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Effects of muscarinic M_2 and M_3 receptor stimulation and antagonism on responses to isoprenaline of guinea-pig trachea *in vitro*

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1 In guinea-pig and canine airway smooth muscle, there is reduced β-adrenoceptor agonist sensitivity in tissues pre-contracted with muscarinic agonists when compared to tissues pre-contracted with other spasmogens, such as histamine or leukotriene D_4. This reduced sensitivity may be the result of an interaction between muscarinic receptors and β-adrenoceptors. In this study the effects of M_2 receptor antagonism and stimulation have been investigated on the relaxant potency of isoprenaline in guinea-pig isolated tracheal smooth muscle.

2 (+)-*cis*-Dioxolane contracted isolated tracheal strips in a concentration-dependent manner (EC_{50} = 11.5 ± 0.9 nM). The rank order of antagonist apparent affinities (with pA_2 values in parentheses) was atropine (9.4 ± 0.1) > zamifenacin (8.2 ± 0.1) > para-fluoro-hexahydro-siladiphenidol (p-F-HHSiD, 7.2 ± 0.1) > pirenzepine (6.5 ± 0.1) > methoctramine (5.5 ± 0.1). Schild slopes were not significantly different from unity. This was consistent with a role of muscarinic M_3 receptors in mediating contraction.

3 In tissues pre-contracted to 3 g isometric tension using (+)-*cis*-dioxolane (0.2 μM, approximately EC_{80}), the relaxant potency of isoprenaline was significantly ($P<0.05$) increased by 0.3 μM methoctramine (control EC_{50} = 32.2 ± 4.3 nM, plus methoctramine EC_{50} = 19.1 ± 4.5 nM). This concentration of methoctramine had no effect on contractile responses to (+)-*cis*-dioxolane (control, EC_{50} = 17.6 ± 3.2 nM, plus methoctramine, EC_{50} = 21.0 ± 4.4 nM).

4 When acetylcholine (non-selective), (+)-*cis*-dioxolane (non-selective), L-660,863 ((±)-3-(3-amino-1,2,4-oxadiazole-5-yl)-quinuclidine, M_2-selective) or SDZ ENS 163 (thiopilocarpine, mixed M_2 antagonist, partial M_3 agonist) were used to achieve isometric tensions of 3 g, the relaxant potency of isoprenaline ranged from 3.7 ± 0.3 nM (SDZ ENS 163) to 49.4 ± 3.2 nM ((+)-*cis*-dioxolane). Reducing the concentration of these agonists (and therefore the level of developed tension to 2 g), significantly ($P<0.05$) increased the relaxant potency of isoprenaline. In contrast, when histamine was used to pre-contract tissues to either 2 or 3 g (EC_{50} = 4.2 ± 0.6 and 3.8 ± 1.1 nM, respectively), there was no significant effect on the relaxant potency of isoprenaline.

5 There was a slight but significant ($P<0.05$) reduction in the relaxant potency of isoprenaline, in tissues pre-contracted to 3 g using histamine in combination with (+)-*cis*-dioxolane (30 nM). This effect was reversed by M_2 receptor antagonism, using methoctramine (1 μM).

6 These data suggest that in guinea-pig isolated trachea, the relaxant potency of isoprenaline may depend not only on the level of developed tension but also, on the level of muscarinic M_2 receptor stimulation/blockade of the spasmogen inducing the tension. However, the lack of selective M_2 agonist and the low M_2/M_3 selectivity of antagonists in this tissue do not permit definitive conclusions to be made about the role of these receptors in modulating isoprenaline potency.

Keywords: Muscarinic cholinoceptors; β-adrenoceptors; tracheal smooth muscle; M_2/M_3 receptor agonists and antagonists

Introduction

In guinea-pig and canine airway smooth muscle, the relaxant potency of β-adrenoceptor agonists depends both upon the nature of the agonist used to elevate the resting tension and the magnitude of the contracture from which relaxations are elicited (Torphy, 1984; Russell, 1984; Torphy *et al.*, 1985). Thus, from equivalent levels of developed isometric tension, isoprenaline is less potent in relaxing tissues pre-contracted with muscarinic agonists than tissues pre-contracted with either histamine or leukotriene D_4, (Torphy, 1984; Russell, 1984; Koenig *et al.*, 1989). One explanation for the difference in isoprenaline relaxant potency, is an inhibitory action of muscarinic receptor stimulation on β-adrenoceptor function (Torphy, 1984; Gunst *et al.*, 1989).

Activation of muscarinic receptors in smooth muscle results in inhibition of adenylyl cyclase activity and stimulation of phosphoinositide specific phospholipase C activity (Jones *et al.*, 1987; Sankary *et al.*, 1988; Yang *et al.*, 1991; Pyne *et al.*, 1992). This is due to activation of two muscarinic receptor subtypes, M_2 (80–88%) and M_3 (12–20%) respectively (Fryer & El Fakahany, 1990; Mahesh *et al.*, 1992). Muscarinic M_3 receptors mediate smooth muscle contraction (Roffel *et al.*, 1990; Ten Berge *et al.*, 1993), but the role, if any, of the majority M_2 receptor population is unclear.

In smooth muscle, adenylyl cylcase activity is enhanced by β-adrenoceptor activation, resulting in relaxation. Activation of muscarinic M_2 receptors inhibits this stimulation, by coupling to a pertussis toxin-sensitive guanine nucleotide binding protein, G_i (Sankary *et al.*, 1988; Yang *et al.*, 1991; Griffin & Ehlert, 1992; Pyne *et al.*, 1992). In canine isolated trachea pre-contracted with muscarinic agonists, selective antagonism of M_2 receptors enhances the relaxant potency of isoprenaline (Fernandes *et al.*, 1992). Pertussis toxin, which ADP-ribosylates and thereby inactivates the alpha subunit of G_i, has a similar effect (Mitchell *et al.*, 1993). Thus, activation of M_2 receptors may attenuate β-adrenoceptor-mediated relaxation, thereby facilitating M_3 receptor-mediated contraction (Torphy *et al.*, 1985; Sankary *et al.*, 1988).

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Alternatively, it is also possible that attenuation of relaxation to β-adrenoceptor activation involves M_3 receptors, directly. Stimulation of M_3 receptors activates a phosphoinositide specific phospholipase C, via a pertussis toxin-insensitive G protein, G_q. This results in the formation of inositol (1,4,5) trisphosphate and 1,2 diacylglycerol (see Chilvers & Nahorski, 1990, for review), which causes intracellular release of calcium and activation of a protein kinase C, respectively. These two processes may lead to phosphorylation of β-adrenoceptors, guanine nucleotide binding proteins (G_s) or adenylyl cyclase (Van Amsterdam *et al.*, 1989; 1990). Functional antagonism by M_3 receptor activation may, therefore, offset relaxations to β-adrenoceptor agonists, without involving M_2 receptors. In support of this hypothesis, Meurs *et al.* (1993) have failed to demonstrate an effect of selective M_2 receptor antagonism on the relaxant potency of isoprenaline in bovine trachea. However, a correlation was demonstrated by these workers, between the inhibitory effects on the relaxant potency of isoprenaline and the potency at M_3 receptors mediating enhanced inositol phospholipid metabolism (Meurs *et al.*, 1993).

The aim of the present studies was to explore further the role of muscarinic receptor subtypes in modulating relaxations of guinea-pig, isolated trachea to isoprenaline. The lack of potent and selective M_2 and M_3 receptor agonists (see Caulfield, 1993, for review) mandated that indirect approaches be employed. These were, firstly, the use of a non-selective muscarinic agonist, (+)-*cis*-dioxolane, in the presence of selective M_2 receptor antagonism using methoctramine. Secondly, the use of tissues pre-contracted with muscarinic agonists possessing varying intrinsic efficacies at muscarinic receptors. Thirdly, by studying the effects of selective M_2 receptor stimulation using (+)-*cis*-dioxolane in the presence of M_3 receptor antagonism by *p*-F-HHSiD (*para*-fluoro-hexahydrosiladiphenidol). A preliminary account of this work was presented to the British Pharmacological Society (Watson & Eglen, 1993).

Methods

General

Male, Dunkin-Hartley guinea-pigs (250–350 g) were killed by exposure to CO_2. Tracheae were isolated and placed in aerated, modified Krebs solution (composition mM: KCl 4.6, KH_2PO_4 1.2, $MgSO_4$ 1.2, NaCl 118.2, glucose 10.0, $NaHCO_3$ 24.3 and $CaCl_2$ 2.5) and cleaned of extraneous tissue. Tracheae were opened along their ventral surface and strip preparations were cut transversely, with each strip containing 3–4 cartilaginous rings. Silk (4–0) sutures were attached to the cartilaginous portions on either side of the smooth muscle bands and the preparations were suspended, at a resting tension of 1 g, in 10 ml organ baths containing aerated, modified Krebs solution (pH 7.4, 37°C). This 1 g applied tension was considered the baseline, from which all further tension charges were recorded. Indomethacin (1 μM) was present in the Krebs solution throughout, to inhibit prostaglandin synthesis. Tetrodotoxin (0.1 μM) was also present throughout, to eliminate pre-junctional effects of muscarinic agonists. Corticosterone (30 μM) was present in all studies with β-adrenoceptor agonists, to inhibit extraneuronal monoamine uptake. All preparations were allowed 60 min to equilibrate, prior to construction of concentration-effect curves. These were established in a cumulative manner, using incremental concentrations at 0.5 $\log_{10}$ intervals. Each successive concentration was added once a sustained contracture to the previous concentration was attained.

Receptor characterization

Concentration-effect curves to (+)-*cis*-dioxolane, were constructed and tissues were washed and re-equilibrated, for 60 min, in the presence of a single concentration of one of the following antagonists: atropine (10, 30 or 100 nM), pirenzepine (1, 3 or 10 μM), methoctramine (1, 3 or 10 μM), p-F-HHSiD (0.03, 0.3, 1 or 3 μM) and zamifenacin (10–100 nM). A second concentration-effect curve to (+)-*cis*-dioxolane was then established in the presence of antagonist. Parallel studies were undertaken in the absence of antagonist to correct for temporal changes in sensitivity.

*The effect of muscarinic M_2 receptor antagonism, on the relaxant potency of isoprenaline in tissues pre-contracted with (+)-*cis*-dioxolane*

A concentration-effect curves was initially obtained to (+)-*cis*-dioxolane (1 nM–1 μM) in all tissues, to establish both the maximal contractile response and the concentration required to give approximately a 3 g increase in isometric tension. During this initial exposure to (+)-*cis*-dioxolane, methoctramine (0.3 μM) was present to inhibit M_2 receptor desensitization occurring at high (+)-*cis*-dioxolane concentrations. Tissues were then washed at 15 min intervals over the following 120 min period and during the final 60 min, separate tissues were equilibrated in the absence or presence of a single concentration of methoctramine (0.3 μM). Tissues were then pre-contracted to 3 g using (+)-*cis*-dioxolane (0.2 μM) and concentration-effect curves to isoprenaline (01. nM–1 μM) were established.

The effect of muscarinic agonists and histamine on the relaxant potency of isoprenaline

Concentration-effect curves to L-660,863 (1 nM–0.3 μM), SDZ ENS 163 (0.1–30 μM), acetylcholine (ACh, 1 nM–10 μM), (+)-*cis*-dioxolane (1 nM–1 μM) or histamine (0.1–0.3 mM) were obtained to establish both the maximal response to these agonists and the concentration required to increase isometric tension by approximately 3 g. Tissues were then washed at 15 min intervals over a 60 min period and allowed to re-attain baseline isometric tension. The tension was then increased to 3 g by addition of either muscarinic agonist or histamine. Each tissue was exposed to only one agonist. Once a stable contracture was attained, relaxant concentration-effect curves to isoprenaline (0.1 nM–1 μM) were established. Similar experiments were also undertaken, in separate tissues pre-contracted with a lower concentration of the above agonists, to an isometric tension of 2 g. Physostigmine (0.3 μM) was present in the studies with acetylcholine, to inhibit acetylcholinesterase activity.

The effect of M_2 receptor stimulation and antagonism on the relaxant potency of isoprenaline in tissues pre-contracted with histamine

In this series of experiments, the effect of muscarinic M_2 receptor activation, using (+)-*cis*-dioxolane, was investigated on the relaxant potency of isoprenaline. In short, this was achieved by raising the level of isometric tension to 3 g with histamine and, in the presence of M_3 receptor antagonism (p-F-HHSiD, 0.3 μM), activating M_2 receptors with (+)-*cis*-dioxolane (0.1 μM).

Concentration-effect curves to histamine (0.1 μM–0.3 mM) were obtained in all tissues, to establish both the maximal contractile response and the concentration required to give approximately a 3 g isometric tension change. Tissues were then washed and re-equilibrated for 60 min in the absence or presence of a single concentration of methoctramine (1.0 μM). (+)-*cis*-Dioxolane (0.1 μM) was added to all but control tissues and the tone was raised to a final isometric tension of 3 g using histamine. A concentration-effect curve to isoprenaline was then constructed.

The apparent affinity (pA_2) of p-F-HHSiD at M_2 receptors is 6.0 (Lambrecht *et al.*, 1988; Eglen *et al.*, 1990). Therefore, at the concentration of p-F-HHSiD (0.3 μM) used to inhibit

M_3 receptor-mediated contractions in this experiment, 23% of M_2 receptor would be occupied. This level of M_2 receptor occupancy may compromise putative inhibitory effects of (+)-*cis*-dioxolane on the isoprenaline relaxant potency. Therefore, these experiments were repeated in the absence of p-F-HHSiD. To retain M_2 receptor activity but reduce M_3 receptor-mediated contractions, the concentration of (+)-*cis*-dioxolane was reduced from 0.1 μM to 30 nM. The final 3 g increase in isometric tension was therefore achieved by a combination of histamine and (+)-*cis*-dioxolane (test tissues) or histamine alone (control tissues).

Measurement and analysis of responses

All responses were recorded as changes in isometric tension (g). Contractile responses were normalized to the maximal contractile responses in each tissue during the first exposure to agonist. Relaxant responses were expressed as a percentage of the isometric tension induced by the agonist, before application of isoprenaline. Data were analysed by the relationship of Parker & Waud (1971), using a non-linear iterative curve fitting procedure (Kaleidagraph, Synergy software, Reading, PA 19606, U.S.A., Leung *et al.*, 1992). The potency (defined as the EC_{50}) and maximal responses determined by this procedure were corrected for changes in sensitivity with time, where necessary. Apparent antagonist affinities (pA_2) were determined, where appropriate, by Schild regression analysis (Arunlakshana & Schild, 1959). Values quoted are those obtained when the slope, not being significantly different from unity, was constrained to unity.

Statistical analysis of the data was performed using paired and unpaired Student's *t* tests where appropriate, with $P < 0.05$ being considered significant. All values quoted are the mean ± s.e.mean from five animals, unless otherwise stated.

Compounds used

(+)-*cis*-Dioxolane (L-(+)-*cis*-2-methyl-4-trimethylammonium methyl-1,3-dioxolane iodide, a 60:40 mixture of *cis*:*trans*), atropine, pirenzepine, methoctramine, p-F-HHSid (*para*-fluoro-hexa-hydro-sila-diphenidol) and histamine were obtained from Research Biochemicals Inc. (Natick, MA, USA). L-660,863 ((±)-3-(3-amino-1,2,4-oxadiazole-5-yl)-quinuclidine) was synthesized in the Institute of Organic Chemistry (Syntex Discovery Research, Palo Alto, CA, U.S.A.). Tetrodotoxin, physostigmine, indomethacin, corticosterone, isoprenaline, acetylcholine and ascorbic acid were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). SDZ ENS 163 (thiopilocarpine) was a gift from Sandoz Pharma Ltd (Basel, Switzerland) and zamifenacin was generously provided by Pfizer Ltd. (Sandwich, U.K.).

Indomethacin was made up as a 1 mg ml^{-1} solution in propylene glycol and solubilized by a brief period (2–3 min) of sonication. Corticosterone was made up as a 0.1 M solution in dimethyl sulphoxide. Tetrodotoxin was made up as a 1.0 mM stock solution in 0.01 M acetic acid, which was then divided into 200 μl volumes and frozen until use. Ascorbic acid (22 μM) was added to solutions of histamine and isoprenaline as an anti-oxidant and these solutions were kept on ice for the duration of the experiments.

Results

Receptor characterization

(+)-*cis*-Dioxolane caused concentration-dependent contractions of tracheal strips with a maximal response of 4.2 ± 0.1 g and potency (EC_{50}) of 11.5 ± 0.9 nM. No significant time-dependent shift in the concentration-effect curves to (+)-*cis*-dioxolane was observed. All muscarinic antagonists caused parallel concentration-dependent dextral shifts in the concentration-effect curves to (+)-*cis*-dioxolane. The apparent antagonist affinities (pA_2), were atropine 9.4 ± 0.1, pirenzepine 6.5 ± 0.1, methoctramine 5.5 ± 0.1, p-F-HHSiD 7.2 ± 0.1 and zamifenacin 8.2 ± 0.1 The Schild slopes were not significantly different from unity.

The effect of muscarinic M_2 receptor antagonism, on the relaxant potency of isoprenaline in tissues pre-contracted with (+)-cis-dioxolane

Isoprenaline caused concentration-dependent relaxations in tissues pre-contracted with 0.2 μM (+)-*cis*-dioxolane to approximately 3 g (Figure 1). Isoprenaline completely reversed contractions induced by (+)-*cis*-dioxolane, with a potency (EC_{50}) of 32.2 ± 4.3 nM. In the presence of methoctramine (0.3 μM), the concentration-effect curve to isoprenaline was shifted to the left in a parallel fashion (Figure 1) with a potency (EC_{50} value) of 19.1 ± 4.5 nM ($P < 0.05$). There was no significant difference in the magnitude of the developed tension in either of these two groups, prior to performing concentration-effect curves to isoprenaline (2.9 ± 0.2 g controls and 3.2 ± 0.2 g plus methoctramine). There was no significant effect of this concentration of methoctramine (0.3 μM) on the potency of (+)-*cis*-dioxolane (control, EC_{50} = 17.6 ± 3.2 nM, plus methoctramine, EC_{50} = 21.0 ± 4.4 nM) nor the magnitude of the maximum response to agonist ($n = 6$).

The effect of muscarinic agonists and histamine on the relaxant potency of isoprenaline

All muscarinic agonists produced concentration-dependent contractions of tracheal strips (Table 1) and were full agonists with respect to (+)-*cis*-dioxolane, with the exception of SDZ ENS 163 (Figure 2, Table 1). The rank order of potency was L-660,863 ≥ (+)-*cis*-dioxolane > ACh > SDZ ENS 163 (Table 1). Physostigmine (0.3 μM), added 15–20 min prior to the addition of acetylcholine, increased isometric tension by 1.4 ± 0.2 g (see Figure 3).

Concentration-dependent relaxations to isoprenaline were seen in all tissues pre-contracted to either 3 or 2 g (Figure 4, Table 2). In tissues pre-contracted with the higher concentration of muscarinic agonist, to approximately 3 g, isoprenaline was most potent when SDZ ENS 163 was used to induce the

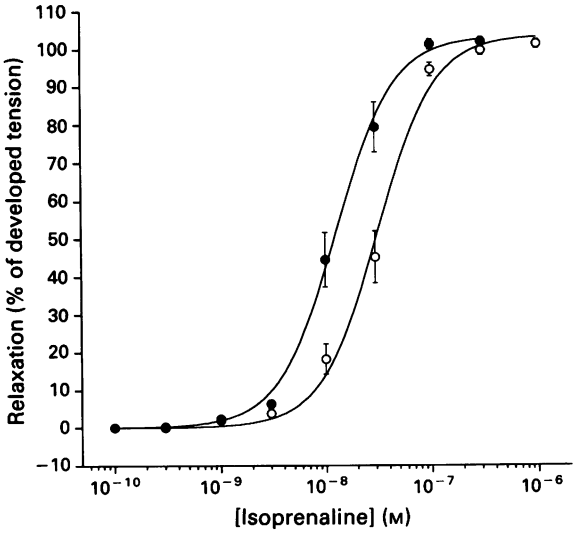

Figure 1 Isoprenaline-induced relaxation in tissues pre-contracted with (+)-*cis*-dioxolane in the absence (○) and presence of 0.3 μM (●) methoctramine. Relaxations are expressed as a percentage of the developed tension induced by (+)-*cis*-dioxolane, which were not significantly different between groups. Values are the mean ± s.e. mean, $n = 6$.

Table 1 The contractile potency (EC_{50}) and maximal tension changes associated with a range of muscarinic agonists in guinea-pig tracheal smooth muscle

	SDZ ENS 163	*L-660,863*	*Acetylcholine*	*(+)-cis-dioxolane*
Maximal response (g)	2.4 ± 0.3*	3.8 ± 0.2	3.7 ± 0.2	3.8 ± 0.2
Potency (EC_{50} nM)	1000 ± 100	5.7 ± 0.8	159 ± 19	8.1 ± 1.6
n	(15)	(15)	(15)	(18)

Values are the mean ± s.e.mean, where *n* = number of experiments. *$P<0.05$ Student's unpaired *t* test ((+)-*cis*-dioxolane maxima v SDZ ENS 163 maxima).

Table 2 The relaxant potency (EC_{50}) of isoprenaline in tissues pre-contracted using a range of muscarinic agonists

A *Pre-contracted to approximately 3 g*

	SDZ ENS 163	*L-660,863*	*Acetylcholine*	*(+)-cis-dioxolane*
Developed tension (g)	2.8 ± 0.3	2.8 ± 0.2	3.0 ± 0.1	3.0 ± 0.2
Potency (EC_{50} nM)	3.7 ± 0.3†	7.0 ± 0.8†	20.8 ± 4.3†	49.4 ± 3.2†
n	(3)	(5)	(6)	(12)

B *Pre-contracted to approximately 2 g*

	SDZ ENS 163	*L-660,863*	*Acetylcholine*	*(+)-cis-dioxolane*
Developed tension (g)	2.1 ± 0.1	2.0 ± 0.1	2.0 ± 0.1	2.0 ± 0.1
Potency (EC_{50} nM)	1.1 ± 0.3	3.6 ± 0.7	4.5 ± 1.6	6.2 ± 1.1
n	(5)	(5)	(6)	(5)

The concentrations of agonist required to achieve these developed tensions were: (A) SDZ ENS 163 = 30 μM, L-660,863 = 30 nM, ACh = 10 μM, (+)-*cis*-dioxolane = 0.1 μM and (B) SDZ ENS 163 = 12.6 ± 1.2 μM, L-660,863 = 9.6 ± 1.4 nM, ACh = 0.4 ± 0.2 μM, (+)-*cis*-dioxolane = 21.2 ± 5.4 nM. Values are the mean ± s.e.mean, where *n* = the number of experiments. *$P<0.05$. Student's paired *t* test, †$P<0.02$, Student's unpaired *t* test.

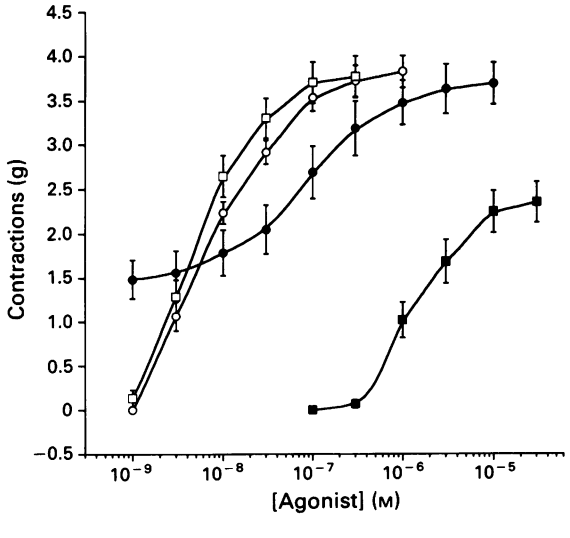

Figure 2 Contractions of the trachea induced by a range of muscarinic agonists; (+)-*cis*-dioxolane (○), acetylcholine (●), L-660,863 (□) and SDZ ENS 163 (■), given as absolute changes in isometric tension. The potencies of these agonists are shown in Table 2. Values are the mean ± s.e.mean, *n* = 15–18 experiments.

contracture and least potent when (+)-*cis*-dioxolane was used (Table 2). The relaxant potency of isoprenaline was significantly different in all cases, although the initial level of isometric tension, induced by the muscarinic agonists, were not significantly different. Isoprenaline completely reversed contractions induced by all the muscarinic agonists, with the exception of ACh in which case the contracture was reversed by 71 ± 5% (Figures 3 and 4).

When tissues were pre-contracted with the lower concentration of agonist, to approximately 2 g, isoprenaline was again most potent against contractures induced by SDZ ENS 163 and least potent against (+)-*cis*-dioxolane-induced contracture (Table 2). Under these conditions, isoprenaline completely reversed the contracture induced by all agonists (Figure 4) and the relaxant potency of isoprenaline was significantly increased when compared to that obtained in tissues pre-contracted with the higher concentration of agonist (to approximately 3 g).

Histamine produced concentration-dependent contractions of tracheal strips with a potency (EC_{50}) of 5.1 ± 0.9 μM and maximal tension change of 3.5 ± 0.1 g. Isoprenaline caused concentration-dependent relaxations of tissues pre-contracted with high and low concentrations of histamine, to approximately 3 g (2.6 ± 0.2 g) or 2 g (2.1 ± 0.1 g). These two levels of initial contracture were significantly different from each other, but were not significantly different from those elicited by the high and low concentrations of muscarinic agonists described above. Isoprenaline completely reversed histamine-induced contractions at either 3 or 2 g isometric tension, with potencies (EC_{50}) of 4.2 ± 0.6 nM and 3.8 ± 1.1 nM, respectively. These values were not significantly different from one another, despite the fact that the initial levels of isometric tension were significantly different from each other (2.6 ± 0.2 g vs 2.1 ± 0.1 g).

In tissues pre-contracted to 3 g, isoprenaline was significantly more potent at relaxing tissues contracted by histamine than L-660,863, (+)-*cis*-dioxolane or acetylcholine. However, at this level of isometric tension, there was no significant difference between the relaxant potency of isoprenaline in tissues pre-contracted with either histamine or SDZ ENS 163. In tissues pre-contracted to 2 g, there was no significant difference between the potency of isoprenaline in tissues pre-contracted with muscarinic agonists or with histamine.

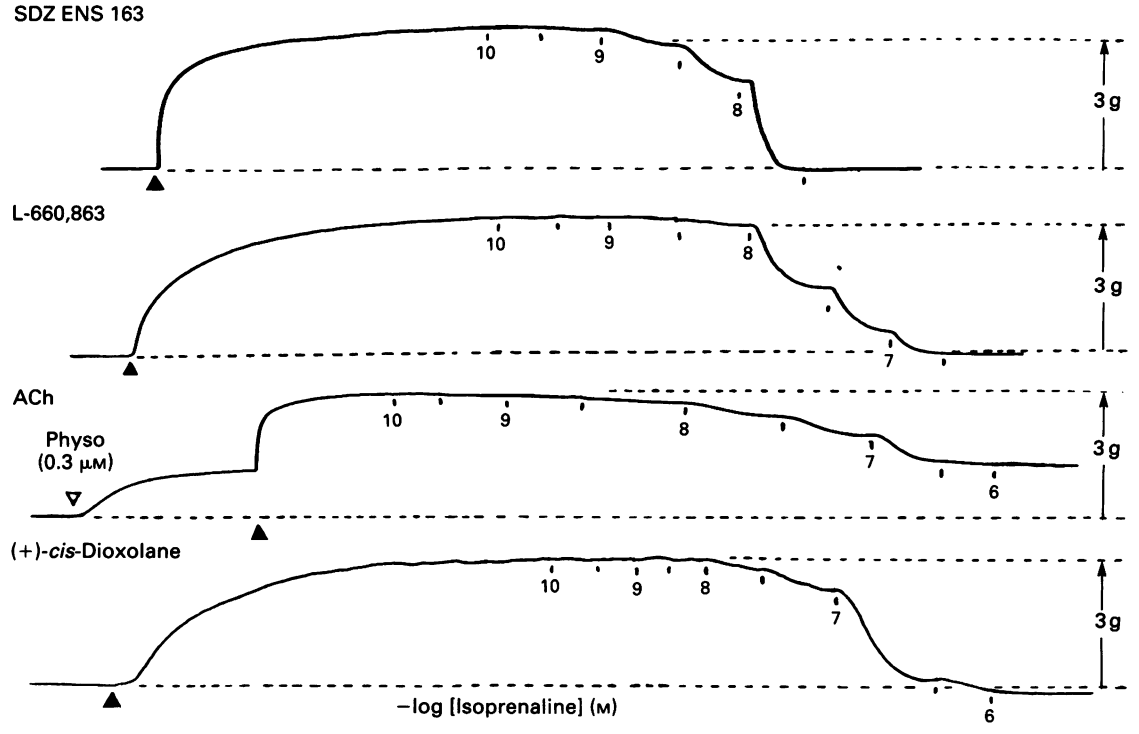

Figure 3 Representative results showing relaxations to isoprenaline in tracheal strips pre-contracted to 3 g by a range of muscarinic agonists. The broken lines indicate 3 g isometric tension and the concentrations of isoprenaline in 0.5 $\log_{10}$ increments are indicated.

The effect of M_2 receptor stimulation and antagonism, on the relaxant potency of isoprenaline in tissues pre-contracted with histamine

In the presence of p-F-HHSiD (0.3 μM), the relaxant potency (EC_{50}) of isoprenaline against histamine pre-contracture, was not significantly altered by agonism of M_2 receptors using (+)-*cis*-dioxolane (0.1 μM). Similarly, M_2 receptor antagonism using methoctramine (1 μM), did not significantly alter the relaxant potency of isoprenaline in tissues pre-contracted with histamine in the presence of (+)-*cis*-dioxolane (Figure 5; Table 3). Despite the presence of 0.3 μM p-F-HHSiD, (+)-*cis*-dioxolane (0.1 μM) caused a significant increase in isometric tension (0.4 ± 0.1 g). However, the concentration of histamine used to induce tone was adjusted, such that the magnitude of the contracture prior to performing concentration-effect curves to isoprenaline, was not significantly different between the three groups (Figure 5; Table 3).

In the absence of p-F-HHSiD, the relaxant potency of isoprenaline was significantly reduced in tissues pre-contracted to 3 g with a combination of histamine and (+)-*cis*-dioxolane (30 nM), when compared to tissues pre-contracted to equivalent isometric tension with histamine alone. This effect was reversed by 1.0 μM methoctramine (Figure 5; Table 3). In the absence of p-F-HHSiD, the increase in isometric tension induced by (+)-*cis*-dioxolane (30 nM) was 3.1 ± 1.0 g. However, the concentration of histamine used to induce tone was adjusted, such that the magnitude of the contracture prior to performing concentration-effect curves to isoprenaline, was not significantly different between the three groups (Figure 5; Table 3).

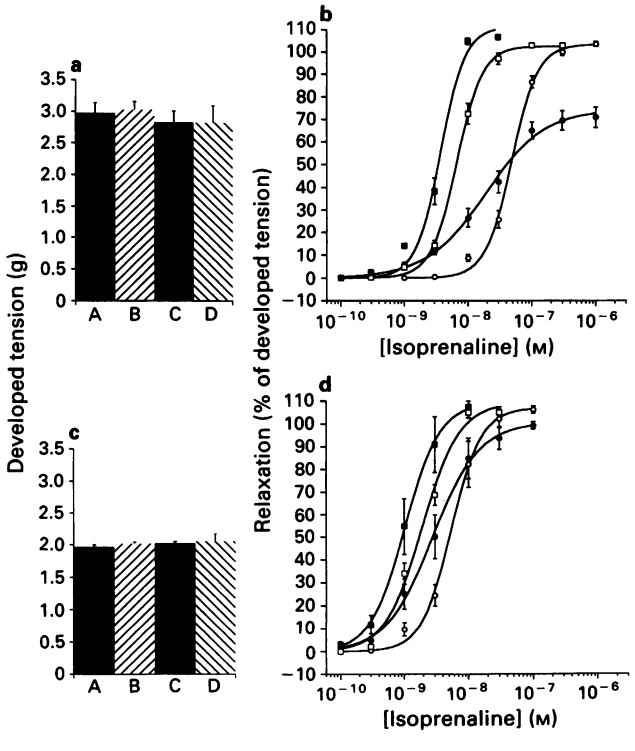

Figure 4 Isoprenaline-induced relaxation of tissues pre-contracted to 3 g (b) and 2 g (d) isometric tension by a range of muscarinic agonist; (+)-*cis*-dioxolane (○ and A), acetylcholine (● and B), L-660,863 (□ and C) and SDZ ENS 163 (■ and D). Relaxations are expressed as a % of the developed tension induced by these agonists and are shown in absolute values in the histograms (a) and (c) (see also Table 3). Values are the mean ± s.e.mean. The number of experiments for each group are shown in Table 3, along with the EC_{50} values for isoprenaline-induced relaxation under these conditions.

Discussion

In airway smooth muscle, muscarinic receptors mediate contraction and appear to modulate the relaxant potency of β-adrenoceptor agonists. In all species studied to date, M_2

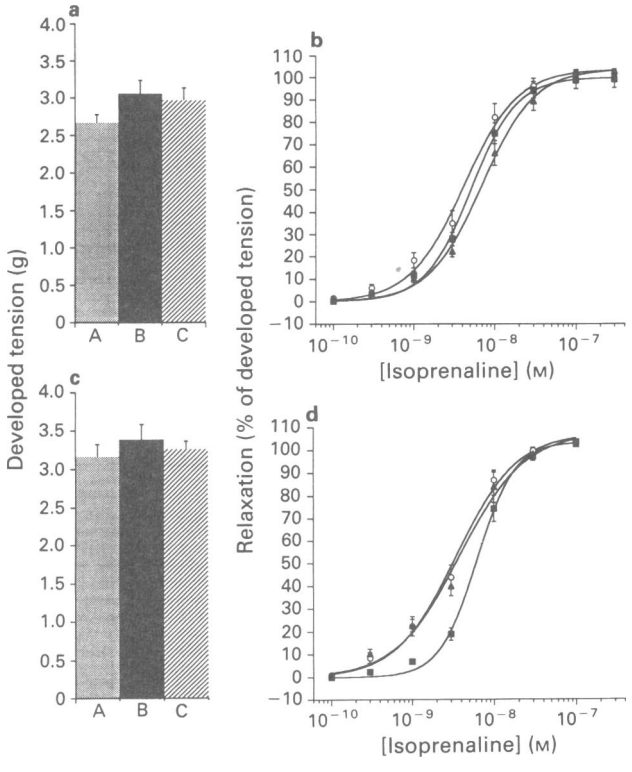

Figure 5 Isoprenaline-induced relaxation in tissues pre-contracted with histamine under the following conditions; in the absence of (+)-*cis*-dioxolane and methoctramine (○ and A), in the presence of 0.1 μM (b) or 30 nM (d) (+)-*cis*-dioxolane (■ and B) and in the presence of (+)-*cis*-dioxolane (as above) and 1 μM methoctramine (▲ and C). (a) and (b) show responses when 0.3 μM p-F-HHSiD was present to antagonize M_3 receptors and (c) and (d) show responses in the absence of p-F-HHSiD. Relaxations are expressed as a percentage of the developed tension induced either by histamine alone or in combination with (+)-*cis*-dioxolane. The developed tensions for each group are shown in absolute values in the histogram (a) and (c) (see also Table 1). Values are the mean ± s.e.mean, $n = 6$ for each of the six treatment groups.

Table 3 The effect of (+)-*cis*-dioxolane (0.1 μM or 30 nM), alone or in the presence of methoctramine (1 μM) on the relaxant potency of isoprenaline in tissues pre-contracted with histamine in the presence and absence of p-F-HHSiD (0.3 μM)

	Control	*Test 1*	*Test 2*
A *In the presence of p-F-HHSiD*			
Developed tension (g)	2.7 ± 0.1	3.1 ± 0.2	3.0 ± 0.2
Potency (EC_{50} nM)	4.8 ± 0.7	5.5 ± 0.6	6.0 ± 1.1
B *In the absence of p-F-HHSiD*			
	Control	*Test 1*	*Test 2*
Developed tension (g)	3.2 ± 0.2	3.4 ± 0.2	3.3 ± 0.2
		**	
Potency (EC_{50} nM)	4.4 ± 0.6	6.7 ± 0.8	3.6 ± 0.9
		NS	

Control tissues received neither (+)-*cis*-dioxolane nor methoctramine and the developed tension was achieved with histamine at a concentration of (A) 49 ± 17 μM and (B) 27 ± 2 μM. Test 1 tissues received (+)-*cis*-dioxolane (A) 0.1 μM and (B) 30 nM and the developed tension was achieved by the addition of histamine (A) 21 ± 4 μM and (B) 2.8 ± 0.7 μM. Test 2 tissues received (+)-*cis*-dioxolane in the concentrations already noted, along with methoctramine (1 μM). In test 2 tissues the developed tension was achieved by the addition of histamine (A) 20 ± 5 μM and (B) 2.8 ± 0.8 μM. Values are the mean ± s.e.mean, $n = 6$ per treatment group. $^{**}P < 0.02$ and NS: not significant, by Student's paired t test.

and M_3 receptors are present in airway smooth muscle in the ratio approximately 4:1 (Fryer & El Fakahany, 1990; Mahesh *et al.*, 1992). Both these muscarinic receptor subtypes have been implicated in the muscarinic receptor-mediated inhibition of β-adrenoceptor agonist-mediated relaxation (Van Amsterdam *et al.*, 1989; Fernandes *et al.*, 1992). The aim of the present study was to characterize further the role of muscarinic M_2 receptors in modulating relaxations of guinea-pig, isolated trachea to isoprenaline. In order to achieve this, three separate approaches were used, after first establishing an affinity profile for a range of muscarinic antagonists at the post-junctional receptor mediating contraction.

Receptor characterization

The antagonist affinity profile was consistent with stimulation of M_3 receptors causing contraction with no involvement of M_2 receptors (Ten Berge *et al.*, 1993). The pA_2 for the novel M_3 receptor antagonist, zamifenacin, was similar to that previously reported by Wallis *et al.* (1993). The pA_2 value for p-F-HHSiD (7.2) was lower than reported at M_3 receptors in other smooth muscles, including guinea-pig ileum (7.8–8.0, Lambrecht *et al.*, 1988) but consistent with previous findings in our laboratory (Eglen *et al.*, 1990). The M_3 receptor in guinea-pig trachea may be atypical, since p-F-HHSiD, RDS 129 (Saihin & Ilhan, 1986) and zamifenacin (Wallis *et al.*, 1993; this study) discriminate between M_3 receptors in guinea-pig trachea and ileum. However, in the absence of evidence for structural heterogeneity of M_3 receptors, the reasons for these differences in pA_2 values remain unknown (Caulfield, 1993). A consequence of the atypical nature of the tracheal M_3 receptor is that studies attempting to characterize M_2 or M_3 receptor function alone in this tissue are compromised by the low tracheal M_2:M_3 selectivity of p-F-HHSiD and zamifenacin.

The effect of muscarinic M_2 receptor antagonism on the relaxant potency of isoprenaline in tissues pre-contracted with (+)-cis-dioxolane

The first approach to study the role of M_2 receptors in guinea-pig trachea reproduced the findings of Fernandes and colleagues (1992) in canine trachea. Methoctramine (0.3 μM) increased the relaxant potency of isoprenaline, in (+)-*cis*-dioxolane pre-contracted tissues. The small but statistically significant increase in isoprenaline relaxant potency (1.7 fold shift) in the presence of methoctramine may be an underestimate, since comparison with the potency of isoprenaline in tissues not previously exposed to methoctramine indicates a 2.6 fold shift induced by methoctramine (see below). As the apparent affinities of methoctramine at M_2 and M_3 receptors are 7.8 and 5.8, respectively (Melchiorre *et al.*, 1987; Eglen *et al.*, 1988), it is unlikely that M_3 receptor antagonist properties of methoctramine account for the effect observed. To support this conclusion, 0.3 μM methoctramine had no significant effect on M_3-mediated contractile responses (this study). Furthermore, higher concentrations of methoctramine (1 and 3 μM), which increase M_3 receptor occupancy, did not further augment the potency of isoprenaline (data not shown).

The effect of muscarinic agonists and histamine on the relaxant potency of isoprenaline

The second approach was to stimulate the M_2 receptors with agonists possessing different intrinsic activities at muscarinic receptors. Acetylcholine and (+)-*cis*-dioxolane are agonists of high intrinsic efficacy, although non-selective between M_2 and M_3 receptors (Ford *et al.*, 1991). L-660,863 is a relatively selective muscarinic M_2 receptor agonist (Eglen *et al.*, 1992). However, its efficacy at this receptor is low and therefore, in poorly coupled M_2 receptor systems it acts as an antagonist (Freeman *et al.*, 1990; Eglen *et al.*, 1992). SDZ ENS 163, is a

full agonist at M_1 receptors, a partial M_3 receptor agonist and an M_2 receptor antagonist (Enz *et al.*, 1992). Under the present experimental conditions, agonist effects of SDZ ENS 163 at M_1 receptors in the parasympathetic ganglia (Bloom *et al.*, 1987), were inhibited by the inclusion of tetrodotoxin, while post-junctional M_1 receptors have not been detected in airway smooth muscle (Mak & Barnes, 1990). Therefore, the use of SDZ ENS 163 permitted the relaxant potency of isoprenaline to be assessed under conditions of partial M_3 receptor stimulation and M_2 receptor antagonism. This agent, unlike L660,863 shows no selectivity between receptor subtypes on the basis of affinity, although it is 'functional selective' (atria M_2 $-\log K_B = 5.9 \pm 0.2$; tracheal $-\log K_A = 5.8 \pm 0.1$; Watson & Eglen, 1993).

All compounds were full agonists at M_3 receptors mediating contraction of tracheal strips, with the exception of SDZ ENS 163 which acted as a partial agonist. This finding is consistent with previous reports (Eglen *et al.*, 1990; Ford *et al.*, 1991; Enz *et al.*, 1992). The isoprenaline relaxant potencies were not greatly different between preparations contracted with the muscarinic agonists to 2 g isometric tension. Moreover, the isoprenaline relaxant potencies were not significantly different from potency estimates in tissues pre-contracted with histamine. This suggests that at low concentrations of muscarinic agonists, there is little effect of muscarinic receptor activation on the attenuation of relaxant responses to isoprenaline. Therefore, despite activation of M_3 receptors, to elicit a 2 g increase in tension, no inhibition of β-adrenoceptor-mediated relaxant responses could be detected.

However, increasing the level of both M_2 and M_3 receptor activation, significantly reduced the relaxant potency of isoprenaline. Under this condition, significant differences were seen in the relaxant potency of isoprenaline between tissues pre-contracted with full muscarinic agonists ((+)-*cis*-dioxolane, acetylcholine and L-660,863) and those pre-contracted with the partial M_3 agonist/M_2 antagonist, SDZ ENS 163. These differences may suggest that differential agonist activity at M_2 or M_3 receptors, rather than the developed tension *per se*, may influence the isoprenaline relaxant potency. In contrast, differences in isoprenaline relaxant potency were not seen in tissues pre-contracted with histamine to 2 or 3 g. Since histamine does not interact at muscarinic receptors, these data implicate a specific role for muscarinic receptors in the attenuation of relaxant responses to isoprenaline, which is in agreement with the work of others (Torphy, 1984; Jones *et al.*, 1987; Fernandes *et al.*, 1992).

The effect of M_2 receptor agonism and antagonism, on the relaxant potency of isoprenaline in tissues pre-contracted with histamine

The final approach in the elucidation of the role of M_2 receptors was to stimulate the M_2 receptors in the absence of M_3 receptor activation. Ideally, an agonist with high potency and selectivity at M_2 receptors, which lacks intrinsic efficacy at M_3 receptors, is required. However, such a compound is unavailable. Thus, the alternative was to use (+)-*cis*-dioxolane, to activate M_2 receptors, in the presence of M_3 receptor antagonism by p-F-HHSiD. In this manner, the effect of M_2 receptor activation on relaxant potency of isoprenaline was examined in tissues pre-contracted to 3 g using histamine in the presence of 0.3 μM p-F-HHSiD. Under these conditions, there was no effect of (+)-*cis*-dioxolane on the relaxant potency of isoprenaline. This finding may imply that activation of M_3 receptors is required to mediate the reduction in isoprenaline relaxant potency, supporting conclusions reached by Meurs *et al.* (1993).

Alternatively, an involvement of M_2 receptors cannot be excluded, since in guinea-pig trachea, p-F-HHSiD shows relatively low selectivity between M_3 and M_2 receptors (16 fold Eglen *et al.*, 1990). Assuming a pA_2 of 6.0 for p-F-HHSiD (Lambrecht *et al.*, 1988; Eglen *et al.*, 1990) 23% of M_2 receptors would be occupied at the concentration required to antagonize M_3 receptor-mediated contractions (0.3 μM). This may be sufficient, in a poorly coupled system, to antagonize the effect of M_2 receptor-mediated inhibition of isoprenaline-induced relaxations. In an attempt to clarify the problem, experiments were repeated in the absence of p-F-HHSiD, and with a lower concentration of (+)-*cis*-dioxolane (30 nM), to reduce effects at M_3 receptors, while maintaining detectable effects on isoprenaline relaxant potency. In these studies, (+)-*cis*-dioxolane reduced the relaxant potency of isoprenaline, an effect which was reversed by methoctramine. The modest effect of (+)-*cis*-dioxolane (30 nM) was unsurprising given our previous observations that low concentrations of muscarinic agonists have small effects on isoprenaline relaxant potency. Taken together these data may suggest that the level of isometric tension achieved, is less important in determining the relaxant potency of isoprenaline compared to the level of muscarinic receptor activation.

Some data obtained in these studies require further explanation; (1) In the first study, an equilibration step with methoctramine, was used during construction of concentration-effect curves to (+)-*cis*-dioxolane. As discussed above, in tissues treated in this way, the relaxant potency of isoprenaline was 1.5 fold greater than in tissues not exposed to methoctramine. This suggests that residual M_2 receptor antagonism by methoctramine may have occurred. (2). There was no significant difference in the relaxant potency of isoprenaline in tissues pre-contracted to 2 g with either muscarinic agonists or histamine. This indicates a lack of muscarinic inhibition of isoprenaline relaxant responses. (3) In preparations pre-contracted to 3 g with L-660,863, the relaxant potency of isoprenaline was 1.7 fold less than in tissues pre-contracted with histamine (lacking M_2 activity) and 7 fold greater than in tissues pre-contracted with (+)-*cis*-dioxolane. (4) In the presence of M_3 receptor antagonism by p-F-HHSiD (0.3 μM) approximately 23% of the M_2 receptors would be occupied and no effect of M_2 receptor stimulation by (+)-*cis*-dioxolane (0.1 μM) could be demonstrated.

One explanation for all four observations is that muscarinic inhibition of β-adrenoceptors is mediated by M_2 receptors that are poorly coupled. This would be predicted to have the following consequences. Firstly, residual M_2 receptor antagonism (as a result of prior exposure to methoctramine) would reverse inhibitory effects of (+)-*cis*-dioxolane and thus increase the potency of isoprenaline. Secondly, high concentrations of agonists would be required to achieve adequate M_2 receptor occupancy in order to detect a functional inhibition. Thirdly, agonists with low intrinsic efficacy at M_2 receptors, such as L-660,863 or SDZ ENS 163, would behave as antagonists in such a system. Finally, antagonist occupation of a small porportion of M_2 receptors would have a large effect on the functional response. However, without direct measurement of the receptor reserve associated with M_2 inhibitory effects, this can only be speculated upon.

These studies have therefore provided some evidence for the involvement of M_2 receptors in the inhibitory effects of (+)-*cis*-dioxolane on the relaxant potency of isoprenaline, although a role for M_3 receptor cannot be excluded. In this respect these data concur with reports that AF-DX 116 or gallamine enhance the potency of isoprenaline in canine and rabbit trachea (Fernandes *et al.*, 1992; Arjona *et al.*, 1993). In the former tissue, Mitchell *et al.* (1993) has also demonstrated a similar effect of pertussis toxin, which functionally uncouples M_2 receptors from inhibition of adenylyl cyclase. In contrast, in bovine and guinea-pig trachea, gallamine has been reported not to augment the relaxant potency of isoprenaline (Meurs *et al.*, 1993; Roffel *et al.*, 1993). The reason for these discrepancies is unclear, although differences in methodology may be important. Thus, differences in the level of basal tension, the presence or absence of epithelium or of indomethacin, and the recording of isometric or isotonic tensions may account for the differences. However, it is important to note that in guinea-pig ileum, when M_3 receptors have been inactivated by alkylation, stimulation of M_2

receptors causes contraction by inhibiting relaxations elicited by isoprenaline, in tissues pre-contracted to histamine (Thomas *et al.*, 1993). However, similar studies have yet to be reported in guinea-pig trachea.

In conclusion, these data suggest that it is the degree of muscarinic receptor activation that is important in determining the relaxant potency of isoprenaline and this is related to the efficacy of the muscarinic agonist at M_2 and M_3 receptors. Since muscarinic M_2 receptor antagonism augments the relaxant potency of isoprenaline, these data provide some evidence for an inhibitory role of M_2 receptors on relaxant responses to isoprenaline. However, the involvement of M_3 receptors cannot be definitively excluded due to (a) the lack of selective M_2 and M_3 receptor agonists and (b) the low M_2/M_3 selectivity of antagonists in guinea-pig trachea.

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