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Muscarinic receptors on rat ileal villus and crypt cells

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Ileal villus and crypt cells exhibit morphological and biochemical differences which may be responsible for functional differences in relation to ion transport. Cholinergic agonists act directly on epithelial cell muscarinic receptors, but it is not known if these receptors exist on both villus and crypt cells. Using the potent muscarinic antagonist [^{3}H](−)-quinuclidinyl benzilate (QNB) we have determined the distribution of muscarinic receptors in rat ileal villus and crypt cells. Plasma membrane preparations from ileal villus and crypt cells possessed a specific, saturable, and high affinity QNB binding site with apparent dissociation constants of 0·23 ± 0·05 and 0·21 ± 0·04 nM (mean ± s.e., n = 6) and densities of 92·2 ± 2·8 and 90·1 ± 16·2 fmol (mg protein)$^{-1}$, respectively. Both types of cells showed similar potencies for agonist and antagonist competition of QNB binding. The muscarinic receptors in membrane fractions from villus cells were found primarily on the basolateral membrane rather than on the brush border membrane. Secretion induced by cholinergic stimulation of the small intestine might, therefore, be due to an effect on both villus and crypt cells as both types contain muscarinic receptors. Furthermore, such stimulation also may result in mucin secretion, as goblet cells were present in the preparation we studied, and receptors on these cells may have contributed to the amount of binding of [^{3}H]QNB.

Muscarinic receptors are found on membranes of intestinal epithelial cells (Rimele et al 1981; Isaacs et al 1982; Zimmerman & Binder, 1982). Net water and electrolyte secretion result from activation of these receptors by either parasympathetic nerve stimulation or by action of muscarinic agonist drugs (Tidball 1961; Browning et al 1978; Hardcastle & Eggenton 1973; Hubel 1976, 1977; Isaacs et al 1976; Morris & Turnberg 1980; Powell & Tapper 1979; Tapper et al 1978). The muscarinic receptors identified in epithelial cell membrane preparations could be associated with one or more of the many cell types in the epithelium of the small intestine (Cheng & Leblond 1974). The major cell types are mature villus cells and proliferating crypt cells, which differ both in morphological appearance and function. Villus cells acquire a well-developed brush border membrane. They appear to be involved in mediating absorption of water and electrolytes since absorptive function decreases following selective villus damage by intraluminal hypertonic Na_2SO_4 (Browning et al 1978). The crypt cells have a relatively undeveloped brush border membrane. These cells appear to be involved in mediating secretion of water and electrolytes since cholinergically elicited secretory function decreases following selective damage to crypts by cycloheximide (Browning et al 1978). Further evidence for a secretory function of crypt but not villus cells was obtained by observing the source of water droplets in mucosal sheets placed under oil and by comparing membrane potentials in the two kinds of cells following induction of secretion with cholera toxin (Welsh et al 1982).

We hypothesized that if crypt cells contain muscarinic receptors which are lost or altered during differentiation into villus cells, this might explain the observation that parasympathetic stimulation of the small intestine produces water secretion from crypt but not villus regions. The present study indicates equal receptor densities in both kinds of cells.

Methods

Isolation of rat ileal villus and crypt cells and preparation of membranes. Cells were isolated using the method of Weiser (1973) with minor modifications. Male Sprague-Dawley rats (8–10 per experiment), 200–300 g were killed by cervical dislocation. The ileum was immediately excised and gently flushed with saline-DTT (154 mM NaCl and 1 mM dithiothreitol at 37 °C) to remove luminal contents. The tissue was everted on a glass rod and tied with suture silk. The everted ileum was immersed for 15 min at 37 °C in solution containing (mM) KCl 1·5, NaCl 96, KH_2PO_4 8, Na_2HPO_4 5·6, and sodium citrate 27 (pH 7·3). The segment was filled with 'separation solution' (EDTA 1·5, dithiothreitol 0·5, NaCl 154, K_2HPO_4, NaH_2PO_4 0·6 mM, pH 7·3) and tied at the other end. The everted segment was then sequentially placed in 50 ml of separation buffer for 4, 2, 2, 3, 4, 5, 7, 10 and 15 min and gently mixed on a reciprocating shaker at 1·25 Hz. At the end of each incubation, the tissue was gently shaken by hand to dislodge any loosened cells. On the basis of marker enzyme activity, the first three fractions (which contained villus cells) were pooled and the last three fractions (which contained crypt cells) were pooled. Clumps of cells were dissociated by aspirating and expelling the suspension using a pasteur pipette. The cell suspension was filtered through a nylon mesh (250 μm) to trap mucus. Villus and crypt cells were then washed three times with phosphate-buffered saline (pH 7·3) by low speed centrifugation, 900 *g* for 10 min, in a Sorvall centrifuge (RC-5B).

Membrane fractions of both villus and crypt cells were isolated according to the methods of Rimele et al (1981). Cells were homogenized in 50 ml buffer (sucrose 250, triethanolamine-HCl 10, and EDTA 0·5 mM) with a Brinkman Polytron at setting 7 and further disrupted

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with a glass/Teflon homogenizer. The homogenates were centrifuged at 2600 *g* for 15 min. The pellet (pellet 1) was discarded, and the supernatant (supernatant 1) was then centrifuged at 10000 *g* for 20 min. The white fluffy top layer of pellet 2 was resuspended, homogenized, and centrifuged at 30000 *g* for 20 min. The white fluffy top layer of pellet 3 was resuspended in 50 mM phosphate buffer (pH 7·4), homogenized and centrifuged at 30000 *g* for 10 min. This membrane fraction was washed twice and then resuspended in an appropriate volume for binding assays.

For separation of brush border and basolateral membranes of villus cells, homogenates of these cells were centrifuged at 450 *g* for 10 min. The pellet was purified as brush border membrane by the method of Forstner et al (1968). The resultant supernatant was centrifuged at 48000 *g* and the pellet, identified as basolateral membrane, was washed twice with 50 mM phosphate buffer.

Protein concentrations were determined by the method of Lowry et al (1951) using bovine serum albumin as a standard. Sucrase activity was assayed by the method of Dahlqvist (1968). Thymidine kinase activity was determined according to the method of Ives et al (1969). Sodium-potassium ATPase activity was determined according to the method of Quigley & Gotterer (1969).

[^{3}H](−)-QNB binding

A slight modification of the method of Rimele et al (1981) was used for membrane binding. Membrane fractions containing 200–500 μg protein were added to 2 ml polystyrene cups containing [^{3}H]QNB in 50 mM phosphate buffer (pH 7·4), total volume 1·5 ml, and incubated at 37 °C for 40 min in a Dubnoff incubator with constant shaking (1·66 Hz). Following incubation, samples were filtered under vacuum through presoaked Whatman GF/B glass fibre filters. The filters were then washed three times with 5 ml ice-cold phosphate buffer, and placed in scintillation vials. Filters were disintegrated using Protosol followed by vigorous shaking, and radioactivity was determined by liquid scintillation counting. Nonspecific binding was determined by including 100 μM atropine in the binding assay. Specific [^{3}H]QNB binding was the mean total binding minus the mean binding in the presence of atropine.

Drugs

[^{3}H](−)-Quinuclidinyl benzilate (QNB), sp. act. 33·1 Ci mmol^{-1}, and [^{3}H]thymidine, sp. act. 16·2 Ci mmol^{-1}, were purchased from New England Nuclear Corporation (Boston, MA). Pirenzepine was obtained from Boehringer Ingelheim (Ridgefield, CT). Ouabain was purchased from Inland Chemical Corp. (Tipton, IN); all other drugs were obtained from Sigma Chemical Co. (St. Louis, MO). Reagents used in the preparation of buffers and other solutions were of the highest grade available.

Results

The relative enrichment of villus and crypt cells in their respective fractions was assayed using the brush border enzyme sucrase as a marker for villus cells and the soluble thymidine kinase enzyme for crypt cells. Sucrase activity in the villus fraction was more than 20-fold higher than in the crypt fraction (76 ± 10 vs 3·5 ± 0·4 nmol (mg protein)$^{-1}$ min^{-1}; mean ± s.e., n = 8), while thymidine kinase activity was more than 10-fold lower (11 ± 0·8 vs 56 ± 11). The results demonstrate a good separation of the two cell types.

The presence of muscarinic receptor binding sites was studied using [^{3}H]QNB binding. The binding variables of B_{max}, K_d, and Hill coefficient were nearly identical for both villus and crypt cell fractions (Table 1). The potencies of both muscarinic agonists and antagonists in competing for QNB binding sites were nearly identical in both cell types (Fig. 1).

Table 1. [^{3}H]QNB binding to crypt and villus cells.

Measurement	Cell type Crypt	Villus
[^{3}H]QNB Binding		
B_{max} (fmol (mg protein)$^{-1}$)	92 ± 3	90 ± 16
K_d (pM)	210 ± 40	230 ± 50
Hill coefficient	1·04 ± 0·04	1·04 ± 0·02

Data are means ± s.e. (n = 6)

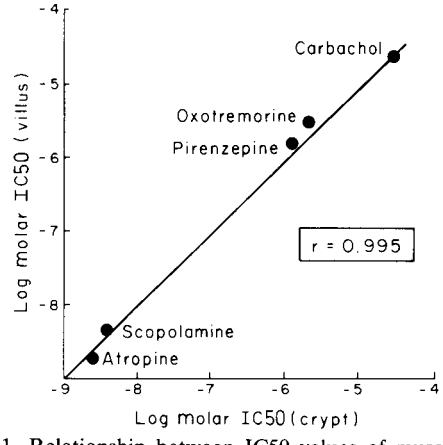

FIG. 1. Relationship between IC50 values of muscarinic antagonists and agonists obtained in villus and crypt cells. Data shown are means of two experiments.

Villus cell membranes were subfractionated into brush border and basolateral enriched preparations. A fraction 3–4-fold enriched in brush border membranes and a fraction enriched to about the same extent in basolateral membranes were obtained (Table 2). The ratio of [^{3}H]QNB binding in the two membrane fractions was close to that of the basolateral membrane marker sodium-potassium ATPase (Table 2). This suggests that the muscarinic binding sites in villus cells are largely localized to the basolateral membrane.

Table 2. Muscarinic receptors and enzyme markers of brush border and basolateral membranes from villus cells.

	Brush border	Basolateral
Receptor density (fmol (mg protein)$^{+1}$)	24·8 ± 1·1**	61·9 ± 0·9
Sucrase activity (μmol (mg protein)$^{-1}$ h^{-1})	14·4 ± 3·1*	4·1 ± 1·5
Na-K ATPase activity (μmol (mg protein)$^{-1}$ h^{-1})	0·8 ± 0·6*	3·1 ± 0·1

Data are means ± s.e. (n = 3).
Values for brush border membranes differed from those of basolateral membranes (*$P < 0.05$; **$P < 0.01$).

Discussion

The goal of the present work was to determine whether parasympathetic stimulation produces secretion by crypt but not villus cells (Browning et al 1978; Welsh et al 1982) because of a different distribution of muscarinic receptors. This does not seem to be the case since our studies demonstrate that [^{3}H]QNB binding was similar in crypt and villus cell membrane preparations. These observations extend the findings of Isaacs et al (1982) that jejunal villus and crypt cells both bind QNB. We obtained a good separation of cell types and then showed similar QNB binding.

Pirenzepine and oxotremorine differentiate between the M1 and M2 muscarinic receptor subtypes (Hirschowitz et al 1984). Using binding conditions like those employed in this study, the ratio of potencies of oxotremorine to pirenzepine in competition of QNB binding is the best indicator of receptor subtypes; a ratio greater than 2 or less than 0·1 occurs, respectively, where M1 or M2 subtypes are predominant (Tien et al 1985). The ratio of 2·6 and 2·9 for villus and crypt cells, respectively, suggests that the receptor subtype is M1 in both kinds of cells (Tien et al 1985).

The observed distribution of muscarinic receptors suggests that cholinergic agonists should be capable of acting on both villus and crypt cells. In addition, anatomical studies suggest that parasympathetic nerves should be capable of acting on both villus and crypt cells. Both cell types appear to have cholinergic neurons in close apposition as suggested by cholinesterase staining (Jacobowitz 1965; Isaacs et al 1976), choline acetyltransferase staining (Furness et al 1983), and by ultrastructural characteristics (Gabella 1972; Furness & Costa 1980; Gershon & Erde 1981). These observations coupled with our results suggest that muscarinic receptors are active on both villus and crypt cells but that receptor activation leads to divergent functional responses in the two cell types. However, since we do not know where the goblet cells are isolated in our separation procedure, some of the muscarinic receptors we measured might have existed on goblet cells and play a role in regulating mucus secretion or immunoglobulin secretion (Bradbury et al 1980; Specian & Neutra 1982; Wilson et al 1982).

In conclusion, using radioligand binding we have obtained evidence for the presence of muscarinic cholinergic receptors in both villus and crypt cell membranes of rat ileum. Further subfractionation of villus cell membranes suggests that the receptors are mainly localized on the basolateral membranes. The data suggest that a proposed secretory function for crypt but not villus cells cannot be explained by a selective distribution of muscarinic receptors limited to only crypt cells.

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REFERENCES

Bradbury, J. E., Black, J. W., Wyllie, J. H. (1980) Eur. J. Pharmacol. 68: 417–425

Browning, J. G., Hardcastle, J., Hardcastle, P. T., Redfern, J. S. (1978) J. Physiol. (Lond.) 281: 15–27

Cheng, H., Leblond, C. P. (1974) Am. J. Anat. 141: 537–561

Dahlqvist, A. (1968) Analyt. Biochem. 22: 99–107

Forstner, G. G., Sebesin, S. M., Isselbacher, K. J. (1968) Biochem. J. 106: 381–390

Furness, J. B., Costa, M. (1980) Neuroscience 5: 1–20

Furness, J. B., Costa, M., Eckstein, F. (1983) Neurosci. Lett. 40: 105–109

Gabella, G. (1972) J. Anat. 111: 69–97

Gershon, M. D., Erde, S. M. (1981) Gastroenterology 80: 1571–1594

Hardcastle, P. T., Eggenton, J. (1973) Biochim. Biophys. Acta. 298: 95–100

Hirschowitz, B. I., Hammer, R., Giachetti, A., Keirns, J. J., Levine, R. R. (eds) (1984) Subtypes of Muscarinic Receptors. Elsevier, Cambridge, UK

Hubel, K. A. (1976) Am. J. Physiol. 231: 252–257

Hubel, K. A. (1977) Proc. Soc. Exp. Biol. Med. 154: 41–44

Isaacs, P. E. T., Corbett, C. L., Riley, A. C., Hawker, P. C., Turnberg, L. A. (1976) J. Clin. Invest. 58: 535–542

Isaacs, P. E. T., Whitehead, J. S., Kim, Y. S. (1982) Clin. Sci. 62: 203–207

Ives, D. H., Durham, J. P., Tucker, V. S. (1969) Analyt. Biochem. 28: 192–205

Jacobowitz, D. (1965) J. Pharmacol. Exp. Ther. 149: 358–364

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, J. (1951) J. Biol. Chem. 193: 265–275

Morris, A. I., Turnberg, L. A. (1980) Gastroenterology 79: 861–866

Powell, D. W., Tapper, E. J. (1979) in: Binder, H. J. (ed.) Mechanisms of Intestinal Secretion, Kroc Foundation Symposium 12. Alan R. Liss, Inc., New York, pp 175–192

Quigley, J. P., Gotterer, G. S. (1969) Biochim. Biophys. Acta. 173: 456–468

Rimele, T. J., O'Dorisio, M. S., Gaginella, T. S. (1981) J. Pharmacol. Exp. Ther. 218: 426–434

Specian, R. D., Neutra, R. A. (1982) Am. J. Physiol. 242: G370–G379

Tapper, E. J., Powell, D. W., Morris, S. M. (1978) Ibid. 235: E402–E409
Tidball, C. S. (1961) Ibid. 200: 309–312
Tien, X. Y., Wahawisan, R., Wallace, L. J., Gaginella, T. S. (1985) Life Sci. 36: 1949–1955
Weiser, M. M. (1973) J. Biol. Chem. 248: 2536–2541
Welsh, M. J., Smith, P. S., Fromm, M., Frizzell, R. A. (1982) Science 218: 1219–1221
Wilson, I. D., Sottis, R. D., Olson, R. E. (1982) Gastroenterol. 83: 881–888
Zimmerman, T. W., Binder, H. J. (1982) Ibid. 83: 1251

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A comparison of the antagonisms by neostigmine and diaminopyridine against the neuromuscular block caused by cobrotoxin and (+)-tubocurarine

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Cobrotoxin was about 11-fold more potent than (+)-tubocurarine on a weight basis in blocking neuromuscular transmission in mouse isolated phrenic nerve-diaphragm preparations. Neostigmine and diaminopyridine increased the concentrations of cobrotoxin for 70% inhibition of indirect contraction by 290 and 320%, and increased those of (+)-tubocurarine by 180 and 230%, respectively. More than additive increases were obtained when neostigmine and diaminopyridine were used simultaneously. Cobrotoxin, however, was only 6-fold more toxic than (+)-tubocurarine after intraperitoneal injection in mice. The lethal dose of (+)-tubocurarine was increased by 80% when both antidotes were used together, but only by 15–20% when used alone. In contrast, the lethality of cobrotoxin was not decreased by these drugs. Unexpectedly, the time to death after treatment with cobrotoxin was shortened when mice were pretreated with these antidotes.

One of the major toxic effects upon envenomation with cobra venom is the respiratory paralysis due to neuromuscular block (see Campbell 1979; Chang 1979). Most cobra venoms contain basic polypeptide neurotoxins (see Karlsson 1979) which block neuromuscular transmission by acting, like (+)-tubocurarine, on the postsynaptic acetylcholine receptor of the motor endplate (see Chang 1979). Neostigmine, a typical anticholinesterase drug used in anaesthesiology to antagonize (+)-tubocurarine-paralysis, was ineffective in overcoming the effects of cobra venom in the completely paralysed dog although there was distinct and immediate improvement if it was administered during the stage of recovery (Gode et al 1968). Similar observations were made in human envenomation (Kumar & Usgaonkar 1968; Banerjee et al 1972). Aminopyridines, which increase the release of acetylcholine by an inhibition of the K^+-conductance, have been introduced as antidotes against (+)-tubocurarine (Bowman 1982). We have compared the antidotal efficacies of neostigmine and 3,4-diaminopyridine against crude cobra (*Naja naja atra*) venom and its purified neurotoxin (cobrotoxin) with those against (+)-tubocurarine in isolated phrenic nerve-diaphragm preparations and lethality in mice. The results have revealed that both antidotes were effective equally against the neuromuscular block caused by cobrotoxin or (+)-tubocurarine in isolated preparations. However, no antidotal effect to cobrotoxin could be observed in-vivo.

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Materials and methods

Nerve-muscle preparations in-vitro. The phrenic nerve-diaphragm preparation, isolated from 20–25 g NIH or ICR mice of either sex, was incubated in 10 ml Tyrode solution (mM: NaCl 137, KCl 2·7, $CaCl_2$ 1·8, $MgCl_2$ 1·1, $NaHCO_3$ 11·9, $NaHPO_4$ 0·33, glucose 11·2) at 37 °C and oxygenated with 95% O_2 and 5% CO_2. Contractile responses were evoked by indirect stimulation of the nerve with supramaximal single pulses (pulse duration ≤0·1 ms) at 0·1 Hz and recorded isometrically by means of strain gauge force-displacement transducers (FT ·03).

Assay of antidote efficacy in-vitro. The isolated diaphragms were treated with appropriate concentrations of either cobrotoxin (0·012 μM) or (+)-tubocurarine (1·2 μM) to produce 70% depression of the contractile response at steady state. Then neostigmine (0·3–1·7 μM) or 3,4-diaminopyridine (4·5–5 μM) was added to restore the twitch contraction. The concentration of cobrotoxin or (+)-tubocurarine was then increased until the twitch contraction was depressed again to the same level. The antidote efficacy is defined as the ratio of the concentration of (+)-tubocurarine (or cobrotoxin) required to produce the same extent of block in the presence over that in the absence of antidotes.

Lethal toxicity in mice. Mice of either sex (NIH strain), 15–20 g were used. Groups of mice were injected (i.p.) with cobrotoxin or (+)-tubocurarine with or without pretreatment (i.p.) with neostigmine methylsulphate (0·25 μg g^{-1}) plus atropine sulphate (10 μg g^{-1}) and/or 3,4-diaminopyridine (1 μg g^{-1}). The LD50 was calcu-