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Characterization of the interaction between muscarinic M_2 receptors and β-adrenoceptor subtypes in guinea-pig isolated ileum

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1 Contraction of guinea-pig ileum to muscarinic agonists is mediated by M_3 receptors, even though they account for only 30% of the total muscarinic receptor population. The aim of this study was to characterize the biochemical and functional effects of stimulation of the predominant M_2 muscarinic receptor (70%) and to investigate the hypothesis that M_2 receptors specifically oppose β-adrenoceptor-mediated effects in the ileum.

2 In guinea-pig ileal longitudinal smooth muscle slices, isoprenaline, a non-selective β-adrenoceptor agonist, and BRL 37344 (sodium-4-[2-[2-hydroxy-2-(3-chlorophenyl)ethylamino]propyl]-phenoxyacetate sesquihydrate), a β_3-adrenoceptor selective agonist, increased cyclic AMP accumulation with $-\log EC_{50}$ values of 6.6 ± 0.1 and 5.8 ± 0.1 respectively. Maximal stimulation by BRL 37344 (10 μM) was $26.4 \pm 5.2\%$ of that observed with isoprenaline (10 μM). Isoprenaline (10 μM)-stimulated cyclic AMP accumulation was significantly, but not completely, inhibited by propranolol (5 μM), with a propranolol-resistant component of $28.2 \pm 6.8\%$ of the maximal stimulation to isoprenaline. In contrast, basal and BRL 37344 responses were resistant to this antagonist. These data provide evidence that both β_1- and β_3-adrenoceptors activate adenylyl cyclase in guinea-pig ileum.

3 Isoprenaline (10 μM)-stimulated cyclic AMP accumulation was inhibited ($67.4 \pm 0.9\%$) by the muscarinic agonist (+)-*cis*-dioxolane ($-\log EC_{50} = 7.3 \pm 0.1$). The rank order of antagonist affinities against the (+)-*cis*-dioxolane response was ($-\log K_B$ values in parentheses): atropine (9.0 ± 0.2) > methoctramine (7.1 ± 0.1) > *p*-fluoro-hexa-hydrosilaphenidol (*p*-F-HHSiD; 6.5 ± 0.2) ≥ pirenzepine (6.3 ± 0.2). (+)-*cis*-dioxolane also significantly inhibited BRL 37344 (10 μM; $56.5 \pm 2.4\%$) stimulated cyclic AMP accumulation. These data suggest that M_2 receptors mediate inhibition of cyclic AMP accumulation in response to both β_1- and β_3-adrenoceptor stimulation in guinea-pig ileum.

4 5-Hydroxytryptamine (5-HT), vasoactive intestinal peptide, prostaglandins E_2 and E_1, all at 10 μM, significantly increased cyclic AMP accumulation. (+)-*cis*-Dioxolane (10 μM) inhibited both basal and agonist-induced cyclic AMP accumulation. Thus the inhibitory effect of M_2 receptor agonism does not appear to be restricted to β-adrenoceptor-stimulated cyclic AMP accumulation.

5 The potential for involvement of activation of M_2 receptors on responses to β-adrenoceptor agonists was also studied functionally. Selective M_3 receptor alkylation was achieved by pretreatment of tissues with 4-DAMP mustard (40 nM), in the presence of methoctramine (1 μM; to protect M_2 receptors). After washing, tissues were pre-contracted with histamine (0.3 μM) and relaxed with isoprenaline (0.6 μM). Under these conditions, oxotremorine M caused concentration-dependent contractions ($-\log EC_{50}$ of 7.8 ± 0.1), that were surmountably antagonized by methoctramine (1 μM) with a $-\log K_B$ estimate of 7.4 ± 0.1. Similar observations were seen versus relaxation produced by BRL 37344 (1 μM), where the $-\log K_B$ value for methoctramine was 7.8 ± 0.2. These data suggest that M_2 receptors mediate a functional inhibition of relaxant responses to isoprenaline and BRL 37344.

6 These findings are consistent with β_1- and β_3-adrenoceptors coupling to stimulation of adenylyl cyclase in guinea-pig ileum; a response that is inhibited by M_2 receptor stimulation. Concordantly, M_2 receptor stimulation also inhibits relaxation to both β_1- and β_3-adrenoceptor stimulation. These results implicate M_2 receptors in the modulation of sympathetic control of ileal motility.

Keywords: M_2 muscarinic receptors; β-adrenoceptors; β_3-adrenoceptors; guinea-pig ileum; cyclic AMP; receptor alkylation

Introduction

Several smooth muscles, including guinea-pig ileum, contain heterogeneous populations of muscarinic receptors (see Eglen *et al.*, 1994 for a review). Radioligand binding studies have demonstrated that the majority of guinea-pig ileal muscarinic receptors are of the M_2 subtype (~70%), while a minority are of the M_3 subtype (~30%), with no measurable quantities of M_1 or M_4 receptors (Giraldo *et al.*, 1987; Ford *et al.*, 1991). Activation by muscarinic agonists of the minority M_3 receptor population is associated with longitudinal and circular smooth muscle contraction and electrolyte transport (Kachur *et al.*, 1990; Ford *et al.*, 1991). Inactivation of M_2 receptors by either selective alkylation (Eglen & Harris, 1993) or by treatment with pertussis toxin, which inactivates the guanine nucleotide regulatory protein G_i (Eglen *et al.*, 1988), has minimal effects on guinea-pig ileal contractile responses to muscarinic agonists. These observations suggest that M_2 receptors are not directly involved in contraction; thus, a role for the majority M_2 receptor population in ileal tissue is unclear.

In some smooth muscle tissues, such as guinea-pig uterus, M_2 receptors directly mediate contractile responses to muscarinic agonists (Eglen *et al.*, 1989; Doods *et al.*, 1993). However, in general, smooth muscle contractile tone can be relaxed by increased levels of adenosine 3':5'-cyclic monophosphate (cyclic AMP) (Berridge, 1975). Consequently, as

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M_2 receptors have been shown to inhibit adenylyl cyclase activity in canine colon, rat ileum and bovine trachea (Zhang & Buxton, 1991; Griffin & Ehlert, 1992; Challiss *et al.*, 1993), it has been proposed that M_2 receptors may modulate ileal relaxation. In guinea-pig and canine tracheal smooth muscle the relaxant potency of isoprenaline in tissues pre-contracted with muscarinic agonists, is less than in tissues precontracted with histamine (Russell, 1984; Watson & Eglen, 1994). Furthermore, the relaxant potency of isoprenaline in tracheal tissues is enhanced by either selective M_2 receptor antagonism or pertussis toxin pretreatment (Fernandes *et al.*, 1992; Mitchell *et al.*, 1993; Watson & Eglen, 1994). Together, these findings indicate an inhibitory effect of M_2 receptors on relaxant responses to β-adrenoceptor stimulation.

Although no direct contractile response to M_2 receptor activation can be demonstrated in guinea-pig ileum (Eglen & Harris, 1993), an indirect influence on contraction via inhibition of β-adrenoceptor-mediated relaxation remains possible. In support of this proposal, Griffin & Ehlert (1992) demonstrated, in rat ileum, that M_2 receptors specifically inhibit β-adrenoceptor-mediated cyclic AMP accumulation. In contractile studies, Thomas *et al.* (1993) have shown that, in guinea-pig ileum, after selective M_3 receptor alkylation, M_2 receptor stimulation offsets β-adrenoceptor-mediated relaxation; an effect which manifests as re-contraction. Non-selective β-adrenoceptor agonists mediate relaxation of guinea-pig ileum via activation of both β_1- and β_3-adrenoceptor subtypes (Grassby & Broadley, 1984; Mian *et al.*, 1984; Bond & Clarke, 1988). The contribution of distinct β-adrenoceptor subtypes involved in the interaction between M_2 receptors and β-adrenoceptors in this tissue has not been addressed.

The aim of this study was to investigate the effect of stimulation of β_1- and β_3-adrenoceptors on guinea-pig ileal adenylyl cyclase activity and to characterize the effect thereupon of muscarinic receptor agonism. A further aim was to investigate the functional interactions occurring between muscarinic M_2 receptors and β_1- and β_3-adrenoceptors at the level of contraction in guinea-pig ileum. A preliminary account of this work has been presented to the British Pharmacological Society (Reddy *et al.*, 1994).

Methods

Measurement of [^{3}H]-cyclic AMP accumulation

Male guinea-pigs (300–400 g) were killed by carbon dioxide asphyxiation and ileal tissue removed into modified Krebs-Henseleit solution (containing mM: KCl 4.6, KH_2PO_4 1.2, $MgSO_4$ 1.2, NaCl 118.2, glucose 10.0, $NaHCO_3$ 24.8 and $CaCl_2$ 2.5). Longitudinal smooth muscle from the ileum was isolated by rubbing the ileum with a cotton swab (Rang, 1964). Slices were then prepared by cross-chopping the tissue on a McIlwain tissue chopper and placed into gassed (95% O_2/5% CO_2) Krebs solution. The slices were then labelled with 1 μM [^{3}H]-adenine (2 μCi ml^{-1}) and subsequently washed, as described by Griffin & Ehlert (1992).

[^{3}H]-adenine labelled slices, in gassed Krebs solution containing 3-isobutyl-1-methylxanthine (1 mM, final assay concentration), were pre-incubated in either the presence or absence of antagonists for 10 min at 37°C. This was followed by a further pre-incubation for 3 min at 37°C in the presence or absence of muscarinic agonists as indicated. In most studies the non-selective muscarinic agonist (Grana *et al.*, 1986) (+)-*cis*-dioxolane was used. Forskolin (0.1 μM) was then added to each sample unless otherwise indicated (this significantly increased the stimulation of cyclic AMP accumulation by isoprenaline (10 μM), above basal levels from 2.5 to 3.1 fold). The assay was started by the addition of β-adrenoceptor agonist (or other agonist as indicated) giving a total assay volume of 300 μl. The accumulation of cyclic AMP was terminated after 10 min, shaking at 37°C, by the addition of 30 μl of 2.2 N HCl containing [^{14}C]-cyclic AMP (~1000 d.p.m./tube). In initial studies, idazoxan (3 μM) was included to minimize the potential for effects of isoprenaline at α_2-adrenoceptors (Drew, 1978). However, as no significant effect of idazoxan (3 μM) on basal or isoprenaline-stimulated cyclic AMP accumulation was observed (results not shown), it was omitted from further studies.

Chromatographic separation of [^{3}H]-cyclic AMP from substrate and other adenine metabolites was achieved by the method of Alvarez & Daniels (1992). An aliquot was removed from each tube as an estimate of the total radioactivity present and the remaining contents applied to columns containing acidic alumina (1.3 g). The columns were washed with 12 ml HCl (0.005 N) followed by 1.5 ml ammonium acetate (0.1 mM; unbuffered) and the eluant discarded. The columns were placed over scintillation vials and [^{3}H]-cyclic AMP was eluted by the addition of 4.5 ml ammonium acetate (0.1 mM; unbuffered). After addition of scintillant, quantitation of radioactivity was determined by liquid scintillation counting (Packard Tri-Carb). Assays were carried out in triplicate and results were corrected for [^{14}C]-cyclic AMP recovery from the alumina columns and expressed as a percentage of total [^{3}H]-adenine counts converted to [^{3}H]-cyclic AMP.

Contractile studies

Whole ileum was obtained from guinea-pigs as described above, and placed into gassed (95% O_2/5% CO_2), modified Krebs solution (the composition of the Krebs solution was as above except that 1.8 mM $CaCl_2$ was used). Ileal segments (~2 cm in length) were suspended, under 0.5 g tension, in 10 ml organ baths containing gassed Krebs solution (pH 7.4, 37°C) and changes in isometric tension recorded. All preparations were equilibrated for 60 min prior to construction of a concentration-response curve to oxotremorine M, a non-selective muscarinic agonist (Grana *et al.*, 1986), and then treated as described below before performing a second concentration-response curve to oxotremorine M. Antagonists when used were equilibrated with tissues for 60 min before construction of the second curve to oxotremorine M.

Certain conditions have been described as optimal for revealing indirect contractile responses to M_2 receptor-activation: selective alkylation of M_3 receptors, followed by induction of tone by histamine and subsequent relaxation with isoprenaline, prior to addition of a muscarinic agonist (Thomas *et al.*, 1993). Under these conditions a reversal of the isoprenaline-induced relaxation can be seen. In the present studies a similar protocol was used and preliminary experiments, to optimize these conditions, were undertaken. For the sake of clarity, these are discussed separately. In addition, in order to adhere to the protocol developed by Thomas *et al.* (1993), oxotremorine M rather than *cis*-dioxolane was employed. Both agonists exhibit a high intrinsic efficacy and do not discriminate between muscarinic receptor subtypes.

Effects of histamine precontraction and β-adrenoceptor relaxation on the contractile response to muscarinic receptor stimulation

Tissues were washed at 15 min intervals for 60 min and then precontracted with histamine (0.3 μM) and relaxed with isoprenaline (0.6 μM). When a steady level of isometric tension was attained under these conditions, a second concentration-response curve to oxotremorine M was constructed.

In some studies, the above protocol was repeated but in the presence of histamine (1 μM) and the β_3-adrenoceptor agonist BRL 37344 (Arch *et al.*, 1964) at 1 μM. All contractile studies with BRL 37344 were carried out in the presence of propranolol (5 μM) to antagonize β_1-adrenoceptors. In order to achieve the same precontractile response, a histamine con-

centration of 1 μM was used in this study since an inhibitory effect of propranolol on the histamine contraction was observed (data not shown; see also Bond & Clarke, 1988).

Effect of selective M_3 receptor alkylation on the contractile response to muscarinic receptor stimulation

Tissues were washed and exposed to the slightly M_3-selective irreversible receptor antagonist 4-DAMP mustard (40 nM), for 60 min, in the presence of methoctramine (1 μM). Tissues were then exposed to sodium sulphite (0.5 mM) for an additional 10 min to inactivate the mustard. Methoctramine was present to protect M_2 receptors from alkylation, thereby enhancing the selective M_3 inactivation by 4-DAMP mustard. Tissues were then washed at 10 min intervals for 90 min to remove both 4-DAMP mustard and methoctramine and a second concentration-response curve to oxotremorine M established.

Effect of selective M_3 receptor alkylation, histamine precontraction and β-adrenoceptor relaxation on the contractile response to muscarinic receptor stimulation

A schematic outline of the protocol used is shown in Figure 1. Concentration-response curves to oxotremorine M were established, tissues washed and then exposed to 4-DAMP mustard (40 nM) under the conditions described above. Tissues were then exposed to histamine (0.3 μM), followed by isoprenaline (0.6 μM), and a second concentration-response curve to oxotremorine M constructed. To investigate the potential interaction between M_2 receptors and β_3-adrenoceptors, the above protocol was repeated in separate tissues with histamine (1 μM) and BRL 37344 (1 μM).

Chemicals

The following compounds were used: vasoactive intestinal peptide was from Bachem Inc. (Torrance, CA, U.S.A.). Prostaglandins E_1 and E_2 were from Cayman Chemical Company (Ann Arbor, MI, U.S.A.). Alumina (Acidic, Activity grade I) was from ICN Biomedicals GmbH (Eschwege, Germany). [2-^{3}H]-Adenine (specific activity 15–25 Ci mmol^{-1}) and [8-^{14}C]-cyclic AMP (specific activity 50–60 mCi mmol^{-1}) were obtained from Moravek Biochemicals Inc. (Brea, CA, U.S.A.). Atropine sulphate, (+)-*cis*-dioxolane (L-(+)-*cis*-2-methyl-4-trimethylammonium methyl-1,3-dioxolane iodide, a 60:40 mixture of *cis*:*trans*), 4-DAMP mustard (4-diphenylacetoxy-N-(2-chloroethyl) piperidine), *p*-F-HHSiD (*para*-fluoro-hexa-hydro-siladiphenidol), histamine, idazoxan hydrochloride, methoctramine tetrachloride, oxotremorine methiodide (oxotremorine M) and pirenzepine dihydrochloride were obtained from Research Biochemicals Inc. (Natick, MA, U.S.A.). Adenine, ammonium acetate, 5-HT, 3-isobutyl-1-methylxanthine, (±)-isoprenaline sulphate, forskolin, (±)-propranolol hydrochloride and sodium sulphite were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). BRL 37344 (sodium-4-[2-[2-hydroxy-2-(3-chlorophenyl)ethylamino] propyl]-phenoxyacetate sesquihydrate (**RR.SS** diastereoisomer) was synthesized by the Institute of Organic Chemistry (Syntex Discovery Research, Palo Alto, CA, U.S.A.).

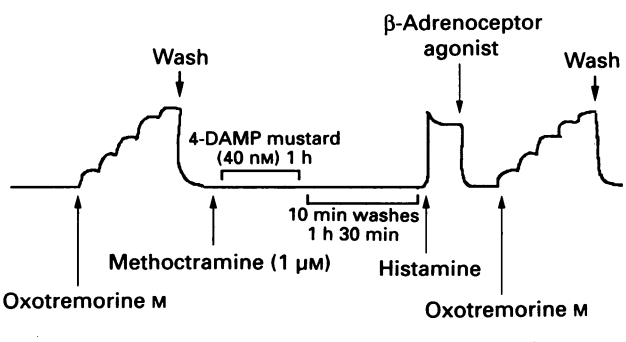

Figure 1 Schematic representation of the protocol used. Concentration-response curves to oxotremorine M were constructed. Tissues were then alkylated with 4-DAMP mustard in the presence of M_2 receptor protection and the response to oxotremorine M assessed in the presence of histamine and β-adrenoceptor agonism.

Solutions were prepared in distilled water with the following exceptions: stock solutions of BRL 37344, *p*-F-HHSiD, prostaglandins E_1 and E_2 were prepared in ethanol. Isoprenaline was dissolved in ascorbic acid (5 mM). Vasoactive intestinal peptide and 4-DAMP mustard were prepared in distilled water and subsequently acidified with dilute acetic acid.

Data analysis

Agonist potencies ($-\log EC_{50}$) were estimated using the relationship of Parker & Waud (1971) by a non-linear iterative curve fitting procedure (Kaleidagraph, Synergy software, Reading, PA, U.S.A.). Antagonist affinities ($-\log K_B$ values) were estimated as described by the relationship of Furchgott (1972). All values quoted are mean ± s.e.mean. Statistical significance was determined by Student's unpaired *t* test, a probability level of $P<0.05$ being considered significant.

Results

β-Adrenoceptor stimulation of [^{3}H]-cyclic AMP accumulation

Isoprenaline caused concentration-dependent increases in cyclic AMP accumulation above basal levels in longitudinal smooth muscle slices (Figure 2a). The potencies ($-\log EC_{50}$) of isoprenaline and BRL 37344 were 6.6 ± 0.1 and 5.8 ± 0.3, respectively ($n = 5$). The maximal stimulation of cyclic AMP accumulation by BRL 37344 was 26.4 ± 5.2% of the maximal response to isoprenaline. Propanolol (1 μM) caused a dextral shift in portions of the concentration-response curve to isoprenaline and the resultant curve was best described by iterative curve fitting to a two-site model (Figure 2a). This analysis revealed a propanolol-resistant component, with a maximum comprising 19 ± 9% of the total response to isoprenaline. The magnitude of the propranolol-resistant component was similar to the response to BRL 37344 alone (26.4 ± 5.2% of the maximum response to isoprenaline). The apparent affinity ($-\log K_B$) of propanolol on the sensitive component of the isoprenaline-response curve was approximately 8.3. Propanolol at a concentration of 5 μM did not significantly alter either basal or BRL 37344 (10 μM)-stimulated cyclic AMP levels (Figure 2b). By contrast, the response to isoprenaline (10 μM) was significantly reduced by 5 μM propranolol, but with a resistant component of 28.2 ± 6.8% of the maximal isoprenaline stimulation (5 μM; Figure 2b).

Effect of muscarinic agonists on β-adrenoceptor stimulated [^{3}H]-cyclic AMP accumulation

(+)-*cis*-Dioxolane (10 μM) significantly inhibited basal, BRL 37344 (10 μM) and isoprenaline (10 μM)-stimulated cyclic AMP accumulation (Figure 3a). Similar results were obtained with oxotremorine M (10 μM) which is also a non-selective muscarinic agonist (results not shown). The inhibition by (+)-*cis*-dioxolane of isoprenaline (67%) and BRL 37344 (56%) stimulated cyclic AMP accumulation was significantly greater than the inhibitory effect on basal (45%). In the absence of forskolin (0.1 μM), (+)-*cis*-dioxolane (10 μM) significantly reduced the cyclic AMP response to basal, BRL 37344 and isoprenaline-stimulated cyclic AMP accumulation (data not shown).

(+)-*cis*-Dioxolane produced a concentration-dependent

inhibition ($-\log EC_{50}$ of 7.3 ± 0.1) of isoprenaline (10 μM)-stimulated cyclic AMP accumulation (Figure 3b). Several antagonists were used to characterize the muscarinic receptor subtype responsible for this inhibitory effect: atropine (10 nM), pirenzepine (1 μM), methoctramine (1 μM) and *p*-F-HHSiD (3 μM) as shown in Figure 3b and Table 1. All antagonists caused parallel dextral shifts in concentration-response curves to (+)-*cis*-dioxolane, with no significant effect on either basal cyclic AMP accumulation or the maximal response to (+)-*cis*-dioxolane. The rank order of apparent antagonist affinities ($-\log K_B$) at receptors mediating the (+)-*cis*-dioxolane response was atropine > methoctramine > *p*-F-HHSiD ≥ pirenzepine (Table 1). Due to the small amount of cyclic AMP accumulation induced by both basal and BRL 37344-stimulation, the muscarinic receptor subtype involved in inhibition of these responses was not investigated.

Specificity of the inhibitory effect of (+)-cis-dioxolane on [^{3}H]-cyclic AMP accumulation

To investigate the specificity of the inhibitory effect of (+)-*cis*-dioxolane on cyclic AMP accumulation, other agents known to stimulate adenylyl cyclase were studied. Thus, 5-HT, vasoactive intestinal peptide, prostaglandin E_1 and E_2, all at 10 μM, caused significant increases in cyclic AMP

Table 1 Muscarinic receptor antagonist affinities against (+)-*cis*-dioxolane inhibition of isoprenaline (10 μM)-stimulated cyclic AMP accumulation

Antagonist	*Concentration*	*Affinity* (estimated $-\log K_B$)	n
Atropine	10 nM	9.0 ± 0.2	4
Methoctramine	1 μM	7.1 ± 0.1	5
p-F-HHSiD	3 μM	6.5 ± 0.2	5
Pirenzepine	1 μM	6.3 ± 0.2	5

Antagonists were studied at single concentrations and $-\log K_B$ values estimated by the method of Furchgott (1972). Values are means ± s.e.mean.

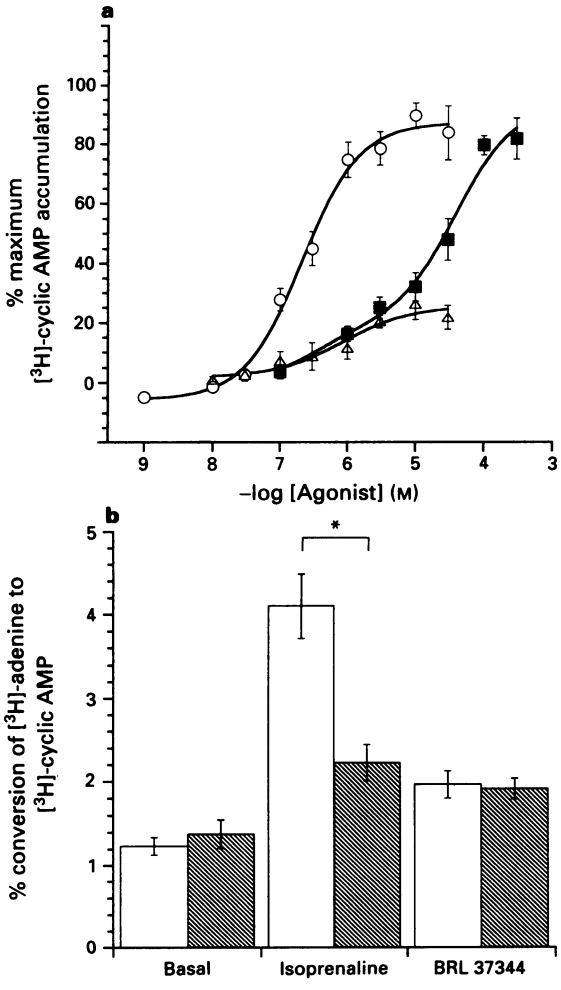

Figure 2 (a) Accumulation of [^{3}H]-cyclic AMP in longitudinal smooth muscle slices in response to β-adrenoceptor agonism: stimulation by isoprenaline in the absence (○) and presence (■) of propranolol (1 μM), or stimulation by BRL 37344 (△). Results are expressed as a percentage of maximal stimulation of isoprenaline (basal levels subtracted). Values are the mean ± s.e.mean, $n = 5$. (b) The effect of propranolol on basal and β-adrenoceptor stimulated [^{3}H]-cyclic AMP accumulation in longitudinal smooth muscle slices. Histograms showing cyclic AMP accumulation in the presence (hatched columns) and absence (open columns) of propranolol (5 μM) on basal, isoprenaline (10 μM) and BRL 37344 (10 μM)-stimulated levels. Values are the mean ± s.e.mean, $n = 5$–6, $^*P < 0.05$.

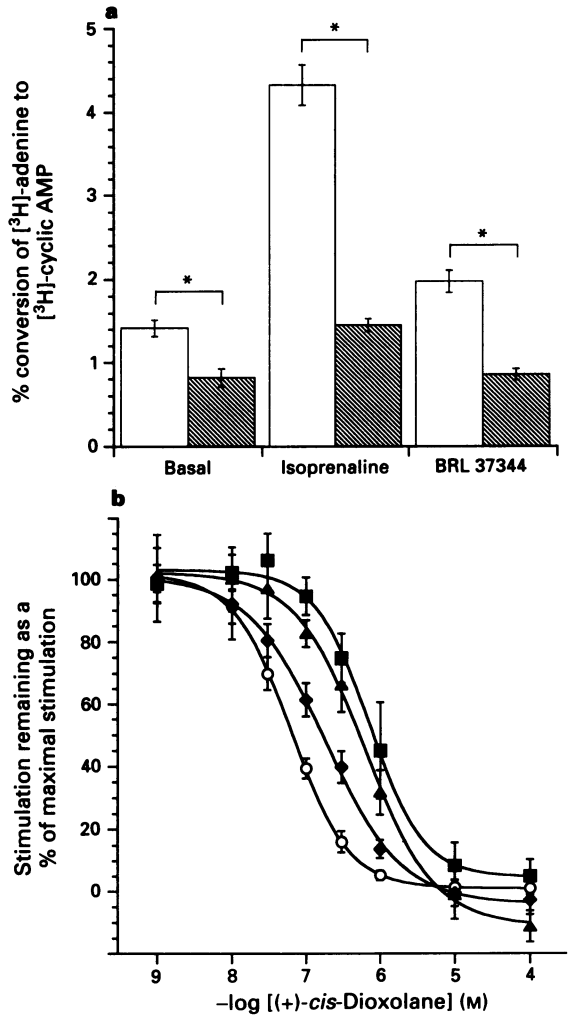

Figure 3 (a) The effect of the presence (hatched columns) and absence (open columns) of (+)-*cis*-dioxolane (10 μM) on basal, isoprenaline (10 μM) and BRL 37344 (10 μM)-stimulated [^{3}H]-cyclic AMP accumulation in longitudinal smooth muscle slices. Values are the mean ± s.e.mean, $n = 5$, $^*P < 0.05$. (b) The effect of muscarinic antagonism on (+)-*cis*-dioxolane inhibition of isoprenaline (10 μM) stimulated [^{3}H]-cyclic AMP accumulation in longitudinal smooth muscle slices. Slices were incubated with isoprenaline (10 μM) and increasing concentrations of (+)-*cis*-dioxolane in the absence (○) and presence of pirenzepine (◆: 1 μM), *p*-F-HHSiD (△: 3 μM) and methoctramine (■: 1 μM). Results are expressed as a percentage of maximal stimulation with isoprenaline (10 μM) with basal levels subtracted. $-\log K_B$ estimates calculated for these antagonists are shown in Table 1.

Table 2 Effect of muscarinic agonism with (+)-*cis*-dioxolane on basal and stimulated cyclic AMP accumulation

Condition	% conversion to cyclic AMP Control	(+)-*cis*-Dioxolane	% inhibition (+)-*cis*-Dioxolane	n
Basal	1.42 ± 0.12	0.82 ± 0.10	45.0 ± 4.0	8
BRL 37344 (10 μM)	1.98 ± 0.13	0.86 ± 0.07	56.4 ± 2.4	6
Isoprenaline (10 μM)	4.33 ± 0.24	1.46 ± 0.08	67.4 ± 0.9	8
5-HT (10 μM)	2.41 ± 0.07	1.30 ± 0.09	46.0 ± 2.3	3
PGE_1 (10 μM)	7.48 ± 0.95	2.96 ± 0.63	61.2 ± 3.7	3
PGE_2 (10 μM)	4.87 ± 0.61	1.83 ± 0.19	62.2 ± 1.7	3
VIP (10 μM)	3.20 ± 0.55	1.95 ± 0.37	38.8 ± 5.1	3

The percentage of total [^{3}H]-adenine counts converted to [^{3}H]-cyclic AMP in response to several agents, in the presence and absence of (+)-*cis*-dioxolane (10 μM); PGE_1, prostaglandin E_1; PGE_2, prostaglandin E_2; VIP, vasoactive intestinal peptide. Values are means ± s.e.mean and n = number of experiments.

Table 3 Affinity of methoctramine (1 μM) at receptors mediating ileal contractions to oxotremorine M under several conditions

Condition	Affinity (estimated $-\log K_B$)	n
Control	6.2 ± 0.2	6
Histamine/isoprenaline	6.5 ± 0.1	6
Histamine/BRL 37344	6.5 ± 0.1	6
4-DAMP mustard	6.4 ± 0.1	5
4-DAMP must/histamine/isoprenaline	7.4 ± 0.1*	6
4-DAMP must/histamine/BRL 37344	7.8 ± 0.2*	5

Histamine/isoprenaline; tissues contracted with histamine (0.3 μM) and relaxed with isoprenaline (0.6 μM) prior to contraction with oxotremorine M. Histamine/BRL 37344; tissues contracted with histamine (1 μM) and relaxed with BRL 37344 (1 μM) prior to contraction with oxotremorine M. 4-DAMP mustard; tissues treated with 4-DAMP mustard (described in Methods) before contraction with oxotremorine M. Values are means ± s.e.mean. *$-\log K_B$ values are significantly greater than control value.

accumulation in longitudinal smooth muscle slices, all of which were inhibited by (+)-*cis*-dioxolane (10 μM; Table 2).

Effects of histamine precontraction and β-adrenoceptor relaxation on the contractile response to oxotremorine M

In contractile studies the potency ($-\log EC_{50}$) of oxotremorine M was 7.7 ± 0.1 and the maximal response was 3.1 ± 0.2 g (n = 6). Methoctramine (1 μM) reduced the potency of oxotremorine M to 7.3 ± 0.1, with no significant effect on maximum contraction. The apparent affinity of methoctramine estimated against contraction to oxotremorine M ($-\log K_B$ value) was 6.2 ± 0.2 (n = 6; Table 3). In tissues precontracted to histamine (0.3 μM) and relaxed with isoprenaline (0.6 μM), there was no significant effect on either potency (7.7 ± 0.1) or the maximal response to oxotremorine M (2.6 ± 0.3 g, n = 6; Figure 4a). Methoctramine antagonized the response to oxotremorine M in the presence of histamine and isoprenaline resulting in a reduced potency (7.1 ± 0.1) with no significant effect on the maximal response (2.8 g ± 0.2 g) (Figure 4a; Table 3). Likewise, the potency and maximal contractile response to oxotremorine M were unaltered by pretreatment with histamine (1 μM) and BRL 37344 (1 μM). (These studies, using BRL 37344 were conducted in the presence of 5 μM propranolol, to antagonize β_1-adrenoceptors.) Methoctramine (1 μM) had no significant effect on the magnitude of the responses to histamine, isoprenaline or BRL 37344 under the conditions discussed above. There was no significant difference in the apparent affinity of methoctramine (1 μM) between control contractions to oxotremorine M and on the contractile responses in the presence of histamine and isoprenaline or BRL 37344 (Table 3).

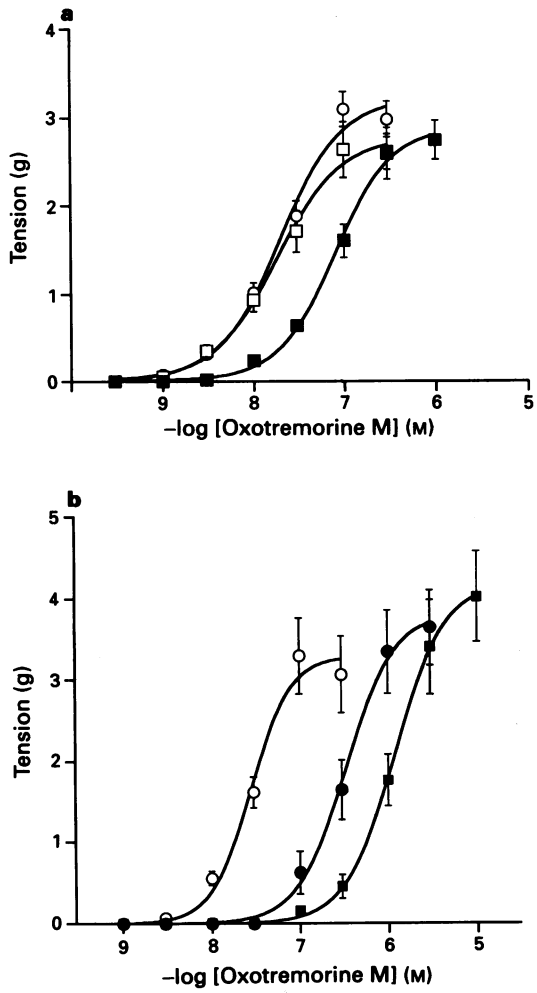

Figure 4 (a) Contractile responses of guinea-pig ileum to oxotremorine M in control tissues (○) and responses to oxotremorine M in tissues pre-exposed to histamine (0.3 μM) and isoprenaline (0.6 μM) in the absence (□) and presence (■) of methoctramine (1 μM). Values are means ± s.e.mean (n = 6). (b) Contractile responses of guinea-pig ileum to oxotremorine M in control tissues (○) and after pretreatment with 4-DAMP mustard (in the presence of M_2 receptor protection) (closed symbols). Effect of the presence (■) and absence (●) of methoctramine (1 μM) on tissues pretreated with 4-DAMP mustard (in the presence M_2 receptor protection). Values are means ± s.e.mean (n = 5).

Effects of M_3 receptor alkylation on the contractile response to muscarinic receptor stimulation

Pretreatment with 4-DAMP mustard (40 nM for 60 min, in the presence M_2 receptor protection; see Methods) significantly reduced the potency of oxotremorine M to a value

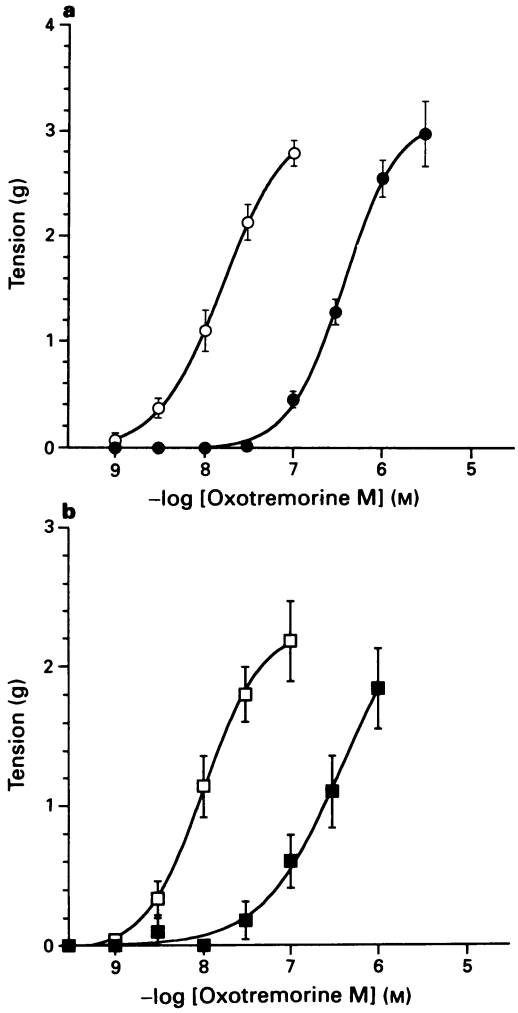

Figure 5 (a) Contractions to oxotremorine M in the absence (○) and presence of methoctramine (1 μM) (●) in guinea-pig ileum pre-exposed to 4-DAMP mustard (in the presence of M_2 receptor protection). Contractions to oxotremorine M were recorded in the presence of histamine (0.3 μM) and isoprenaline (0.6 μM). Values are means ± s.e.mean ($n = 6$). (b) Contractions to oxotremorine M in the absence (□) and presence of 1 μM methoctramine (■) in guinea-pig ileum pre-exposed to 4-DAMP mustard (in the presence of M_2 receptor protection). Contractions to oxotremorine M were recorded in the presence of histamine (1.0 μM), BRL 37344 (1.0 μM) and propranolol (5 μM). Values are means ± s.e.mean ($n = 5$).

of 6.5 ± 0.1, with no significant effect on the maximal contraction (3.6 ± 0.5 g; Figure 4b). Methoctramine (1 μM) surmountably antagonized this response, resulting in a $-\log K_B$ value of 6.4 ± 0.1 ($n = 5$, Table 3). Consequently, there was no significant difference in the affinity ($-\log K_B$) of methoctramine at muscarinic receptors mediating ileal contraction before or after alkylation by 4-DAMP mustard.

Effects of selective M_3 alkylation, histamine precontraction and β-adrenoceptor relaxation on the contractile response to muscarinic receptor stimulation

In separate tissues, the control contractile potency estimate for oxotremorine M was 7.7 ± 0.1, with maximal contraction of 3.1 ± 0.1 g ($n = 6$). In tissues alkylated with 4-DAMP mustard (in the presence of M_2 receptor protection), precontracted with histamine (0.3 μM) and relaxed with isoprenaline (0.6 μM), oxotremorine M gave a contractile potency of 7.8 ± 0.1 ($n = 6$) with a maximum response of 2.7 ± 0.1 g ($n = 6$). Methoctramine (1 μM) surmountably antagonized this concentration-response curve more potently, resulting in a potency estimate for oxotremorine M of 6.4 ± 0.1, yielding a $-\log K_B$ value of 7.4 ± 0.1 (Figure 5a; Table 3).

In separate tissues, the above experimental protocol was repeated except that, after alkylation, tissues were precontracted to histamine (1 μM) and relaxed with BRL 37344 (1 μM). Under these conditions, the potency of oxotremorine M was 8.0 ± 0.1 with a maximal response of 2.4 ± 0.3 g ($n = 5$). Methoctramine (1 μM) also surmountably antagonized this response, reducing the potency of oxotremorine M to 6.2 ± 0.1, yielding a $-\log K_B$ value of 7.8 ± 0.2 (Figure 5b; Table 3). These affinity values for methoctramine are significantly greater than the value obtained on the contractile response to oxotremorine M alone (Table 3).

Discussion

This study has investigated the effect of β-adrenoceptor stimulation on cyclic AMP accumulation in ileal longitudinal smooth muscle of guinea-pig and the inhibitory effect, thereupon, of muscarinic receptor agonism. Furthermore, the functional sequelae of this interaction were characterized by investigation of the effect of selective muscarinic M_2 receptor stimulation on β-adrenoceptor-mediated relaxation.

Isoprenaline and BRL 37344 both stimulated cyclic AMP accumulation in ileal smooth muscle slices, presumably through activation of G_s and stimulation of adenylyl cyclase (Emorine *et al.*, 1991). The potency of isoprenaline ($-\log EC_{50} = 6.6$) at receptors mediating isoprenaline-stimulated cyclic AMP accumulation is lower than the potency of isoprenaline causing relaxation of guinea-pig ileum ($-\log EC_{50} = 7.3$; data not shown). This suggests that there is a receptor reserve for isoprenaline-mediated relaxation; however, it is likely that responses to isoprenaline were mediated through both β_1- and β_3-adrenoceptors (Arch & Kauman, 1993). Further evidence to support the latter was seen in the biphasic nature of concentration-response curve to isoprenaline in the presence of propranolol (1 μM). The affinity of propranolol at the propranolol-sensitive component of the isoprenaline response (8.3) is similar to the affinity of propranolol at either β_1- or β_2-adrenoceptors (~8.7; Farmer & Levy, 1970). Although BRL 37344 was less potent (5.8) than isoprenaline on cyclic AMP accumulation, BRL 37344 is equipotent compared to isoprenaline in relaxing guinea-pig ileum and, furthermore maximally relaxes this tissue (data not shown) despite a sub-maximal stimulation of cyclic AMP accumulation (26% of that observed with isoprenaline). Similar observations regarding BRL 37344 effects on relaxation and cyclic AMP accumulation have been made in rat oesophageal smooth muscle (Ford *et al.*, 1992). In the present study the cyclic AMP response to BRL 37344 was resistant to propranolol (5 μM); this has also been observed with respect to the relaxant effect of BRL 37344 in guinea-pig ileum (Bond & Clarke, 1988). Taken together, these results support the presence of both β_1- and β_3-adrenoceptors in guinea-pig ileum as suggested by Bond & Clarke (1988), and demonstrate a positive coupling of each of these subtypes to adenylyl cyclase.

(+)-*cis*-Dioxolane inhibited basal, isoprenaline and BRL 37344-stimulated cyclic AMP accumulation. This inhibitory effect was partial and was also observed in the absence of forskolin, where the isoprenaline response was inhibited by 33% (data not shown). In rat ileum, an inhibitory effect of a similar magnitude has been reported (Griffin & Ehlert, 1992). Muscarinic receptor agonism inhibited both β_1- and β_3-stimulated cyclic AMP levels but was not specific as an inhibitory effect was also seen against activation of other receptors mediating increases in cyclic AMP. Thus, basal and cyclic AMP levels stimulated by 5-HT, vasoactive intestinal peptide and prostanoid receptors were also inhibited. This suggests that M_2 receptor-mediated inhibition of adenylyl cyclase, via G_i, modulates the responses to other agents which increase cyclic AMP levels. However, in contrast,

Griffin & Ehlert (1992) have shown that rat ileal M_2 receptors interact specifically with β-adrenoceptors. Further experiments are required to define the role of muscarinic receptors in this respect.

The potency of (+)-*cis*-dioxolane at receptors inhibiting cyclic AMP accumulation (7.3, this study) was greater than the potency previously reported at receptors stimulating phosphoinositide hydrolysis in ileum (5.6; Ford *et al.*, 1991). In order to identify the muscarinic subtype responsible for the inhibition of adenylyl cyclase a range of antagonists were investigated. It is unlikely that M_4 receptors are involved, given the lack of expression of mRNA in guinea-pig ileum (Ford *et al.*, 1991) and the low affinity for pirenzepine (this study; Caulfield, 1993). In general M_3 and M_1 muscarinic receptors do not couple preferentially to adenylyl cyclase and therefore M_2 receptors are the most likely subtype responsible for inhibition of adenylyl cyclase in this tissue. The apparent affinity of methoctramine (7.1) obtained in this study is somewhat lower than reported values for methoctramine at other M_2 receptors (7.8; Melchiorre *et al.*, 1993; Eglen *et al.*, 1988). However, the affinity was estimated from only a single concentration and may not be conclusive. Nevertheless, the rank order of antagonist affinities at receptors inhibiting cyclic AMP accumulation was atropine > methoctramine > *p*-F-HHSiD ≥ pirenzepine. From the literature this rank order of antagonist affinities is entirely consistent with the involvement of M_2 receptors (Caulfield, 1993). This conclusion is in agreement with Griffin & Ehlert (1992) who have suggested that M_2 receptors inhibit adenylyl cyclase in rat ileum.

The apparent affinity of methoctramine at receptors mediating contraction to oxotremorine M in guinea-pig ileum was 6.2, a value that is consistent with contraction mediated through M_3 receptors and which is in agreement with previous studies (Ford *et al.*, 1991). M_3 receptor inactivation by 4-DAMP mustard, in conjunction with AFDX-116 to protect M_2 receptors, has been used to study the functional role of M_2 receptors in ileum (Thomas *et al.*, 1993). In the present study the selectivity of 4-DAMP mustard was enhanced by the more selective M_2 antagonist, methoctramine (Melchiorre *et al.*, 1993), which has an M_2/M_3 fold selectivity of approximately 100, compared with the selectivity ratio for AFDX 116 (Micheletti *et al.*, 1987) which is approximately 10 fold. Selective alkylation of M_3 receptors by 4-DAMP mustard dextrally shifted concentration-response curves to oxotremorine M (~16 fold), with no decrease in maxima. Therefore, under the alkylation conditions used, there was incomplete alkylation of the M_3 receptor population. However, use of higher concentrations of 4-DAMP mustard, in an attempt to increase the proportion of M_3 receptors alkylated, would progressively alkylate more M_2 receptors (Thomas *et al.*, 1992). Nonetheless, under the conditions used in the present study, the population of M_3 receptors was reduced sufficiently to reveal a role for M_2 receptors, as described below. After partial M_3 receptor alkylation, contractile responses were antagonized by methoctramine with an affinity of 6.4, suggesting that, after alkylation, the contractile response was still mediated by M_3, and not M_2 receptors. Clearly, even after selective, albeit incomplete, M_3 receptor alkylation and *in the absence of β-adrenoceptor stimulation*, a contractile role for M_2 receptors was absent.

Consequently, studies were also performed using M_3 receptor inactivation followed by relaxation of histamine-induced tone with β-adrenoceptor agonists. Preliminary studies showed that, in the absence of M_3 receptor alkylation, there was no significant effect of pretreatment with histamine and isoprenaline or BRL 37344 on the subsequent responses to oxotremorine M. This showed that functional antagonism of the contractile response to oxotremorine M did not occur under any of the conditions investigated. Furthermore, the affinity of methoctramine under these conditions was not significantly different from tissues treated with oxotremorine M alone. However, in the presence of β-adrenoceptor stimulation with isoprenaline, following partial M_3 receptor inactivation, a role for M_2 receptors was disclosed, since the observed affinity ($-\log K_B$) for methoctramine increased from 6.2 to 7.4, in agreement with the findings of Thomas *et al.* (1993). Similar effects were seen when β_3-adrenoceptors were selectively stimulated by BRL 37344 to relax the histamine-induced tone. The demonstration of a response antagonized with high affinity by methoctramine ($-\log K_B$ values of 7.4 and 7.8), under conditions of partial M_3 alkylation along with β-adrenoceptor stimulation, reflects a role for the M_2, but not the residual M_3 population, in modulation of the response to β-adrenoceptor agonists.

The potency of oxotremorine M at M_2 receptors mediating this 're-contraction' under these conditions was significantly more potent ($-\log EC_{50} = 7.8$) than the potency of oxotremorine M under the alkylation conditions alone ($-\log EC_{50} = 6.5$). Indeed, the potency was similar to that mediating control oxotremorine M contractions ($-\log EC_{50} = 7.7$). Consequently, it is possible that inhibition of β-adrenoceptor relaxation, by M_2 receptors, may occur at similar levels of acetylcholine release from parasympathetic nerves which mediate contraction, assuming, that M_2 and M_3 receptors are equally accessed by neuronally released acetylcholine. Hypothetically then, the role of M_2 and M_3 receptors may vary, depending upon the prevailing level of sympathetic tone, with M_2 receptor agonism inhibiting the action of catecholamines, and thereby aiding contraction to M_3 receptor stimulation. The ability of M_2 receptor activation to inhibit cyclic AMP levels stimulated by a variety of neurotransmitters further suggests that M_2 receptors may provide an important modulatory control over ileal motility under many different circumstances.

In conclusion, evidence has been found that M_2 receptors inhibit β-adrenoceptor stimulation of cyclic AMP accumulation and that, after inactivation of M_3 muscarinic receptors, M_2 receptors inhibit β_1- and β_3-adrenoceptor relaxation of guinea-pig isolated ileum. Thus, both M_2 and M_3 muscarinic receptors play a role in controlling motility in this tissue.

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