



Functional interactions between muscarinic M₂ receptors and 5-hydroxytryptamine (5-HT)₄ receptors and β₃-adrenoceptors in isolated oesophageal muscularis mucosae of the rat

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1 Relaxations of isolated oesophageal muscularis mucosae of rat are mediated by 5-hydroxytryptamine (5-HT), acting at 5-HT₄ receptors, and isoprenaline, principally acting via β₃-adrenoceptors. The aim of this study was to investigate the hypothesis that muscarinic M₂ receptors, also present in this tissue, functionally oppose 5-HT and β-adrenoceptor-relaxant effects in this preparation.

2 Contractions of rat oesophageal muscularis mucosae were induced, in a concentration-dependent manner, by the muscarinic receptor agonist, oxotremorine M (pEC₅₀ = 6.7 ± 0.1). The contractile responses to oxotremorine M were surmountably antagonized by the following compounds, (pK_B values in parentheses): atropine (9.1 ± 0.2), 4-DAMP (4-diphenylacetoxy-N-methyl piperidine methiodide, 8.7 ± 0.1), *p*-F-HHSiD (*para*-fluoro-hexa-hydro-siladifenidol, 7.5 ± 0.1), zamifenacin (8.6 ± 0.3), himbacine (7.2 ± 0.2), pirenzepine (6.8 ± 0.3) and methoctramine (6.2 ± 0.2). These data are consistent with a role for muscarinic M₃ receptors mediating contractions to oxotremorine M. The contractile response was associated with a low receptor reserve, since the responses were shifted to the right and virtually abolished by the alkylating agent, 4-DAMP mustard (4-diphenylacetoxy-N-(2-chloroethyl) piperidine, 40 nM; 60 min equilibration).

3 In tissues precontracted with U46619 (0.7 μM; approx. EC₉₀), isoprenaline (pEC₅₀ = 8.0 ± 0.1) and 5-HT (pEC₅₀ = 7.5 ± 0.2) induced concentration-dependent relaxations. The isoprenaline potency was slightly, but significantly, different in tissues precontracted with oxotremorine M (isoprenaline, pEC₅₀ = 7.4 ± 0.2). In contrast, the potency of 5-HT (pEC₅₀ = 7.5 ± 0.2), in tissues that were precontracted with 1 μM (EC₉₀) oxotremorine M, was identical. When these experiments were repeated in the presence of the muscarinic M₂ receptor antagonist, methoctramine (1 μM), there was no effect on the relaxant potencies to either 5-HT or isoprenaline. Collectively, these data suggest that muscarinic M₂ receptors do not, under these conditions, modulate relaxant potencies to either 5-HT or isoprenaline.

4 In a second protocol, tissues were pre-contracted with U46619 (0.7 μM) and relaxed with either 5-HT (0.1 μM) or isoprenaline (0.1 μM). In these tissues (in which the muscarinic M₃ receptor population was extensively depleted by alkylation), oxotremorine M caused concentration-dependent re-contractions (i.e. reversal of relaxations). In tissues relaxed with 5-HT, the potency of oxotremorine M was 5.9 ± 0.2, while in tissues relaxed with isoprenaline, the potency (pEC₅₀) = 5.6 ± 0.3. These re-contractions were antagonized, in a surmountable fashion, by methoctramine (1 μM; pK_B = 7.6 ± 0.1). Similar observations were seen when relaxations were induced by isoprenaline (1 μM; pK_B = 7.5 ± 0.2). Under these conditions, therefore, the pK_B values are consistent with activation of muscarinic M₂ receptors, and inconsistent with activation of M₃ receptors.

5 It is concluded that in isolated oesophageal muscularis mucosae of rat, muscarinic M₃ receptors mediate direct contractions and are associated with a low receptor reserve. When this population is depleted, and the tissues relaxed via activation of receptors that augment adenylyl cyclase activity, a functional role for muscarinic M₂ receptors is revealed.

Keywords: Muscarinic M₂ receptors; β₃-adrenoceptors; rat oesophageal muscularis mucosae; receptor alkylation

Introduction

Many smooth muscles express both muscarinic M₂ and M₃ receptors. The function of the M₃ receptor in mediating direct postjunctional contraction is well established. In contrast, the function of the M₂ receptor population that, in most smooth muscles, forms the majority, is currently under intensive investigation (see Eglen *et al.*, 1994a,b; Ehlert & Thomas, 1995 for reviews). In isolated ileum of guinea-pig, muscarinic M₂ receptors functionally antagonize relaxations mediated by β-adrenoceptors agonists (Thomas *et al.*, 1993). This effect correlates with a reduction in adenylyl cyclase activity, when initially elevated by isoprenaline or the selective β₃-adrenoceptor agonist, BRL 37344 (Thomas *et al.*, 1993; Thomas & Ehlert, 1994; Reddy *et al.*, 1995). Muscarinic M₂ receptors also func-

tionally antagonize ileal relaxations to forskolin, a direct activator of adenylyl cyclase (Thomas & Ehlert, 1996). These data suggest that an inhibitory effect of muscarinic M₂ receptors can generally attenuate rises in intracellular adenosine 3':5'-cyclic monophosphate (cyclic AMP) and thus reverse muscle relaxation mediated by this signalling system (Berridge, 1975). At a biochemical level, some evidence is available to support this suggestion, since in guinea-pig ileal slices, muscarinic M₂ receptors reduce levels of cyclic AMP, elevated by either 5-hydroxytryptamine (5-HT), prostaglandin E₁ or E₂ or vasoactive intestinal peptide (Reddy *et al.*, 1995). However, functional antagonism of relaxations to these agents via muscarinic M₂ receptors has not been demonstrated.

The occurrence of this 'cross-talk' varies between smooth muscles, although an inhibitory role of M₂ receptors on adenylyl cyclase activity is seen in many smooth muscles from different species (see Eglen *et al.*, 1994a, for review). Based on

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data in guinea-pig trachea and human trachea, for example, evidence for a functional role of muscarinic M₂ receptors is mixed. Thus, while muscarinic M₂ antagonism augments the relaxant potency of β -adrenoceptor agonists in guinea-pig (Watson & Eglén, 1994) and bovine trachea (Fernandes *et al.*, 1992), this has not been seen in human trachea (Watson *et al.*, 1995a). Indeed, augmentation of isoprenaline relaxant potency in bovine trachea has not been confirmed in a subsequent paper (Roffel *et al.*, 1995). Moreover, with a similar protocol to that adopted in guinea-pig ileum (Thomas *et al.*, 1993), muscarinic M₂ receptors have been shown to oppose functionally relaxations induced by forskolin (Thomas & Ehlert, 1996), although this effect has not been seen with isoprenaline (Watson *et al.*, 1995b). In guinea-pig oesophageal muscularis mucosae or stomach fundus, however, a functional role for M₂ receptors is either small or nonexistent, irrespective of the use of isoprenaline or forskolin (Watson *et al.*, 1995c; Thomas & Ehlert, 1996).

Consequently, it is of some interest to characterize other examples of an interaction between muscarinic M₂ receptors and receptors that induce smooth muscle relaxation via augmentation of adenylyl cyclase activity. The isolated oesophageal muscularis mucosae of rat contracts to muscarinic receptor agonists (Bieger & Triggler, 1985), although the nature of the muscarinic receptor subtype mediating contraction has not been defined. The preparation relaxes to either 5-HT, via 5-HT₄ receptors (Baxter *et al.*, 1991) and noradrenaline, principally via β_3 -adrenoceptors (de Boer *et al.*, 1995), both of which are positively coupled to adenylyl cyclase (Ford *et al.*, 1992; de Boer *et al.*, 1995). Taken together, the preparation could provide a simple model to explore the potential cross talk between muscarinic receptors, β -adrenoceptors and 5-HT₄ receptors.

Two approaches were employed to address this possibility. First, the potential for augmentation of 5-HT and isoprenaline relaxant potencies in the presence of muscarinic M₂ receptor antagonism was explored (Fernandes *et al.*, 1992). However, the enhancement of isoprenaline potency has been questioned as definitive evidence to implicate M₂ receptor activation (Ehlert & Thomas, 1995). Thus, antagonist affinities cannot be estimated at M₂ receptors *per se*, as M₃ receptors are also stimulated to elevate the initial resting tension. Alternatively, a role for muscarinic M₂ receptors was evaluated by measuring reversal of relaxations i.e. recontraction (Thomas *et al.*, 1993; Thomas & Ehlert, 1996), under conditions of a depleted muscarinic M₃ receptor population. The advantages of this technique are two fold. First, that resting tension can be elevated by a non-muscarinic receptor agonist. Second, that the nonselective muscarinic agonist, oxotremorine M, can be used to activate selectively muscarinic M₂ receptors. Measurement of antagonist affinities can thus be determined at the M₂ receptor by null methods (Thomas & Ehlert, 1993; 1996; Reddy *et al.*, 1995). A preliminary account of these data has been communicated to the British Pharmacological Society (Eglén *et al.*, 1996).

Methods

General

Male Sprague-Dawley rats (200–250 g) were killed by carbon dioxide asphyxiation, the thoracic oesophagus removed and placed in modified Tyrode solution (composition, mM: NaCl 136.9, dextrose 5.6, NaH₂PO₄ 0.4, KCl 2.4, MgCl₂ 1.1, NaHCO₃ 11.9 and CaCl₂ 1.8). The outer striated smooth muscle was carefully removed, leaving the inner tunica (Kamikawa & Shimo, 1979). Longitudinal segments of this preparation, the muscularis mucosa (2 cm in length) were suspended under 1.0 g tension, in 10 ml organ baths, containing gassed (5% CO₂ in oxygen) Tyrode solution (pH 7.4, 37°C). Responses were measured as changes in isometric tension (g) by use of a Hugo Sachs K30 isometric force transducer and displayed with a Graphtec WR4101 Linearecorder.

The preparations were equilibrated with Tyrode solution for 30 min before 50 mM KCl was used to induce a sustained contraction. This enabled the contractile activity to be assessed and those tissues responding with a developed tension of less than 0.25 g tension were discarded. In practice, however, all tissues used responded to this spasmogen. The tissues were washed and, 15 min later, a single concentration of oxotremorine M (68 nM) added. Once the contracture was established, the tissues were washed and a concentration-response curve to oxotremorine M established 30 min later. This priming procedure was undertaken in all tissues and time-dependent changes in agonist sensitivity were minimized. All concentration-effect curves were established in a cumulative fashion, with concentrations incrementally spaced at 0.5 log intervals. All tissues were washed by overflow, with warmed (37°C), oxygenated Tyrode solution.

Receptor characterization studies

The muscarinic receptor subtype mediating contraction of the tissue was assessed by determining affinities for several selective antagonists (see Eglén *et al.*, 1994a, for review). After construction of an initial concentration-effect curve to oxotremorine M (see above), the tissues were washed and equilibrated, for 60 min, with a single concentration of antagonist. A second concentration-effect curve to oxotremorine M was then established in the presence of antagonist, with each tissue exposed to only one antagonist.

The muscarinic receptor reserve associated with contraction was also determined by use of 4-diphenylacetoxy-N-(2-chloroethyl)piperidine (4-DAMP mustard) (Barlow *et al.*, 1990; Thomas *et al.*, 1992; see Eglén *et al.*, 1994b, for review) as the alkylating agent. After construction of a concentration-effect curve to oxotremorine M, tissues were washed and equilibrated with 4-DAMP mustard (40 nM) for 60 min. They were then washed at 10 min intervals over the succeeding 30 min and a second curve to oxotremorine M established.

Relaxation studies

Thirty min following construction of the initial concentration-effect curve to oxotremorine M, a concentration-response curve to the thromboxane (TP) receptor agonist, U46619, was established. The tissues were washed and allowed to reattain baseline tension for 60 min. In each tissue, the concentration of U46619 inducing 90% of the maximal response was calculated (approx 0.7 μ M) and then re-added to the bath to induce a contracture. In separate tissues, a similar protocol was adopted with oxotremorine M, again titrated to the EC₉₀ concentration. The identification of the EC₉₀ concentrations in each tissue, for each spasmogen, ensured equiactive stimulation of tone. The tissues were allowed to develop sustained contractures to these agonists. This process usually occurred within 20 min of addition of spasmogen and were maintained for the duration of the experiment. Relaxations were then induced by cumulative additions of either 5-HT or isoprenaline. To explore the potential for an involvement of muscarinic M₂ receptors, the experiments were repeated in the presence of methoctramine (1 μ M), following an equilibration period of 60 min.

The rationale behind this approach was that, in tissues precontracted with U46619, an increase in 5-HT or isoprenaline potency, by methoctramine, may suggest that endogenous activation of muscarinic M₂ receptors, by basal release of acetylcholine, functionally antagonized relaxations. In tissues precontracted with oxotremorine M, an increase in 5-HT or isoprenaline potency, in the presence of M₂ receptor blockade, may indicate that exogenous activation of muscarinic M₂ receptors opposes relaxant responses to 5-HT or isoprenaline.

Functional antagonism studies

As described in the introduction, a functional role for muscarinic M₂ receptors in guinea-pig ileum has been disclosed,

after depletion of the muscarinic M₃ receptor population. In the present study, after concentration-effect curves to oxotremorine M were established, the tissues washed and exposed to 4-DAMP mustard (40 nM) and methoctramine (1 μ M). This protection procedure enhanced the selectivity of 4-DAMP mustard, ensuring the preservation of muscarinic M₂ receptors (see Eglen *et al.*, 1994b, for review). After washing, at 10 min intervals over the succeeding 30 min (to remove both 4-DAMP mustard and methoctramine), the tissues were contracted with U46619 (0.7 μ M) and maximally relaxed either by 5-HT (0.3 μ M) or isoprenaline (0.3 μ M). These spasmogens thus elevated tissue tone so that relaxant responses could be observed. A second concentration-effect curve to oxotremorine M was then constructed. Responses to muscarinic agonists under these conditions would be manifested by a reversal of the relaxation i.e. 'a recontraction'.

The hypothesis behind this approach was that, since 4-DAMP mustard alkylated the majority of muscarinic M₃ receptors, (and markedly reduce the direct contraction), it was reasonable to assume that recontractions to oxotremorine M were mediated by activation of muscarinic M₂ receptors alone. To confirm this hypothesis, however, the recontractions to oxotremorine M were studied in the presence of the selective M₂ receptor antagonist, methoctramine (1 μ M), following an equilibration period of 60 min.

Chemicals

Atropine sulphate, 4-DAMP (4-diphenylacetoxy-N-methyl piperidine methiodide), 4-DAMP mustard (4-diphenylacetoxy-N-(2-chloroethyl) piperidine), *p*-F-HHSiD (*para*-fluoro-hexahydro-siladifenidol), histamine, methoctramine tetrachloride, oxotremorine methiodide (oxotremorine M) and pirenzepine dihydrochloride were obtained from Research Biochemicals Inc. (Natick, MA, U.S.A.). U46619 was purchased from Cayman Chemical Co. Ltd (CA, U.S.A.). Himbacine was obtained from Prof. W.C. Taylor, University of Melbourne, Australia. Zamifenacin was generously donated by Pfizer PLC (Sandwich, UK). All remaining chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Solutions were prepared in distilled water except that stock solutions of *p*-F-HHSiD were prepared in ethanol. Isoprenaline was dissolved in ascorbic acid (5 mM). Solutions of 4-DAMP mustard were prepared in dimethylsulphoxide (DMSO) and subsequently diluted in distilled water. Stock solutions of U46619 (11 α , 9 α -epoxymethano-PGH₂) were diluted in DMSO and subsequently diluted in distilled water. All solutions were kept on ice until use.

Data analysis

Agonist potencies (pEC₅₀) were estimated using a relationship of Parker & Waud (1971) by a non-linear iterative curve fitting procedure (Leung *et al.*, 1992). Antagonist affinities (pK_B) values were estimated by the relationship of Furchgott (1972). All values are mean \pm s.e.mean. Statistical significance was determined by an unpaired Student's *t* test and a probability level of *P* < 0.05 was considered significant.

Results

Antagonist studies

Concentration-effect curves to oxotremorine M were antagonized by all compounds in a surmountable fashion (Table 1). The following rank order of antagonist affinity was observed atropine > 4-DAMP = zamifenacin > himbacine > pirenzepine > methoctramine. This rank order, and the absolute values, were in good agreement with pK_B values generated at muscarinic M₃ receptors mediating contraction of other smooth muscles (see Eglen *et al.*, 1994a, for review).

The tissues contracted, in a concentration-dependent man-

ner, to both oxotremorine M (see above) and U46619 (Table 2). The maximum responses to U46619 were less than those seen to oxotremorine M (Table 2). To estimate qualitatively the muscarinic receptor reserve, the effects of the alkylating agent, 4-DAMP mustard, were studied on responses to oxotremorine M. Exposure to this agent resulted in a rightward shift in the concentration-effect curve and a marked depression in the maximum response (Table 2; Figure 1). In contrast, 4-DAMP mustard (40 nM, 60 min equilibration) was without effect on the contractile response to U46619 (Table 2). Given the virtual abolition of the response to oxotremorine M following alkylation, no attempt was made to estimate the affinity of the agonist by null methods.

Relaxant studies

The relaxant potencies of 5-HT were similar (Table 3) in tissues precontracted with either U46619 or oxotremorine M, even though the level of developed tension differed. The potency of isoprenaline was slightly (4 fold) but significantly higher in tissues precontracted with U46619 in comparison to those precontracted with oxotremorine M. When these studies were repeated in the presence of methoctramine (1 μ M), the relaxant potencies of neither 5-HT nor isoprenaline were altered. This lack of effect of methoctramine was seen in tissues precontracted with either U46619 or oxotremorine M (Table 3; Figures 2 and 3).

Functional antagonism studies

As described in the Methods, a second approach was used to assess a functional role for muscarinic M₂ receptors i.e. selective

Table 1 Muscarinic receptor antagonist affinities (pK_B) against oxotremorine M contraction of rat isolated oesophageal muscularis mucosae

Antagonist	Concentration	Affinity (estimated pK _B) oesophagus
Atropine	10 nM	9.0 \pm 0.2
4-DAMP	10 nM	8.7 \pm 0.1
Zamifenacin	10 nM	8.6 \pm 0.3
Himbacine	1 μ M	7.2 \pm 0.2
Methoctramine	1 μ M	6.2 \pm 0.2
<i>p</i> -F-HHSiD	3 μ M	7.5 \pm 0.1
Pirenzepine	1 μ M	6.8 \pm 0.3

Antagonists were studied at single concentrations and pK_B values estimated by the method of Furchgott (1972). 4-DAMP - 4-diphenylacetoxy-N-methyl piperidine methiodide, *p*-F-HHSiD - *para*-fluoro-hexahydro-siladifenidol. Values are mean \pm s.e.mean, *n* = 4-8.

Table 2 Potencies (pEC₅₀) and maximal responses (g) of oxotremorine M and U46619 at muscarinic and TP receptors mediating contraction of isolated oesophagus of rat

	pEC ₅₀	Max. response
Oxotremorine M	6.7 \pm 0.1	1.4 \pm 0.8
Oxotremorine M + 4-DAMP mustard	5.0 \pm 0.1	0.3 \pm 0.06
U46619	7.4 \pm 0.2	0.8 \pm 0.3
U46619 + 4-DAMP mustard	7.2 \pm 0.1	1.0 \pm 0.2

Values are mean \pm s.e.mean, *n* = 6. 4-DAMP mustard (N-(2-chloroethyl)-4-piperidinyl diphenylacetate; 40 nM) was equilibrated with the tissues for 60 min, followed by extensive washing.

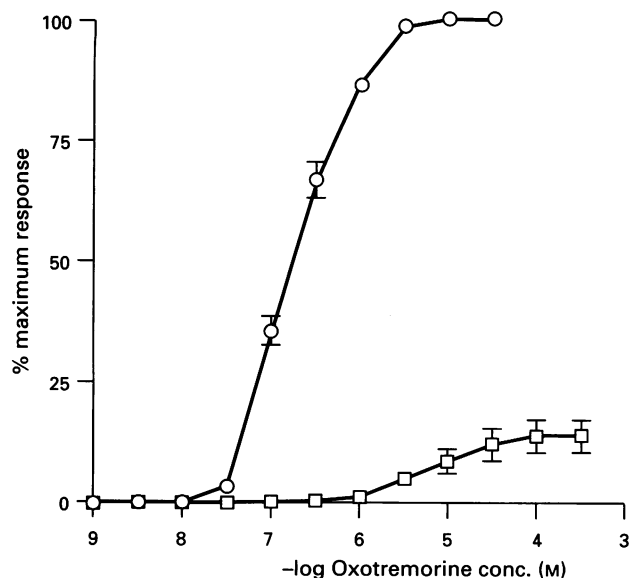


Figure 1 Concentration-effect curves for oxotremorine M in the control (○) and tissues exposed to 4-DAMP mustard (40 nM, 60 min; □) in isolated oesophageal muscularis mucosae of rat. Points shown are means and vertical lines indicate s.e.mean.

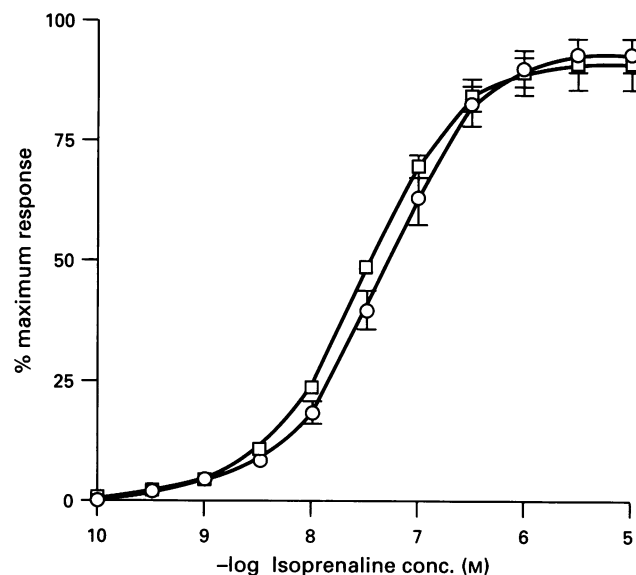


Figure 2 Concentration-effect curves for isoprenaline in the absence (○) and presence (□) of methoctramine (0.1 μM). Tissues were precontracted with oxotremorine M (0.7 μM). Points shown are means and vertical lines represent s.e.mean.

Table 3 Relaxant potencies of 5-HT and isoprenaline in isolated oesophagus of rat, precontracted with either oxotremorine M or U46619

	<i>pEC</i> ₅₀	% max. relaxation
Precontracted with oxotremorine M (1 μM)		
5-HT	7.5 ± 0.2	100
5-HT + methoctramine	7.5 ± 0.1	100
Isoprenaline	7.4 ± 0.2	100
Isoprenaline + methoctramine (1 μM)	7.4 ± 0.3	100
Precontracted with U46619 (0.7 μM)		
5-HT	7.5 ± 0.2	100
5-HT + methoctramine (1 μM)	7.4 ± 0.1	100
Isoprenaline	8.0 ± 0.2	100
Isoprenaline + methoctramine (1 μM)	7.9 ± 0.1	100

Values are mean ± s.e.mean, *n* = 4–8.

muscarinic M₃ alkylation. In tissues so treated, and precontracted with U46619, oxotremorine M caused concentration-dependent reconstrictions (Table 4). These responses were seen in tissues maximally relaxed with either 5-HT (0.1 μM; Figure 4) or isoprenaline (0.1 μM; Figure 5). These concentration-effect curves were antagonized in a surmountable fashion by methoctramine (1 μM; Figures 4 and 5) with *pK_B* values consistent with activation of muscarinic M₂ receptors (relaxed with 5-HT; 7.6 ± 0.2; relaxed with isoprenaline 7.5 ± 0.2; Table 4). Finally, the maximal reconstriction by oxotremorine M was similar to the contracture to U46619 (Table 4), suggesting complete reversal of relaxations to either 5-HT or isoprenaline. However, the reconstriction was significantly less than the maxima to oxotremorine M seen in nonalkylated tissues.

Discussion

This study has investigated the potential for a functional antagonism between muscarinic receptors and 5-HT₄ or β -adrenoceptors in isolated oesophageal muscularis mucosae of rat.

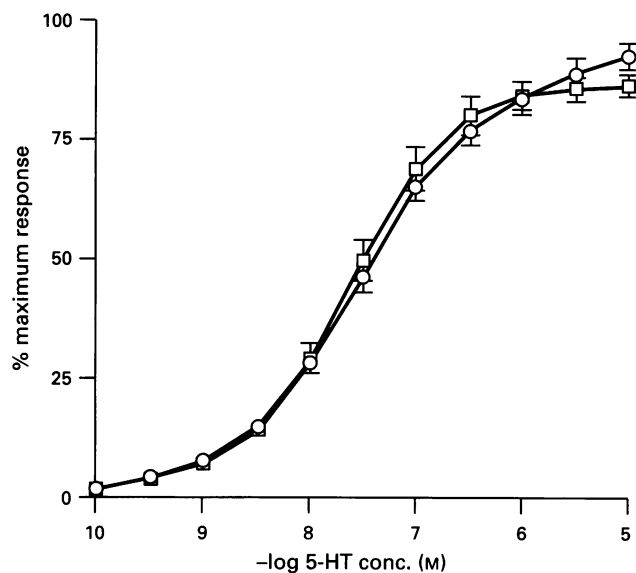


Figure 3 Concentration-effect curves for 5-hydroxytryptamine (5-HT) in the absence (○) and presence (□) of methoctramine (0.1 μM). Tissues were precontracted with oxotremorine M (0.7 μM). Points shown are means and vertical lines represent s.e.mean.

Receptor characterization studies

The muscarinic receptor subtype mediating contraction operationally resembled the muscarinic M₃ receptor subtype (Table 1). Moreover, the *pK_B* values for *p*-F-HHSiD and zamifenacin were similar to those obtained at M₃ receptors in guinea-pig ileum (Lambrecht *et al.*, 1988; Wallis *et al.*, 1993) and oesophageal muscularis mucosae (Eglen *et al.*, 1990; Watson *et al.*, 1995c). The rat oesophageal receptor did not, however, exhibit atypical *pK_B* values found for *p*-F-HHSiD at M₃ receptors in guinea-pig or human trachea and human colon (Eglen *et al.*, 1990; Roffel *et al.*, 1994; Kerr *et al.*, 1995; Watson *et al.*, 1995a). This also held true for the *pK_B* values observed for zamifenacin, a compound that differentiates muscarinic M₃ receptors in guinea-pig ileum from those in urinary bladder (Wallis, 1995; Watson *et al.*, 1995d) or canine iris muscle

Table 4 'Recontraction' potencies in isolated oesophagus of rat, precontracted with U46619 (0.7 μ M) and maximally relaxed with either 5-HT or isoprenaline

	<i>pEC</i> ₅₀	<i>Max.</i> <i>response</i> (g)
Relaxed with 5-HT (0.1 μ M)		
Oxotremorine M	5.9 \pm 0.2	0.64 \pm 0.09
Oxotremorine M + methoctramine (1 μ M)	4.3 \pm 0.5	0.78 \pm 0.11
Relaxed with isoprenaline (0.1 μ M)		
Oxotremorine M	5.6 \pm 0.3	0.76 \pm 0.12
Oxotremorine M + methoctramine (1 μ M)	4.1 \pm 0.4	1.13 \pm 0.14

Values are mean \pm s.e. mean, *n* = 8.

(McIntyre & Quinn, 1995). Taken together, the muscarinic receptor mediating contraction resembled the M₃ receptor described in the isolated ileum or oesophageal muscularis mucosae of guinea-pig. However, the potency of oxotremorine M at muscarinic M₃ receptors in guinea-pig oesophageal muscularis mucosae was greater than the potency at M₃ receptors in rat oesophageal muscularis mucosae (Eglen & Montgomery, unpublished observations). This disparity suggests that the M₃ receptor reserves differ between the two tissues; a hypothesis supported by the alkylation studies, in which 4-DAMP mustard, at 40 nM, virtually abolished the response. In tissues in which the M₃ receptor reserve is higher, such as guinea-pig ileum, oesophagus or trachea, 40 nM 4-DAMP mustard caused smaller shifts in the concentration-effect curve to muscarinic receptor agonists, with only minor depressions in maxima (Reddy *et al.*, 1995; Watson *et al.*, 1995b; 1995c). Detection of a low M₃ receptor reserve is important, in the present study, since it demonstrates that alkylation conditions can be achieved in rat oesophageal muscularis mucosae that extensively (and selectively) deplete the muscarinic M₃ receptor population. In this respect the rat oesophageal muscularis mucosae, and perhaps other smooth muscles with low M₃ reserves, are useful models to explore a functional role for M₂ receptors, since a functional M₂ population can be more easily unmasked.

Relaxation studies

Similar relaxant potencies for 5-HT were seen in tissues precontracted with either U46619 or oxotremorine M. However, isoprenaline was slightly, but significantly more potent in tissues precontracted with U46619, in comparison to those precontracted with oxotremorine M. This difference in isoprenaline relaxant potency resembles the situation in guinea-pig trachea or human bronchus, where the potency of isoprenaline is higher in preparations precontracted with histamine or leukotriene D₄ (Torphy, 1984; van Amsterdam *et al.*, 1989; Watson *et al.*, 1995a), than with a muscarinic receptor agonist. These data have been taken to suggest that muscarinic receptors offset the relaxant potency of isoprenaline (see Eglen *et al.*, 1994a, for review). In the present study, muscarinic M₂ receptors appeared to be specifically uninvolved, since methoctramine (1 μ M) failed to augment the potency of either 5-HT or isoprenaline. This again differs from studies in guinea-pig or canine trachea (Fernandes *et al.*, 1992; Watson & Eglen, 1994) but supports data in bovine trachea (Roffel *et al.*, 1995). It is, however, more probable that the lower developed tension in tissues precontracted with U46619 contributed to the higher isoprenaline relaxant potency. This explanation, however, does not explain the similarities in relaxant potency of 5-HT in tissues precontracted with the two spasmogens. Additional studies are therefore required to explore these small differences in isoprenaline potency.

Using this approach, therefore, a role for muscarinic receptors in the modulation of relaxations to either 5-HT or

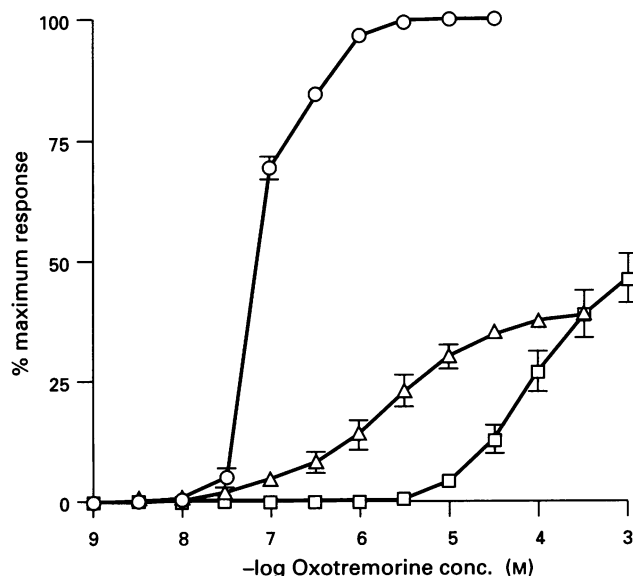


Figure 4 Concentration-effect curves for oxotremorine M in tissues precontracted with U46619 (0.7 μ M) and relaxed with isoprenaline (0.1 μ M). Concentration-effect curves are shown in the absence (Δ) and presence (\square) of methoctramine (1 μ M). These curves were established in tissues in which the muscarinic M₃ receptor population was selectively alkylated (see Methods). For comparative purposes, the control concentration-effect curve is shown (\circ). Points shown are means and vertical lines indicate s.e.mean.

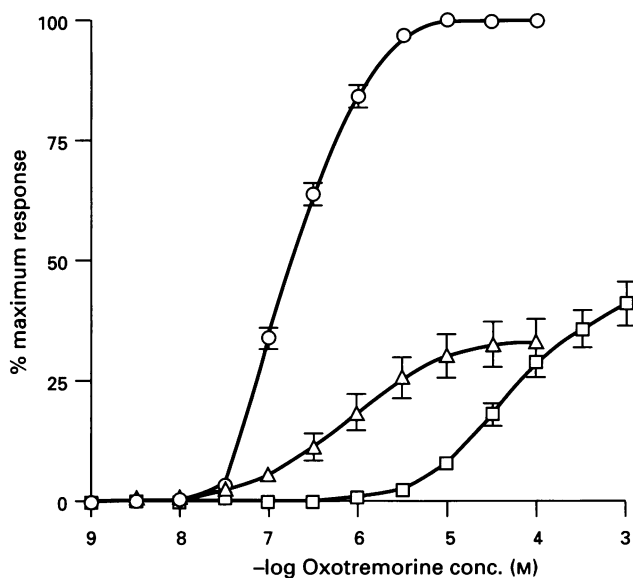


Figure 5 Concentration-effect curves for oxotremorine M in tissues precontracted with U46619 (0.7 μ M) and relaxed with 5-hydroxytryptamine (5-HT; 0.1 μ M). Concentration-effect curves are shown in the absence (Δ) and presence (\square) of methoctramine (1 μ M). These curves were established in tissues in which the muscarinic M₃ receptor population was selectively alkylated (see Methods). For comparative purposes, the control concentration-effect curve is shown (\circ). Points shown are means and vertical lines indicate s.e.mean.

isoprenaline was not disclosed. However, it is possible that the intact muscarinic M₃ receptor population masked the involvement of muscarinic M₂ receptors, as described in guinea-pig ileum (Thomas *et al.*, 1993; Reddy *et al.*, 1995). Consequently, further experiments were undertaken in which the muscarinic M₃ receptor population was alkylated by 4-DAMP mustard.

Functional antagonism studies

In the presence of U46619 (to contract the tissues) and 5-HT or isoprenaline (to provide full relaxation via elevations in intracellular cyclic AMP), oxotremorine M induced concentration-dependent reconstrictions. The maximal reconstriction to oxotremorine M, while fully reversing the contracture to U46619, remained less than that mediated by direct muscarinic M₃ receptor activation. However, the magnitude of the response was markedly larger than those seen in the post alkylation, M₃ receptor-mediated curves (see above). These data provide some evidence that M₂ rather than M₃ receptors mediated the response. Evidence to implicate further M₂ receptors was obtained from studies with methoctramine. Thus, the reconstrictions were surmountably antagonized by methoctramine with pK_B values (7.5, 7.6) consistent with activation of muscarinic M₂ receptors and inconsistent with activation of muscarinic M₃ receptors (pK_B = 6.2; Table 1).

These studies, taken together, provide a further example of an interaction between muscarinic M₂ receptors and β₃ adrenoceptors. Moreover, these data extend the current literature, by showing that muscarinic M₂ receptors functionally antagonize relaxant responses to 5-HT₄ receptor activation. This demonstration of a role for M₂ receptors in rat oesophageal muscularis mucosae differs from studies in guinea-pig oesophageal muscularis mucosae, in which M₂ receptors were not unmasked by alkylation (Watson *et al.*, 1995c). Although the reason for this difference is unclear, species variation clearly influences detection of a role for this muscarinic receptor subtype, even within the same tissue type. This conclusion underlines the importance of conducting future studies of this kind in human gastrointestinal smooth muscle.

Some additional points may be worth emphasizing. First, functional antagonism by muscarinic M₂ receptors was apparent only when the muscarinic M₃ receptors were extensively alkylated. However, this was not apparent when comparing relaxant potencies with different spasmogens, particularly with 5-HT. Moreover, selective M₂ receptor antagonism did not

augment the relaxant potencies to 5-HT or isoprenaline. One may conclude, therefore, that differences in the relaxant potency *per se* of relaxant agonists provide somewhat ambiguous evidence for an involvement of muscarinic M₂ receptors. Selective alkylation of muscarinic M₃ receptors, in contrast, may be a preferred technique to unmask a functional role for muscarinic M₂ receptors (see Ehlert & Thomas, 1995 for further discussion).

A second point concerns the muscarinic M₂ and M₃ receptor reserves in this tissue. It has already been discussed that the reserve associated with the muscarinic M₃ receptor was low, a conclusion supported by the small difference between contractile potency (pEC₅₀ = 7.1) and affinity for oxotremorine M (pK_A = 5.8). Furthermore, the potency of oxotremorine M, in terms of the muscarinic M₂ mediated reconstriction (pEC₅₀ = 5.7) was lower and, in fact, similar to the affinity (pK_A = 5.8), thus indicating complete absence of reserve. However, the low potency of oxotremorine M at the M₂ mediated reconstrictions may also reflect functional antagonism by the relaxations induced by 5-HT or isoprenaline. It is difficult, therefore, to make firm conclusions concerning the magnitude of the M₂ receptor reserve associated with reconstriction. Finally, these data, although indicating that both muscarinic M₂ and M₃ receptors are functional in rat oesophagus, provide no information regarding their relative proportions. Additional studies are clearly required to resolve this issue and, indeed, to demonstrate that muscarinic M₂ receptors serve to inhibit adenylyl cyclase in this tissue.

Conclusion

These data show that activation of muscarinic M₂ receptors in rat oesophageal muscularis functionally opposes relaxant responses to 5-HT₄ and β₃-adrenoceptor activation. The isolated oesophageal muscarinic mucosae of rat, therefore, provides a useful model with which to explore the interaction of muscarinic M₂ receptors with several receptors mediating cyclic AMP-induced relaxation.

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