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Receptors for neurotransmitters in opossum oesophagus muscularis mucosa

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1 Muscularis mucosa of the distal oesophagus of the opossum contains nerves which release acetylcholine and substance P(SP)-like material on field stimulation. The release of SP-like material appeared to be inhibited by the presence of exogenous muscarinic agonists and potentiated by muscarinic antagonists. Analysis of the postjunctional receptors involved using carbachol, McNeil A-343 (McN A-343), atropine and pirenzipine suggested that the receptors were not typical M_2-muscarinic receptors. The potency of agonists and antagonists were consistent with some receptor properties resembling M_1-muscarinic receptors.

2 Prejunctional receptors to opiates, adenosine, agonists at α_2-adrenoceptors and prostaglandins were not detected.

3 Receptors for tachykinins were present on the muscle in this tissue, but did not resemble clearly either SP-E or SP-P type receptors. They appear to be undifferentiated since most tachykinins were of similar potency.

4 These results suggest that not all postjunctional muscarinic receptors in intestinal smooth muscle are M_2 in type. There may be a gradation of types between M_1 and M_2.

Introduction

Recently (Domoto *et al.*, 1983; Robotham *et al.*, 1985), we showed that longitudinal strips of muscularis mucosa of oesophagus from opossum (*Didelphis marsupialis*) responded to low frequency (5 pulses per second) field stimulation of intrinsic nerves by a phasic contraction and to higher frequencies (20 to 50 pulses s^{-1}) by a phasic followed by a prolonged tonic ('off') contraction. Subsequent analysis shows that the phasic response was mediated by acetylcholine while the tonic contraction was mediated by a substance P(SP)-like substance and modulated (inhibited) by muscarinic cholinoceptor agonists. Immunohistochemical studies and unpublished radioimmunoassays revealed a high density of nerves with SP-like immunoreactivity. In ultrastructural studies, the nerves present were shown to have varicosities containing small agranular vesicles and a few large granular vesicles. Capsaicin was also shown to release a SP-like material and to inhibit the tonic response to field stimulation. The tonic response to field stimulation recovered one hour after capsaicin was removed and could be abolished by a second addition of capsaicin. The contraction from release of SP-like material by capsaicin did not recover. No ultrastructural damage to nerves was detected after abolition of tonic responses to nerve stimulation by capsaicin.

The purpose of the present study was to characterize the muscarinic receptors present on smooth muscle in this tissue and the SP or tachykinin receptors on this smooth muscle. A recent study has confirmed our findings that receptors to SP-like substance as well as to acetylcholine are present (Christensen & Percy, 1984) on muscle. A survey was also made of other prejunctional receptors present in this tissue.

Methods

As previously described (Domoto *et al.*, 1983), longitudinal strips were prepared of the muscularis mucosa of distal oesophagus from opossum. Six to eight strips (2 × 15 mm) could be made from each animal. They were mounted in 3 ml baths through concentric Pt electrodes 1 cm apart, attached to Grass

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FT 03C transducers. All dissections and procedures were carried out in Krebs Ringer solution with the composition (mM): Na 139, Cl 124.6, K 4.16, HCO_3 21.9, Ca 2.5, Mg 1.16, H_2PO_4 1.6, SO_4 1.6 and glucose 11.1.

Procedures

Several procedures were used to compare the actions of agonists and antagonists. Cumulative dose-effect curves for carbachol or McN A-343 were obtained

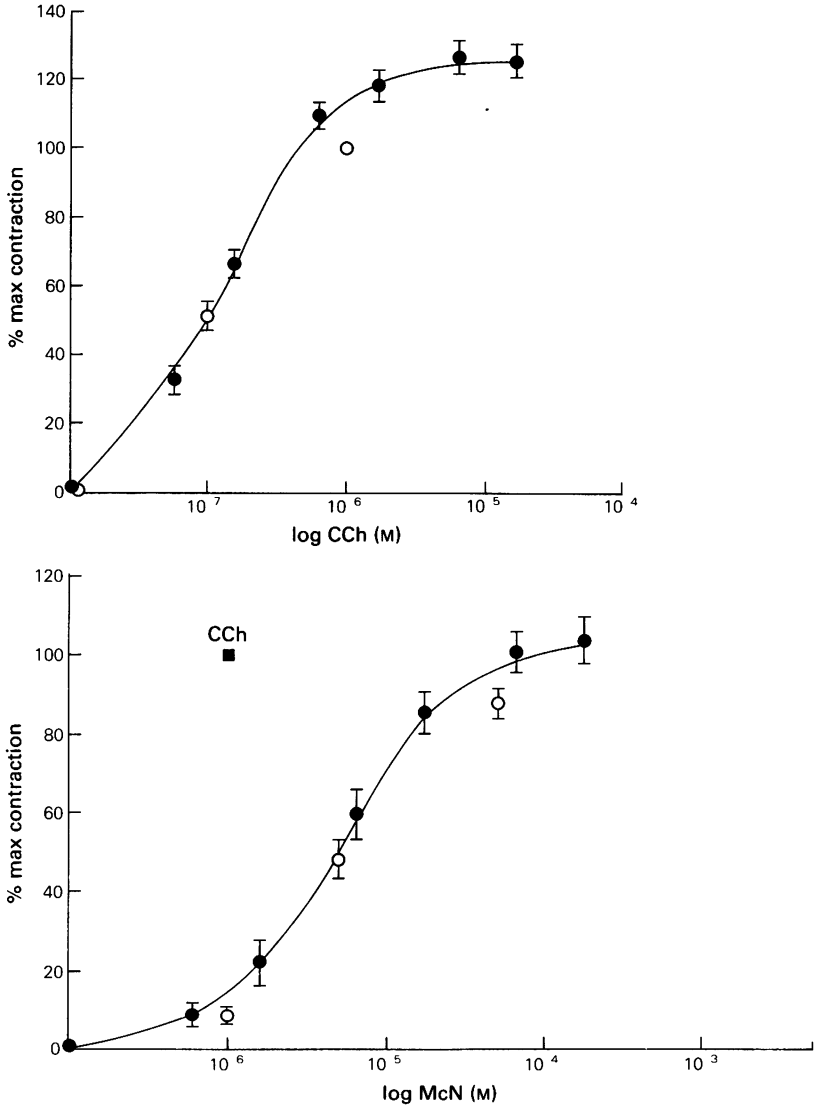

Figure 1 (a) Dose-response relationship (○) obtained with individual doses of carbachol with washout between doses followed by testing of field stimulated responses (40 pulses^{-1}, 0.5 ms, 40 V) until these returned to control levels. Standard errors for 8 strips are shown, expressed in relation to the initial response to 10^{-6}M carbachol which was taken as 100%. The values for a just sub-threshold, half maximum and the initial maximum response are shown and compared to cumulative concentration-effect curves (●; s.e. shown by vertical lines). The cumulative concentration was used for these graphs. Note that there was no evidence of tachyphylaxis during cumulative dose-effect curves. (b) Dose-effect curves obtained from individual doses of McNeil A-343 (○; s.e. shown by vertical lines) obtained as in (a). A just threshold dose, a half maximum dose and a near maximum dose are shown. These values are compared to values obtained in the same eight strips as used for individual doses. Note that no evidence of tachyphylaxis during dose-effect curves was obtained. The response to a 10^{-6}M dose of carbachol (CCh, ■) was taken as 100%.

before and after increasing concentrations of atropine or pirenzepine. Increasing agonist concentrations were added after the responses to the lower ones had reached a plateau. Controls in which dose-effect curves were carried out repetitively without any antagonists were always used. Since there were sometimes inconsistent shifts to the right in these time control curves when these curves were determined over many hours, we used the cumulative dose-effect curves to shorten the duration of the experiments. We discarded the few experiments in which major shifts of control curves were found and corrected the experimental values in which minor shifts occurred (see Baron *et al*; 1972). Washout of agonist contractile effects required approximately 60 min at doses which yielded 50% or greater maximal responses. Washing was continued until the response to field stimulation had recovered to its control value; this was done because reduced tonic and phasic responses on field stimulation were a sensitive index of a residual tachyphylaxis due to previous exposures to muscarinic agonists. Thus, after dissection recovery time, establishment of responses to field stimulation, running a control dose-effect curve followed by another after each of two antagonist concentrations, required over 10 h using cumulative dose-effect curves. This limitation and the limitations on doses imposed by limited quantities and specificity of McN A-343 made use of a Schild plot unjustified for the data obtained in this study. Instead, we calculated the K_B and pA_2 values, equal to negative logarithm of K_B, by the method of Furchgott (1972); i.e., we used the relationship $\log(DR - 1) = \log[B] - \log K_B$, when DR is the dose-ratio for 50% responses after and before the antagonist, [B] is the antagonist concentration and K_B is the dissociation constant for antagonist and receptor. These values were approximately the same at all antagonist concentrations as would be expected of a Schild plot with a slope of 1.0 or unity.

The use of cumulative dose-effect curves did not cause marked tachyphylaxis. The responses achieved during cumulative dose-effect curves in 28 strips treated with 1×10^{-6}M carbachol were $87.6 \pm 19.4\%$ of the initial maximal response to this concentration of carbachol. Also, the maxima achieved after 5×10^{-5}M McN A-343 in 26 strips were $76.0 \pm 24.2\%$ of the initial maximum to 1×10^{-6}M carbachol. To evaluate further the occurrence of tachyphylaxis, we compared responses to concentrations of carbachol and McN A-343 which produced threshold, approximate half or full maximum responses when given in a cumulative fashion to the responses to the same concentrations given first as single concentrations. The results obtained are summarized in Figure 1a and b and show no significant differences. As reported previously, the maximum response to carbachol averaged about 6 g tension increment (Robotham *et al.*, 1985).

To estimate whether various non-muscarinic and non-tachykinin agonists affected tonic and phasic responses to field stimulation, the maximum response to carbachol was determined, the control responses to high frequency (30 to 50 pulses s^{-1}) field stimulation were determined and then increasing doses of agonists were given. None of the agonists studied (see below) caused contraction acting alone.

To estimate the relative doses of substance P and its analogues and fragments required to activate tension in muscularis mucosa strips, cumulative dose-effect curves were obtained and ED_{50} values obtained. Comparisons were made of effects on different strips and of effects of two agonists run sequentially on the same strip. Data are presented as mean dose-ratios (ED_{50} substance P/ED_{50} analogue).

Drugs

Drugs used in this study were: carbachol (Sigma); atropine (Sigma); McNeil A-343 (McN A-343) (3-*m*-chlorophenylcarbamoyloxy - 2 - butynyltrimethylam - monium) gift of McNeil Lab Inc.; pirenzepine gift of Boehringer-Ingelheim; Met-enkephalin (Sigma); morphine (Ingram-Bell Ltd); [D-Ala^2,D-Leu^5]enkephalin (Sigma); dynorphin (Sigma); clonidine (Sigma); adenosine (Sigma); and the following substance P (SP) fragments and analogues: penta, hexa, hepta, octa SP (Peninsula); eledoisin, physalaemin and kassinin (Peninsula).

Statistical procedures

Results involving distribution of values in a population of results are given as means ± standard deviation (s.d.). Results involving comparisons of means are given as means ± s.e.mean. In the case of contractions to muscarinic agonists, results are given as percentage of the initial carbachol maximum response (to 1×10^{-6}M). In the case of responses to field stimulation, results are given in terms of percentage of the control values to the appropriate stimulation. Means were compared for significance using a one- or two-tailed *t* test as appropriate and accepting differences with a probability of <0.05.

Results

Nature of smooth muscle muscarinic receptors

Table 1 summarizes the results of studies of the interaction of muscarinic agonists and antagonists. Both carbachol and McN A-343 were full agonists with carbachol about 30 times more potent than McN A-343. Atropine had nearly identical pA_2 values (9.5 to 9.6) as an antagonist against each agonist (see

Figure 2). Pirenzepine also was similarly effective against both agonists (pA_2 about 8.0).

Other prejunctional receptors

A number of other agonists which activate neural receptors in other systems were tested for their ability to inhibit phasic or tonic responses to field stimulation. None was effective. Ineffective agonists (and the maximum concentrations tested) were dynorphin (1–13) 10^{-6}M; [Met^5]enkephalin 1×10^{-6}M; [D-Ala^2-D-Leu^5]enkephalin 1×10^{-6}M; clonidine 1×10^{-6}M; phenylephrine 1×10^{-4}M; adenosine 1×10^{-4}M.

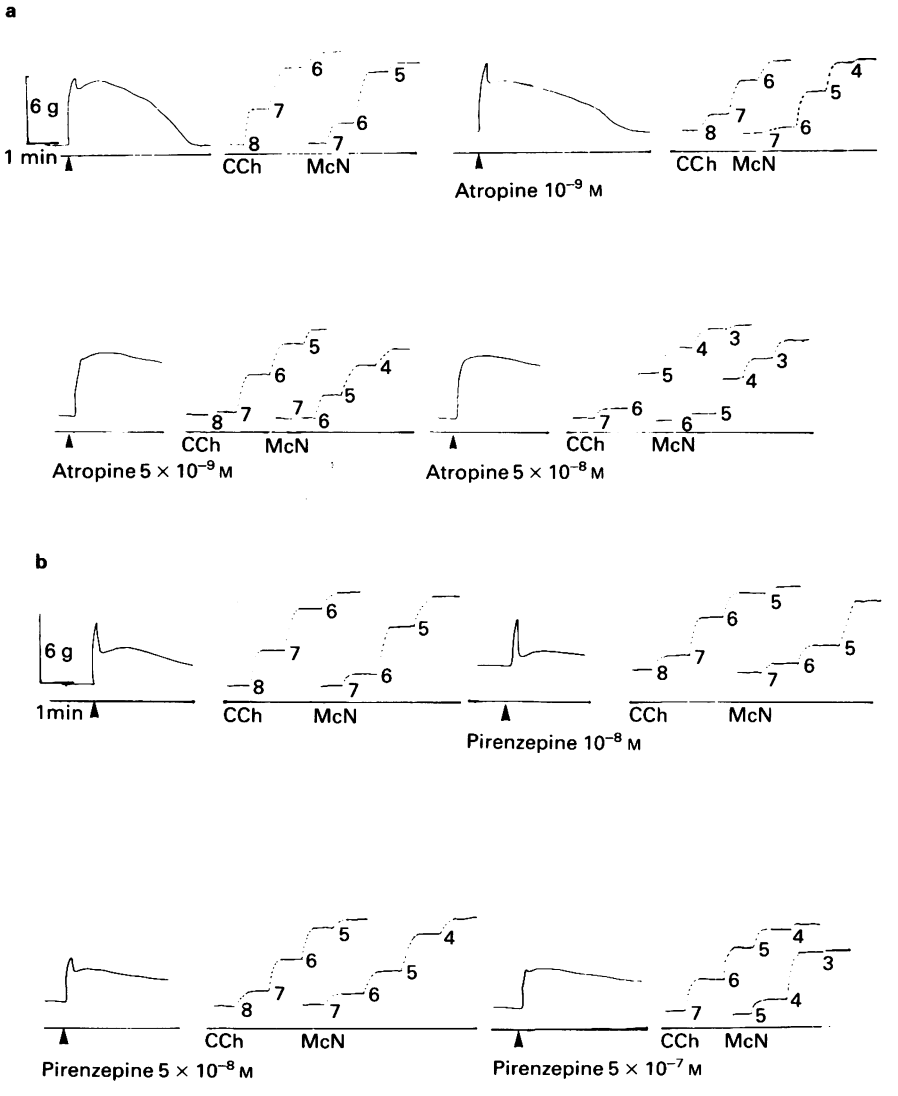

Figure 2 Samples of responses of three strips during cumulative dose-response curves to carbachol (CCh) and McNeil A-343 (McN) and effects of antagonists. A test response to 40 pulses s^{-1} field stimulation was obtained between each curve and some are shown. Only the tension level achieved in response to each cumulative dose is shown and not the true time course. Each curve required about 30 min to complete and washout between curves more than 60 min. Total duration was over 14 h. Concentrations added are shown as negative logarithm of molarity; note that these are cumulative concentration-effect curves, but only the concentration added is shown in the figure. (a) Atropine: 10^{-9}, 5×10^{-9}, 5×10^{-8}M. (b) Pirenzepine: 10^{-8}, 5×10^{-8}, 5×10^{-7}M.

Table 1 Comparison of interactions with smooth muscle muscarinic receptors

Potency of agonists:	*Carbachol*	*McN A-343*
$-\log ED_{50}$	7.31 ± 0.47 ($n = 46$)	5.80 ± 1.46* ($n = 46$)
pA_2 values for antagonists against agonists:	*Atropine*	*Pirenzepine*
Against carbachol	9.53 ± 0.68 ($n = 30$)	7.95 ± 0.45** ($n = 28$)
Against McN A-343	9.60 ± 0.48 ($n = 25$)	8.08 ± 0.50** ($n = 26$)

(Atropine concentrations; 10^{-9} to 10^{-7}M; pirenzepine concentrations; 10^{-8} to 10^{-6}M).
*Significantly different from values for carbachol.
**Significantly different from the values for atropine but not different using carbachol or McN A-343 as agonists.

Table 2 Comparison of potencies of analogues and fragments of substance P (SP)*

	Ratios of ED_{50} values	
Analogues	n	
Substance P (repeat)	14	4.65 ± 1.24
Eledoisin	10	0.40 ± 0.09**
Kassinin	10	0.96 ± 2.70**
Physalaemin	7	3.40 ± 1.2
Fragments	n	
Substance P (repeat)	14	1.90 ± 0.51
Octa-SP	12	1.10 ± 0.24
Hepta-SP	9	3.70 ± 2.18
Hexa-SP	13	1.79 ± 0.52
Penta-SP	5	0.0029 ± 0.0007**

*ED_{50} for substance P divided by ED_{50} for test substance.
**Significantly different from ratio for SP.

Smooth muscle tachykinin receptor

Table 2 shows the relative potencies of various SP analogues or fragments compared to SP itself. In all experiments described here one strip in which SP dose-response curves were repeated several times served as a control. In the remaining strips dose-response curves for the SP fragments were constructed, i.e., each strip was exposed to each fragment in turn. Ratios represent the concomitant comparison of the appropriate control strip with each treated strip. This ensured that tachyphylaxis and other changes in sensitivity during the experiment did not confound the results. Although some significantly different ratios were found, the differences with one exception were trivial relative to those expected (see Discussion). The one exception was the penta-C-terminal fragment of SP which was much less potent but still a full agonist. The absolute values of the ED_{50} values of SP were $1-5 \times 10^{-8}$M. These values are neither very high nor very low compared to those at other tachykinin receptors (see Discussion), suggesting that this receptor may not be selective for SP and may be unselective among tachykinins.

Discussion

Our results show that the muscarinic receptors on smooth muscle of muscularis mucosa have some of the characteristics of M_1-receptors; i.e., atropine has a pA_2 value of about 9.5 while that of pirenzepine is about 8.0. The same values were obtained whether carbachol or McN A-343 was used as the agonist; both were full agonists. These values are typical for some receptors in autonomic ganglia (Brown *et al.*, 1980a,b; Halim *et al.*, 1982; Mitchelson, 1984; Kilbinger, 1984). The potency difference between atropine and pirenzepine is also in the range observed for M_1-receptors (Mitchelson, 1984). In typical M_2-receptors on smooth muscle (Hammer *et al.*, 1980; Barlow *et al.*, 1981) and on nerves (Brown *et al.*, 1980b; Halim *et al.*, 1982; Kilbinger, 1984; Mitchelson, 1984) the pA_2 value for pirenzepine was between 6 and 7 and McN A-343 was ineffective or a partial agonist. In a recently reported study of longitudinal strips of muscularis mucosa from guinea-pig oesophagus, Kamikawa *et al.* (1985) found pA_2 values of 9.4 for atropine and 7.4 for pirenzepine from Schild-plots with slopes near 1. In guinea-pig ileum, pA_2 values were significantly lower than in muscularis mucosa, 8.8 for atropine and 6.9 for pirenzepine, but Schild-plots had slopes of about 1.5. We could not construct Schild-plots but our pA_2 values (from $-\log K_B$) to opossum oesophagus were similar for atropine and slightly higher for pirenzepine compared to those for guinea-pig oesophagus. These authors did not use McN A-343 as an agonist, but the pD_2 value, 6.8, for carbachol was similar to that obtained in this study (Table 2). In our experiments, the potency difference between carbachol and McN A-343 was about 30 fold (carbachol more potent). Its potency was difficult to compare with that observed in other systems with M_1-type receptors studied *in vitro*; in sympathetic ganglia it had complex effects (Brown *et al.*, 1980b).

The criteria used for classifying smooth muscle

muscarinic receptors as M_1 in the present study were similar to those used in other studies (Hammer *et al.*, 1980; Hammer, 1982; Hammer & Giachetti, 1982), but were incompletely applied. Our inability to evaluate Schild-plots in the present study heightens the possibility of misinterpretation. Proof may require more careful evaluation of tissue factors such as the presence of spare receptors and comparisons of binding and functional studies as well as possibly isolation of receptors. If all smooth muscle receptors are M_2 in subtype, as presumed in most recent publications, then the receptors in muscularis mucosa muscle are a distinct subtype at which both McN A-343 and pirenzepine had affinities comparable to those for M_1-receptors in brain and ganglia. This would be consistent with the findings of Kamikawa *et al.* (1985) in muscularis mucosa of guinea-pig based on the evidence cited above. Thus, muscularis mucosa of oesophagus may contain unusual muscarinic receptors (not typical M_2).

The nerves mediating release of SP-like material in muscularis mucosa of opossum may be afferent since capsaicin also releases such a material and blocks the tonic contraction mediated by it after field stimulation (Robotham *et al.*, 1985). In this study, no prejunctional inhibitory receptors operated by opiates, α-adrenoceptor agonists or adenosine were observed affecting these nerves. As previously reported (Domoto *et al.*, 1983) there were presynaptic inhibitory receptors of muscarinic type which inhibited release of substance P. The nature of these receptors will be described elsewhere; they are not typical M_2-receptors since McN A-343 is a full agonist and pirenzepine a potent antagonist. The origin of nerves mediating release of acetylcholine in this tissue is unknown, but the release of acetylcholine (as judged by phasic contractions) was not affected by apparent inhibition of release of SP-like material by capsaicin (Robotham *et al.*, 1985). Thus, it is likely that acetylcholine release was independent of a requirement for similar release of SP-like material, either by virtue of its occurrence in separate nerves or by virtue of its occurrence by an independent release mechanism. Release of acetylcholine from these nerves, too, is modulated by prejunctional muscarinic receptors which resemble those on smooth muscle (Daniel, E.E.; unpublished).

Recently, Rattan & Goyal (1984) reported *in vivo* studies of opossum oesophagus which they interpreted as indicating that M_1-muscarinic receptors were present on inhibitory intrinsic neurones mediating lower esophageal sphincter relaxation and that excitatory M_2-muscarinic receptors were present on circular muscle of the sphincter. Their hypothesis that opossum muscle muscarinic receptors are similar to M_2-receptors elsewhere, considered in light of our findings, suggests that muscularis mucosa may differ from circular muscle of the oesophagus in this ligand. However, the existence of M_2-receptors on muscle was deduced by these authors despite the fact that after tetrodotoxin (to block inhibitory nerves), McN A-343, like bethanechol, produced full sphincter contractions. Pirenzepine was reported not to shift sphincter responses to bethanechol or McN A-343, whereas atropine and 4-dephenylacetoxy-*N*-methylpiperidine (see Barlow *et al.*, 1976; 1980; 1981) shifted responses to bethanechol to higher doses. However, the doses of pirenzepine used did not cover a sufficient range (only 10 fold) relative to effective doses of atropine to conclude that it was ineffective. Also, the possibility cannot be ignored that the pressure changes recorded in the study within the sphincter were contributed to by contraction of the very thick, longitudinally oriented, muscularis mucosa in this region (see Domoto *et al.*, 1983). The complexity of possible loci of various muscarinic receptors and their various additive and opposing actions *in vivo* as well as the lack of equilibrium conditions when agonists are given intra-arterially make such studies difficult to interpret as regards receptor type. Further study will obviously be required to resolve the nature of muscarinic receptors on circular and other muscles of opossum oesophagus and to test whether other muscarinic receptors in opossum gut are like those in muscularis muscosa of opossum and guinea-pig; i.e., M_1 in type or an unusual subtype of M_2-receptors.

The possibly unusual nature of receptors on opossum gut smooth muscle was further emphasized by our findings with regard to receptors for tachykinins related to SP. Neither the tachykinin analogues of SP (eledoisin, kassinin, physalaemin) nor the C-terminal fragments (except the less potent penta fragment) of SP were discriminated by this receptor, despite such discrimination in other systems (Teichberg *et al.*, 1981; Lee *et al.*, 1982; Watson *et al.*, 1983a,b,c; Growcott *et al.*, 1983; Lin & Musacchio, 1983; see Watson 1984 for review). SP selective antagonists inhibit responses both to SP and the endogenous material released by field stimulation, but they were weak antagonists or partial agonists (Domoto *et al.*, 1983; Rossell *et al.*, 1983; Mizrahi *et al.*, 1984; Robotham *et al.*, 1985). To differentiate further and classify tachykinin receptors, more potent and selective agonists and antagonists appear necessary. Since the absolute potencies of all the tachykinins for this receptor were in the intermediate range (ED_{50} order of 10^{-8}M), the possibility emerges that it may be selective for another tachykinin other than the ones tested in this study or that this receptor is relatively undifferentiated. In any case, our results imply that the SP receptor of opossum muscularis mucosa was neither of the SP-P nor of the SP-E type (Lee *et al.*, 1982; Watson, 1984).

In conclusion, our findings suggest that excitatory muscarinic receptors on smooth muscle of opossum

oesophagus muscularis mucosa were M_1 in type or a subtype of M_2 receptor at which pirenzepine had a high affinity and McN A-343 was a full agonist. No prejunctional receptors were identified in this tissue other than muscarinic receptors. The tachykinin receptor on this muscle did not discriminate between various tachykinin molecules or SP, C-terminal fragments down to the penta fragment. This tachykinin receptor may be undifferentiated.

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