Carbachol is a cholinergic agonist which, at high concentrations, has been shown to activate both muscarinic and nicotinic receptors (Tapper et al 1978). O'Malley et al (1995) demonstrated that the effects of carbachol were additive, with 30% of the cholinergic regulation of rat colonic ion transport being attributed to nicotinic receptors, the remainder being mediated by the muscarinic subtype. Our experiments with DMPP and bethanechol provide functional evidence for the presence of both nicotinic and muscarinic receptors in the rat small intestinal mucosa. Therefore, the action of carbachol in rat small intestine is also likely to be the result of combined activation of both cholinergic subtypes. The sensitivity of DMPP-induced responses to tetrodotoxin is in agreement with previous studies which have shown that nicotinic receptors are localized primarily to enteric neural tissues (Buckley & Burnstock 1984; Frieling et al 1991). Although the existence of muscarinic receptors on enteric neurons and epithelial cells has been demonstrated (Rimele et al 1981; Dharmsathaphorn & Pandol 1986; Dickinson et al 1992; Przyborski & Levin 1993; O'Malley et al 1995), there is no evidence of the epithelial expression of nicotinic receptors (Rimele et al 1981). Because we recorded all data for carbachol-stimulated responses in the presence of tetrodotoxin, such induced secretion is most probably mediated by post-synaptic muscarinic receptors alone. Indeed, our characterization of such receptors mediating the secretory activity of carbachol was identical with the outcome achieved with the muscarinic agonist bethanechol. Similar results for both agonists have also been reported in porcine jejunum (Chandan et al 1991), and guinea-pig (Kuwahara et al 1987) and rat (O'Malley et al 1995) colon. Together with information about the expression of muscarinic receptors on the intestinal epithelium (Rimele et al 1981; Dharmsathaphorn & Pandol 1986; Dickinson et al 1992; Przyborski & Levin 1993; O'Malley et al 1995), these data clearly indicate the ability of exogenous cholinomimetics to activate directly muscarinic receptors positioned on enterocytes.

More definitive as tools in muscarinic receptor classification are antagonists which have contrasting affinities towards different subtypes (see reviews by Caulfield (1993) and by Eglen & Watson (1996)). Accordingly, we determined the pA_2 value of various muscarinic antagonists as inhibitors of cholinomimetic-stimulated Cl^- secretion. Such experiments were performed in the presence of tetrodotoxin hence circumventing any neuronal interactions and therefore enabling estimation of the affinity of the enterocyte receptor for each antagonist tested. We report a rank order of antagonist affinity of atropine > 4-DAMP > HHSiD > pirenzepine > methoctramine, which is indicative of interactions with the M_3 muscarinic subtype. This agrees with similar functional characterization performed in porcine jejunum (Chandan et al 1991) and rat colon (O'Malley et al 1995) and compares favourably with ligand-binding studies on gastrointestinal epithelial cells (Kopp et al 1989; Chandan et al 1991; Dickinson et al 1992; O'Malley et al 1995). Moreover, we have previously demonstrated the localization of m3 receptor mRNA to enterocytes positioned on rat jejunal villi (Przyborski & Levin 1993). Our current data, therefore, suggests the translation of this m3 message and its expression as a functional M_3-receptor protein on the mucosal epithelium of rat mid-jejunum, which serves to mediate cholinergically induced Cl^- secretion. The activation of such M_3 epithelial receptors in-situ is most likely induced by acetylcholine released from submucosal nerves of the enteric nervous system. Evidence for cholinergic nerve endings projecting to the intestinal epithelium was demonstrated by positive immunostaining for choline acetyltransferase (Bornstein & Furness 1988). Several previous investigations have also reported that the depolarization of intrinsic nerves by

electrical-field stimulation (EFS) or treatment with scorpion venom increases electrogenic Cl^- secretion and these effects were inhibited by atropine and undergo partial desensitization to carbachol (Cooke et al 1983; Cooke 1984; Carey et al 1987; Kuwahara et al 1987; Chandan et al 1991). Our experiments with the muscarinic agonist McN-A-343 demonstrate its sensitivity to tetrodotoxin and therefore suggest that its secretory action is mediated via submucosal neurons. The inability of the ganglionic blocker hexamethonium to influence the activity of McN-A-343 indicates that its neuronal target site was restricted to muscarinic receptors located on post-ganglionic fibres. Although neural transmission within submucosal ganglia might involve interaction of acetylcholine at these muscarinic receptors, our current results together with previous observations (Carey et al 1987; O'Malley et al 1995) indicate that the contribution of these neural receptors to the overall secretory response is relatively minor compared with the action of cholinergic agonists at the neuro-epithelial junction.

Muscarinic receptors on submucosal nerves appear different from those on enterocytes. McN-A-343 is frequently cited as a selective M_1 muscarinic-receptor agonist; however, responses to this agonist are not conclusive evidence for activation of an M_1 receptor because it has a potency contingent upon the prevailing receptor reserve (Eglen et al 1987). Unfortunately, no information is available about the receptor reserve for the neuronal McN-A-343-sensitive muscarinic sites. Nevertheless, electrophysiological studies in guinea-pig ileum demonstrated that pirenzepine-sensitive receptors localized to post-ganglionic fibres which serve to depolarize neurons were indicative of the M_1 subtype (North et al 1985). Furthermore, we noted the complete inhibition of McN-A-343-induced responses by pirenzepine (10^{-6} M) which had no effect on the secretory activity of bethanechol. In agreement, antagonism of McN-A-343-evoked secretion by pirenzepine has also been reported in rat colon (O'Malley et al 1995) and guinea-pig ileum (Carey et al 1987). Therefore, despite the questionable selectivity of this agonist, these data as a whole suggest that such neuronal McN-A-343-sensitive receptors are probably of the M_1 subtype.

As previously discussed, the activities of McN-A-343 indicate that it does not have a direct agonist action at the enterocyte receptor. However, our experiments show that bethanechol-induced secretory responses were competitively antagonized by McN-A-343 and that it had weak affinity towards the enterocyte receptor. This is consistent with a study of the effect of McN-A-343 in guinea-pig ileum in which the authors reported that it competed for mucosal quinuclidinyl benzilate binding sites as did carbachol and bethanechol, and significantly attenuated the cholinergic portion of EFS-induced responses (Carey et al 1987). Differing effects of McN-A-343 have also been noted in other peripheral tissues; it has been shown to act as an antagonist in rat ileal smooth muscle (Brown et al 1980) and as a partial agonist in rat adrenal medulla (Wakade et al 1986). In relation to its effect in rat small intestine, the secretory activity of McN-A-343 is complex in that it is probably the result of the interaction between McN-A-343-induced release of acetylcholine from enteric nerves and subsequent antagonism of such endogenous acetylcholine by McN-A-343 at the enterocyte receptor.

The current study has also provided evidence of variation in cholinergically-induced ion transport along the length of the rat small intestine. We have shown that in each of the regions tested there is a direct muscarinic action on the epithelium and a cholinergically mediated neural component. Whereas both mechanisms are most probably generally conserved along the small gut, subtle variations in either pathway might explain the observed regional differences in secretory response. Accordingly, the direct activation of the enterocyte receptor which resulted in a progressively larger secretion in a proximal-to-distal direction might reflect a gradual change in drug-receptor interaction (e.g. intrinsic activity) or more simply be due to increased receptor density in more distal regions. Although this study has functionally demonstrated that the M_3-receptor subtype is located on the enterocyte in both jejunum and ileum, no information is available about comparative muscarinic receptor density in these regions. Furthermore, our data indicate that an epithelial muscarinic receptor exists in the duodenum; however, its subtype identity remains unknown. Secretory responses induced through the stimulation of neuronal receptors by McN-A-343 also showed variation along the small intestine, suggesting regional modifications in the neural component of ion transport regulation. Differences in the involvement of neuronal receptors towards an intestinal secretory response have been documented previously. We and others (Carey et al 1987) have noted that tetrodotoxin did not alter responses to bethanechol and carbachol in the small intestine; however, the neural blocker significantly reduced such stimulated secretion in rat colon (O'Malley et al 1995). Moreover, studies with 5-HT have provided evidence for both pro-secretory and anti-secretory neural pathways and the balance between these processes varies along the length of the gut (Franks et al 1996). Clearly, further investigation is required to assess the relative importance of these neural cholinergic pathways and to establish their role in the physiological regulation of intestinal transport.

In summary, the results of this study describe the functional characterization of cholinomimetic-induced electrogenic Cl^- secretion in rat isolated small intestine. Secretory responses appear to be mediated by two dissimilar populations of muscarinic receptor differentially localized within the intestinal mucosa. M_3 muscarinic receptors positioned on enterocytes modulate the direct action of exogenously applied cholinergic substances and probably convey the effects of endogenous acetylcholine, whereas muscarinic receptors sited on submucosal neurons are most likely of the M_1 subtype and also appear to play a role in the regulation of small intestinal secretion. Whereas such a model might operate generally throughout the small intestine, subtle differences in either of these pathways might explain regional variation in the cholinergic control of electrolyte transport.

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