No evidence for a role of muscarinic M₂ receptors in functional antagonism in bovine trachea

¹Ad F. Roffel, Herman Meurs, Carolina R.S. Elzinga & Johan Zaagsma

Groningen/Utrecht Institute for Drug Exploration, Department of Medicinal Chemistry and Molecular Pharmacology, University of Groningen, A. Deusinglaan 2, 9713 AW Groningen, The Netherlands

1 The functional antagonism between methacholine- or histamine-induced contraction and β -adrenoceptor-mediated relaxation was evaluated in bovine tracheal smooth muscle *in vitro*. In addition, the putative contribution of muscarinic M₂ receptors mediating inhibition of β -adrenoceptor-induced biochemical responses to this functional antagonism was investigated with the selective muscarinic antagonists, pirenzepine (M₁ over M₂), AF-DX 116 and gallamine (M₂ over M₃), and hexahydrosiladiphenidol (M₃ over M₂).

2 By use of isotonic tension measurement, contractions were induced with various concentrations of methacholine or histamine, and isoprenaline concentration-relaxation curves were obtained in the absence or presence of the muscarinic antagonists. Antagonist concentrations were chosen so as to produce selective blockade of M₂ receptors (AF-DX 116 0.1 μ M, gallamine 30 μ M), or half-maximal blockade of M₃ receptors (pirenzepine 0.1 μ M, AF-DX 116 0.5 μ M, hexahydrosiladiphenidol 0.03 μ M). Since these latter antagonist concentrations mimicked K_B values towards bovine tracheal smooth muscle M₃ receptors, antagonist-induced decreases in contractile tone were compensated for by doubling the agonist concentration.

3 It was found that isoprenaline-induced relaxation of bovine tracheal smooth muscle preparations was dependent on the nature and the concentration of the contractile agonist used. Thus, isoprenaline pD₂ (–log EC₅₀) values were decreased 3.7 log units as a result of increasing cholinergic tone from 22 to 106%, and 2.4 log units by increasing histamine tone over a similar range. Furthermore, maximal relaxability of cholinergic tone decreased gradually from 100% at low to only 1.3% at supramaximal contraction levels, whereas with histamine almost complete relaxation was maintained at all concentrations applied. As a result, isoprenaline relaxation was clearly hampered with methacholine compared to histamine at equal levels of contractile tone.

4 In the presence of gallamine, isoprenaline relaxation was facilitated for most concentrations of methacholine, and for all concentrations of histamine. These changes could be explained by the decreased contraction levels for both contractile agonists in the presence of gallamine.

5 Isoprenaline-induced relaxation of cholinergic contraction was also facilitated by AF-DX 116 as well as by pirenzepine and hexahydrosiladiphenidol, and these (small) changes were again related to the (small) decreases in cholinergic contraction levels that were present in these experiments despite the additional administration of the agonist to readjust contractile tone. Similarly, changes in isoprenaline relaxation of histamine-induced tone could be explained by different contraction levels.

6 These results can be explained by the sole involvement of muscarinic M₃ receptors, and provide no evidence for a role of muscarinic M₂ receptors in functional antagonism in bovine trachea. Furthermore, they stress the importance of taking into account non-cholinergic controls as well as contraction levels in these experiments.

Keywords: Muscarinic M₂ receptors; functional antagonism; β -adrenoceptor-mediated relaxation; smooth muscle tone; bovine tracheal smooth muscle

Introduction

Muscarinic cholinergic binding sites in airways smooth muscle of mammalian species, including guinea-pig, rat, cow, dog and rabbit, have been shown to consist of both M₂ and M₃ receptor subtypes, and recent biochemical evidence suggests that this is also true for man (Widdop *et al.*, 1993). The M₂ type binding sites represent the major population (75–90%) of muscarinic receptors (Roffel *et al.*, 1988; Fryer *et al.*, 1990; Lucchesi *et al.*, 1990; Schaefer *et al.*, 1992; Fernandes *et al.*, 1992; Mahesh *et al.*, 1992), except in guinea-pig trachea where both populations are approximately equal (Haddad *et al.*, 1991). Among these receptor subtypes, muscarinic M₃ receptors are unanimously found to mediate contraction under normal conditions *in vitro* (Mutschler *et al.*, 1988; Roffel *et al.*, 1988; 1990a; Yang *et al.*, 1991; Gardier *et al.*, 1991; Mahesh *et al.*, 1992), presumably via activation of phospholipase C

(Roffel *et al.*, 1990b; Yang *et al.*, 1991). The M₂ receptors have been shown to inhibit β -agonist- or forskolin-stimulated cyclic AMP production in intact canine, bovine and human airways smooth muscle cells (Sankary *et al.*, 1988; Yang *et al.*, 1991; Schaefer *et al.*, 1992; Challiss *et al.*, 1993; Widdop *et al.*, 1993), as well as in membrane preparations of canine, bovine and guinea-pig trachealis (Jones *et al.*, 1987; Meurs *et al.*, 1992; Pyne *et al.*, 1992), the latter demonstrating a direct inhibitory effect on adenylyl cyclase, presumably via the inhibitory G-protein, G_i (Sankary *et al.*, 1988; Pyne *et al.*, 1992; Widdop *et al.*, 1993). As an additional biochemical response, G_i-mediated inhibition of calcium-dependent potassium channel activity has been put forward (Kotlikoff *et al.*, 1994).

Concerning a functional role for this major population of muscarinic M₂ receptors in airways smooth muscle, it has been demonstrated recently that they may mediate contraction of guinea-pig longitudinal ileum smooth muscle under conditions of elevated cyclic AMP concentrations and in the presence of a non-cholinergic contractile agonist (i.e. histamine) *in vitro*

¹ Author for correspondence.

(Thomas *et al.*, 1993). Furthermore, these M₂ receptors have been implicated in the functional antagonism between cholinergic airways smooth muscle contraction and β -adrenoceptor-mediated relaxation in canine (Fernandes *et al.*, 1992; Mitchell *et al.*, 1993), rabbit (Arjona *et al.*, 1993) and guinea-pig (Watson & Eglén, 1994) trachea. Thus, muscarinic M₂ receptor-mediated inhibition of adenylyl cyclase activity (or potassium channel closure) may contribute to the phenomenon that isoprenaline-mediated relaxation of cholinergic tone is hampered compared to similar contraction levels induced by leukotriene D₄ (Torphy, 1984) or histamine (Russell, 1984; Van Amsterdam *et al.*, 1989), and that such relaxation is dependent on the level of cholinergic tone *per se* (Torphy *et al.*, 1983; Russell, 1984; Van Amsterdam *et al.*, 1989). However, the increases in isoprenaline relaxation potency after muscarinic M₂ receptor blockade or inactivation of G_i, using pertussis toxin (Mitchell *et al.*, 1993), i.e. 2 to 6 fold shift in isoprenaline pD₂ values, were rather small compared to the maximal changes in isoprenaline relaxant potency that can be obtained as a result of varying cholinergic contraction levels (85 and 490 fold shift for guinea-pig and canine trachea, respectively, cf. Torphy *et al.*, 1983; Roffel *et al.*, 1993). Moreover, in guinea-pig trachea such a role for muscarinic M₂ receptors was found to be completely absent when gallamine was used as the M₂-selective antagonist (Roffel *et al.*, 1993).

Transductional cross-talk between receptor-mediated phospholipase C activation and β -adrenoceptor function has also been proposed as a mechanism involved in functional antagonism between (cholinergic) airways contraction and β -adrenoceptor-mediated relaxation, showing a correlation between the production of inositol phosphates and the shift in isoprenaline potencies (Van Amsterdam *et al.*, 1989; 1990). Such cross-talk may involve protein kinase C-mediated uncoupling of β -adrenoceptors (as observed in bovine trachea, Grandordy *et al.*, 1993) through functional inactivation of G_s, (as reported for guinea-pig trachea, Pyne *et al.*, 1992), as has also been suggested for blood lymphocytes of asthmatic patients after allergen provocation (Meurs *et al.*, 1987), and may explain why contractions induced by partial muscarinic receptor agonists are more easily relaxed by isoprenaline than those induced by full muscarinic agonists (Gunst *et al.*, 1989; Van Amsterdam *et al.*, 1989; Al-Hassani *et al.*, 1993).

The present study was undertaken to investigate in detail functional antagonism between methacholine- or histamine-induced contraction and isoprenaline-mediated relaxation in bovine trachea. Furthermore, the putative role of muscarinic M₂ receptors in this process was investigated. This may be especially interesting since bovine tracheal smooth muscle has been reported to comprise a higher proportion of muscarinic M₂ binding sites (Roffel *et al.*, 1988; Lucchesi *et al.*, 1990; Schaefer *et al.*, 1992) than guinea-pig trachea (Haddad *et al.*, 1991). This latter question was addressed by comparing the influence of the selective muscarinic receptor antagonists, AF-DX 116 and gallamine (M₂/M₃-selective, cf. Fernandes *et al.*, 1992; Arjona *et al.*, 1993), pirenzepine (M₁/M₂-selective, cf. Mitchell *et al.*, 1993), and hexahydrosiladiphenidol (M₃/M₂-selective, cf. Fernandes *et al.*, 1992), on isoprenaline-induced relaxation of methacholine- and histamine-induced contractile tone, the latter serving as a control. Part of this work was presented to the British Pharmacological Society (Meurs *et al.*, 1993).

Methods

General

Fresh bovine tracheae were obtained from the local slaughterhouse and transported to the laboratory in room temperature-Krebs-Henseleit buffer solution that had been pre-gassed with 95% O₂/5% CO₂; pH 7.4. The tracheal smooth muscle was carefully dissected and smooth muscle strips (10 × 1 mm) were prepared free of mucosa and connective tissue in Krebs-

Henseleit solution gassed with 95% O₂/5% CO₂ at room temperature. These strips were mounted in 20 ml organ baths (Krebs-Henseleit, 37°C) under isotonic recording with a preload of 0.5 g. After a 90 min equilibration period the strips were precontracted twice by cumulative administration of methacholine (0.1, 1, 10 and 0.1, 1, 10, 100 μ M, respectively) with washing periods of 60 min, between which maximal relaxation was established with isoprenaline (0.1 μ M) immediately followed by a 30 min washing period.

Gallamine experiments

The preparations were subsequently contracted with different concentrations of methacholine (0.01–1000 μ M) or histamine (0.1–1000 μ M), building up smooth muscle tone in 2–4 concentration steps, and cumulative concentration-relaxation curves were obtained with isoprenaline. When the maximal response to isoprenaline had been obtained, the preparations were washed twice and maximal relaxation was re-established with 10 and 100 μ M isoprenaline. The effect of muscarinic M₂ receptor blockade on isoprenaline-induced relaxation was determined with the M₂/M₃-selective muscarinic receptor antagonist, gallamine (30 μ M), administered 30 min before building up tone with methacholine or histamine. This concentration was chosen so as to block selectively muscarinic M₂ receptors (pK_i on bovine heart 6.5; fractional receptor occupancy 99%) compared to muscarinic M₃ receptors (apparent pK_B on bovine trachea 4.1; fractional receptor occupancy 28%) (Roffel *et al.*, 1988).

Stability of the contractions for the duration of the isoprenaline relaxation curve was not monitored in paired controls. However, from independent experiments it was inferred that the methacholine- and histamine-induced contractions are stable for at least 45 and 25 min, respectively i.e. for the largest part of the relaxation curve.

Readjusted tone experiments

In another type of experiment, contractile tone was first induced with methacholine (0.1, 0.3 or 1.0 μ M) or histamine (10 μ M) and then the M₂/M₃-selective muscarinic receptor antagonist, AF-DX 116 (0.1 or 0.5 μ M), the M₁/M₂-selective antagonist, pirenzepine (0.1 μ M), or the M₃/M₂-selective antagonist, hexahydrosiladiphenidol (0.03 μ M) was added. After 30 min of antagonist incubation, the original agonist concentration was added once again to compensate for antagonist-induced decrease of contractile tone, if any (cf. Fernandes *et al.*, 1992), and isoprenaline relaxation curves were obtained as described above. The antagonist concentrations were chosen so as to produce selective blockade of M₂ receptors (AF-DX 116 0.1 μ M; fractional receptor occupancy 50% for M₂ and 17% for M₃), or half-maximal blockade of M₃ receptors (AF-DX 116 0.5 μ M, pirenzepine 0.1 μ M, hexahydrosiladiphenidol 0.03 μ M; fractional M₂ receptor occupancy 83%, 14%, and 7%, respectively), based on pK_B and pK_i values for muscarinic M₃ and M₂ receptors in bovine trachea and heart, respectively (Roffel *et al.*, 1988). Readjustment of tone was achieved by a second dose of the original agonist concentration because antagonist concentrations (except AF-DX 116 0.1 μ M) had been chosen to reflect K_B values towards bovine tracheal smooth muscle M₃ receptors (Roffel *et al.*, 1988), thereby also mimicking the pirenzepine concentration applied in the previous study (Mitchell *et al.*, 1993).

Data analysis

Contractile responses were expressed as percentages of the response to 100 μ M methacholine in the second precontraction in each experiment. Maximal relaxant effects of isoprenaline (E_{max}) were expressed as percentages of the full relaxation obtained with isoprenaline at the end of each experiment. Isoprenaline pD₂ (–log EC₅₀) and E_{max} values in the absence and presence of selective muscarinic antagonists were com-

pared by Student's paired *t* test. Statistical significance was assumed at the 5% level.

Drugs

Methacholine chloride, histamine dihydrochloride, (–)-isoprenaline hydrochloride and gallamine triethiodide were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Pirenzepine dihydrochloride and AF-DX 116 (11-[[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5, 11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepin-6-one) were kind gifts from Thomae GmbH (Biberach an der Riss, Germany) and hexahydrosiladiphenidol hydrochloride was a gift from Dr G Lambrecht (Frankfurt am Main, Germany). All other chemicals were of reagent grade. Fresh solutions of isoprenaline were made up every day in Krebs-Henseleit buffer solution containing 0.5 mM ascorbic acid as the anti-oxidant. Fresh solu-

tions of the other drugs were made up every day in Krebs-Henseleit buffer solution (methacholine, histamine) or ultra-pure water (muscarinic antagonists).

Results

Functional antagonism in bovine trachea and the influence of gallamine

Isoprenaline-induced relaxation of bovine tracheal smooth muscle preparations was dependent on the nature and the concentration of the contractile agonist used (Figure 1). Both with methacholine and histamine, isoprenaline pD₂ values decreased with increasing contractile tone. However, the effect was more pronounced when methacholine was used, i.e. a maximum shift of 3.7 log units compared to 2.4 log units with histamine (Table 1). Maximum relaxation was also decreased with increasing contractile tone in the case of methacholine, but hardly in the case of histamine. Thus, methacholine-induced contractions were almost fully (99%) relaxed up to 70% contractile tone (0.1 µM methacholine) but E_{max} gradually decreased to only 1.3% at higher contraction levels. With histamine, only the highest contraction level (103%, 1 mM) showed a small (4%) decrease in isoprenaline E_{max} values. As a result, isoprenaline pD₂ and E_{max} values were higher with histamine compared to methacholine at equal levels of contractile tone (Table 1, Figure 2a and c). In the presence of the M₂/M₃-selective muscarinic receptor antagonist, gallamine (30 µM), isoprenaline relaxation curves were shifted to the left (by 0.5 to 1.1 log units) for the lower concentrations of methacholine (0.03–3.0 µM) and by 0.1 to 0.4 log units for all concentrations of histamine (Table 1). Maximal relaxation was increased for all concentrations of methacholine. Furthermore, the contraction levels induced by a given concentration of either agonist were decreased in the presence of gallamine, in such a way that a 3 fold higher concentration of agonist induced approximately the same level of contraction in the presence of the antagonist (Table 1). No contraction was obtained with methacholine 0.01 µM and histamine 0.1 µM in the presence of gallamine, whereas contraction levels with histamine 0.3 µM in the presence of gallamine were variable. As

Figure 1 Relaxation of bovine tracheal smooth muscle preparations by isoprenaline following contraction by increasing concentrations of methacholine (a) and histamine (b) in the absence of gallamine; methacholine 0.01 µM (◆), 0.03 µM (◇), 0.1 µM (▲), 0.3 µM (△), 1.0 µM (■), 3.0 µM (□), 10 µM (▼), 100 µM (▽), 1000 µM (●); histamine 0.1 µM (▲), 0.3 µM (△), 1.0 µM (■), 3.0 µM (□), 10 µM (▼), 100 µM (▽), 1000 µM (●). Contraction levels are expressed as percentage of the response to 100 µM methacholine in the second precontraction (see Methods). Data represent means ± s.e. mean of 4 or 5 experiments.

Figure 2 Relationships between contractile tone induced by various concentrations of methacholine (a and b) or histamine (c and d), in the absence (a and c) and presence (b and d) of the M₂-selective muscarinic antagonist, gallamine (30 µM), and isoprenaline relaxation potencies (pD₂) in bovine trachea. Each data point represents one agonist concentration (methacholine or histamine), according to Table 1. Contractile tone expressed as in Figure 1. Error bars (Table 1) have been omitted for reasons of clarity.

shown in Table 1 and seen in Figure 2, the increase in isoprenaline pD₂ values at a given concentration of both methacholine and histamine in the presence of gallamine paralleled the decrease in contractile tone under these conditions to an important extent, and this was similarly true for isoprenaline E_{max} values (not shown).

The influence of selective muscarinic antagonists on functional antagonism in bovine trachea

Isoprenaline-induced relaxation of bovine tracheal smooth muscle strips in the absence of muscarinic receptor antagonists in this independent series of experiments was in good agreement with the controls of the gallamine experiments (cf. Tables 1 and 2). Isoprenaline pD₂ values were increased in the presence of *all* types of selective muscarinic antagonist, i.e. pirenzepine 0.1 µM, AF-DX 116 0.1 and 0.5 µM and

hexahydrosiladiphenidol 0.03 µM, for all concentrations of methacholine investigated (0.1, 0.3 and 1.0 µM). With histamine 10 µM, isoprenaline pD₂ values decreased in the presence of the muscarinic antagonists, except for pirenzepine (Table 2). Amongst these changes in isoprenaline pD₂ values in the presence of muscarinic antagonists, only the increase with hexahydrosiladiphenidol at 1.0 µM methacholine and the decrease at 10 µM histamine were statistically significant (*P* < 0.05 and 0.02, respectively). Maximum relaxation of 1.0 µM methacholine-induced contractile tone by isoprenaline was also increased by all types of selective muscarinic antagonist (Figure 3), from 66.9 ± 4.4% in controls (mean ± s.e.mean, *n* = 12) to 76.8 ± 4.1% in the presence of 0.1 µM AF-DX 116 (*n* = 8; not significantly different from control), 87.2 ± 3.3% with 0.5 µM AF-DX 116 (*n* = 8; *P* < 0.005 compared to control), 89.8 ± 4.2% with 0.03 µM hexahydrosiladiphenidol (*n* = 8; *P* < 0.005), and 93.4 ± 2.2% in the presence of 0.1 µM piren-

Table 1 Isoprenaline pD₂ and E_{max} values obtained after contraction of bovine tracheal smooth muscle preparations with different concentrations of methacholine and histamine in the absence or presence of an M₂-selective concentration of the muscarinic receptor antagonist, gallamine (30 µM)

[Methacholine] (µM)		Contraction level (%)		pD ₂ (-log M)		E _{max} (%)	
Control	Gallamine	Control	Gallamine	Control	Gallamine	Control	Gallamine
0.01	0.03	22.4 ± 3.9	16.6 ± 8.3	9.19 ± 0.03	9.27 ± 0.24	98.8 ± 0.8	100.0 ± 0.0
0.03	0.1	46.2 ± 4.5	37.6 ± 3.6	8.70 ± 0.13	8.92 ± 0.19	99.4 ± 0.05	98.8 ± 0.2
0.1	0.3	70.2 ± 4.0	70.2 ± 2.5	7.92 ± 0.10	8.18 ± 0.15	98.8 ± 0.6	97.8 ± 1.1
0.3	1.0	90.9 ± 1.6	92.3 ± 1.2	7.09 ± 0.10	7.11 ± 0.12	95.2 ± 0.9	84.3 ± 4.7
1.0	3.0	96.7 ± 2.1	97.6 ± 1.8	6.60 ± 0.12	6.68 ± 0.21	71.6 ± 3.6	65.2 ± 9.6
3.0	10	99.8 ± 0.9	101.6 ± 1.1	6.12 ± 0.09	5.88 ± 0.20	39.4 ± 9.6	25.3 ± 4.7
10	100	104.8 ± 2.1	102.8 ± 0.9	5.96 ± 0.18	5.33 ± 0.12	10.0 ± 2.7	8.4 ± 2.4
100	1000	104.4 ± 1.4	103.9 ± 1.4	5.50 ± 0.23	5.46 ± 0.21	3.0 ± 0.8	3.0 ± 0.1
1000		106.1 ± 1.2		5.74 ± 0.31		1.3 ± 0.3	
[Histamine]							
0.1	0.3	21.5 ± 3.9	^a	9.73 ± 0.27	^a	99.1 ± 0.9	^a
0.3	1.0	40.6 ± 6.9	39.4 ± 5.1	9.35 ± 0.03	9.19 ± 0.18	99.3 ± 0.7	100.0 ± 0.0
1.0	3.0	69.6 ± 6.5	63.8 ± 4.6	8.87 ± 0.07	8.91 ± 0.16	99.6 ± 0.4	99.8 ± 0.1
3.0		72.8 ± 6.1		8.51 ± 0.10		99.2 ± 0.5	
10	10	89.2 ± 4.9	83.5 ± 3.4	8.21 ± 0.08	8.47 ± 0.14	98.4 ± 0.7	99.4 ± 0.3
100	100	99.6 ± 1.9	97.7 ± 1.5	7.57 ± 0.16	7.67 ± 0.26	98.5 ± 1.0	96.7 ± 2.0
1000	1000	102.6 ± 2.6	98.0 ± 3.5	7.33 ± 0.05	7.63 ± 0.24	95.3 ± 1.5	92.4 ± 6.1

Results are means ± s.e.mean of 4–5 (control) or 7 (gallamine) experiments, except methacholine 0.03 mM in the presence of gallamine (3 experiments). Contraction levels are expressed as percentage of the response to 100 µM methacholine in the second precontraction (see Methods). Note that methacholine and histamine concentrations in the absence and presence of gallamine have been arranged so as to compare equal contraction levels.

^aData highly variable (see text).

Table 2 Isoprenaline pD₂ values for relaxation of bovine tracheal smooth muscle, contracted by selected concentrations of methacholine and histamine, in the absence or presence of selective muscarinic receptor antagonists

	Methacholine 0.1	Methacholine 0.3	Methacholine 1.0	Histamine 10 µM
Control	8.11 ± 0.10 (8) (73.7 ± 2.2)	7.33 ± 0.11 (12) (89.8 ± 1.4)	6.59 ± 0.12 (12) (97.8 ± 1.0)	8.72 ± 0.13 (8) (71.9 ± 4.8)
Pirenzepine 0.1 µM	8.13 ± 0.17 (4) (76.2 ± 5.0)	7.75 ± 0.21 (4) (84.0 ± 2.0)	7.06 ± 0.19 (4) (92.9 ± 2.3)	8.77 ± 0.17 (4) (74.8 ± 2.0)
AF-DX 116 0.1 µM	8.20 ± 0.09 (4) (66.6 ± 6.6)	7.63 ± 0.12 (8) (84.0 ± 2.6)*	6.88 ± 0.15 (8) (95.7 ± 2.2)	8.59 ± 0.26 (4) (74.0 ± 11.9)
AF-DX 116 0.5 µM	8.44 ± 0.07 (4) (61.6 ± 6.1)*	7.70 ± 0.12 (8) (85.0 ± 3.8)	6.84 ± 0.12 (8) (92.1 ± 1.6)***	8.60 ± 0.24 (4) (73.7 ± 9.6)
Hexahydrosiladiphenidol 0.03 µM	8.22 ± 0.16 (4) (64.7 ± 5.2)	7.68 ± 0.10 (8) (82.9 ± 2.5)***	6.95 ± 0.11 (8)* (93.2 ± 1.8)***	8.23 ± 0.13 (4)** (81.7 ± 4.0)

Results are expressed as means ± s.e.mean of (*n*) experiments. Values in parentheses represent contraction levels after 30 min of antagonist incubation and readjustment of tone, if necessary (see Methods), and are expressed as in Table 1. Significantly different from control: **P* < 0.05, ***P* < 0.02, ****P* < 0.01.

Figure 3 Relaxation of bovine tracheal smooth muscle preparations by isoprenaline after contraction by 1.0 μ M methacholine in the absence (●) and presence of muscarinic antagonists (AF-DX 116 0.1 μ M, ▽; AF-DX 116 0.5 μ M, ▼; hexahydrosiladiphenidol 0.03 μ M, □; pirenzepine 0.1 μ M, ■), with readjustment of tone if necessary (see Methods). Contraction levels (means of 4–12 experiments as indicated in Table 2) expressed as in Figure 1; error bars did not exceed 7% and have been omitted for reasons of clarity.

Figure 4 Relationship between contractile tone induced by various concentrations of methacholine (indicated as the antilog of the molar concentration on the right hand ordinate scale) in the absence (●) and presence of the selective muscarinic antagonists, AF-DX 116 (M₂, 0.1 μ M, □; 0.5 μ M, ■), pirenzepine (M₁, 0.1 μ M, ▲), and hexahydrosiladiphenidol (M₃, 0.03 μ M, ○), and isoprenaline relaxation potencies (pD₂) in bovine trachea. Contractile tone (expressed as in Figure 1) was readjusted after 30 min of antagonist incubation, if necessary. Control data for 0.03 μ M methacholine were taken from pirenzepine experiments (not shown) and gallamine experiments (Table 1).

enzepine ($n=4$; $P<0.02$). Furthermore, the contraction levels induced by either agonist were decreased by the addition of muscarinic receptor antagonists in almost half of the experiments, dependent on both the agonist and the antagonist concentration involved, i.e. more frequently with methacholine 0.1 μ M and AF-DX 116 0.3 μ M or hexahydrosiladiphenidol 0.03 μ M. The decrease in tone was largely but not fully corrected by doubling (after 30 min of antagonist incubation, see Methods) the original agonist concentration. Agonist concentrations were not increased further to exclude the risk of introducing additional inhibition of the β -adrenergic signalling system through M₃ receptor-mediated cross-talk (see below),

which would easily obscure the putative effects of M₂ receptor blockade on isoprenaline relaxation potencies. As shown in Figure 4, the increase in isoprenaline pD₂ values at a given concentration of methacholine in the presence of selective muscarinic antagonists paralleled the decrease in contractile tone under these conditions to an important extent, and this was similarly true for isoprenaline E_{max} values (not shown).

Discussion

The aim of the present study was to establish the nature and the extent of the functional antagonism between contractile agonists, i.e. methacholine and histamine, and isoprenaline in bovine tracheal smooth muscle *in vitro*, and the putative role that muscarinic M₂ receptors inhibiting β -adrenoceptor-stimulated adenylyl cyclase (or potassium channel) activity play in this process. It was found that gradually increasing cholinergic tone from low to supramaximal levels resulted in a pronounced, methacholine concentration-dependent, decrease in the ability of isoprenaline to relax the smooth muscle, both regarding the concentration giving half-maximal effect (4,900 fold shift) as well as the magnitude of the maximal effect (from 100 to 1%). Interestingly, a clear decrease in E_{max} only started to emerge when contractile tone rose over 90%, when the shift in pD₂ values was already more than 2.0 log units. Increasing histamine-induced tone in bovine trachea also resulted in a marked decrease in the ability of isoprenaline to relax the tissue, but in contrast to methacholine-induced tone this hardly affected E_{max} values, and the shift in pD₂ values was markedly smaller than in the former case (250 fold). These observations may be related to quantitative differences in receptor reserve between muscarinic and histamine receptors, as have indeed been demonstrated in bovine trachea (Grandordy & Barnes, 1987). Such difference in receptor reserve may also explain the differential ability of isoprenaline to relax identical levels of contractile tone induced by methacholine and histamine, as signified in Figure 2a and c.

Comparing bovine trachea to guinea-pig (Torphy, 1984; Van Amsterdam *et al.*, 1989; Roffel *et al.*, 1993; Watson & Eglen, 1994) and dog trachea (Torphy *et al.*, 1983; Russell, 1984; Gunst *et al.*, 1987; 1989; Mitchell *et al.*, 1993), and to human bronchial smooth muscle (Van Amsterdam *et al.*, 1990), it appears that functional antagonism by isoprenaline can be quite different quantitatively, both regarding the difference between histamine and cholinergic agonists, and the influence of raising cholinergic tone on isoprenaline pD₂ and E_{max} values. These differences may be related to receptor reserve (as discussed above) but also to receptor cross-talk between contractile and relaxant agonists, and/or to a differential importance of muscarinic M₂ receptors inhibiting isoprenaline-induced biochemical responses as discussed below.

Gallamine experiments

The question whether muscarinic M₂ receptors inhibiting β -adrenoceptor-mediated biochemical responses contribute to the functional antagonism between methacholine (but not histamine) and isoprenaline in bovine tracheal smooth muscle *in vitro* was first addressed by use of the muscarinic M₂/M₃-selective receptor antagonist, gallamine. This compound was chosen because it is more selective for M₂ over M₃ receptors than AF-DX 116 and methoctramine (Roffel *et al.*, 1988; Lee & El-Fakahany, 1991), which is of major importance when studying mixed M₂/M₃ receptor systems. As shown in Table 1, it was observed that isoprenaline relaxation in the presence of gallamine was facilitated both in terms of pD₂ and E_{max} values. This would indeed suggest that muscarinic M₂ receptors play a role in this functional antagonism, as has recently also been reported using gallamine in rabbit trachea (Arjona *et al.*, 1993). However, isoprenaline relaxation of histamine-induced tone, which should not involve muscarinic M₂ receptors in any fashion, was also facilitated in the presence of gallamine, al-

though to a smaller extent (average pD₂ shift for isoprenaline 0.3 versus 0.7 log units in case of methacholine). Moreover, gallamine did influence contraction levels obtained with all methacholine and histamine concentrations, the effect being rather marked at lower levels, and it was concluded that the increase in isoprenaline relaxant action in the presence of gallamine could be fully attributed to these decreases in contractile tone for *both* agonists (Figure 2). As a result, these experiments do not provide any evidence for a role of muscarinic M₂ receptors in the functional antagonism between methacholine and isoprenaline in bovine trachea. Furthermore, they stress the importance of performing non-cholinoceptor controls (as also incorporated in the study of canine trachea by Fernandes *et al.* (1992)), and taking into account differences in contraction levels.

It should of course be questioned why gallamine in apparent M₂-selective concentration influenced cholinoreceptor as well as histamine-induced contractile tone, especially since this concentration of gallamine hardly shifted methacholine concentration-response curves in previous experiments (Roffel *et al.*, 1988), which was confirmed in the course of the present study (not shown). In fact, we do not have a satisfactory explanation for this, although the observation that gallamine affected both methacholine- and histamine-induced contractions suggests that some unspecific property of this compound is involved.

Readjusted tone experiments

The putative role of muscarinic M₂ receptors in functional antagonism in bovine trachea was next investigated by adopting the experimental paradigm applied successfully in canine trachea (Fernandes *et al.*, 1992). Thus, the M₂/M₃-selective muscarinic receptor antagonist AF-DX 116, in concentrations giving selective (half-maximal) M₂ receptor blockade (0.1 µM) or half-maximal M₃ receptor blockade (0.5 µM), the M₃/M₂-selective antagonist hexahydrosiladiphenidol, in a concentration giving half-maximal M₃ receptor blockade (0.03 µM, concentrations based on pK_B and pK_i values taken from Roffel *et al.*, 1988), or vehicle were added after contractile tone had been induced, and contractile tone was readjusted after 30 min of antagonist incubation to compensate for antagonist-induced inhibition of tone, if any (see Methods and Results). Pirenzepine was also subjected to this protocol because this compound had also been reported to facilitate isoprenaline relaxation in canine trachea (Mitchell *et al.*, 1993), which was explained by functional uncoupling of M₃ receptors from adenylyl cyclase. Finally, in contrast to previous studies, three different methacholine concentrations (and thus contraction levels) in addition to one histamine concentration (serving as a control) were investigated, in order to increase the possibility of detecting a specific role for M₂ receptors.

It was found that isoprenaline relaxation potencies were increased, up to 3 fold and in large part not significantly, by all types of selective muscarinic receptor antagonist and for all concentrations of methacholine, whereas for histamine-induced contractions isoprenaline relaxation was somewhat decreased, except with pirenzepine. Maximum relaxation to the highest concentration of methacholine was also increased by all antagonists (Figure 3). Although the results with AF-DX 116 and pirenzepine are in apparent agreement with those obtained in canine trachea (Fernandes *et al.*, 1992; Mitchell *et al.*, 1993), they do not appear to suggest a *specific* role for muscarinic M₂ receptors in the functional antagonism between methacholine and isoprenaline in bovine trachea. This conclusion is based on the observation that, in contrast to the dog (Fernandes *et al.*, 1992), the M₃/M₂-selective muscarinic receptor antagonist hexahydrosiladiphenidol also produced increased relaxant action by isoprenaline in bovine trachea, and on the fact that the effect of the M₁/M₂-selective antagonist pirenzepine cannot be attributed to selective blockade of M₂ muscarinic receptors, the affinity towards this receptor subtype being (somewhat) lower than for M₃ and of course M₁ re-

ceptors (Roffel & Zaagsma, 1995). By contrast, the (small) effects of the selective muscarinic receptor antagonists on isoprenaline relaxation in bovine trachea can be fully explained (similar to the gallamine experiments discussed above) by the (small) differences in contractile tone that were encountered in these experiments. Thus, despite the readjustment of contractile tone following antagonist incubation, contractile levels were generally slightly, although only occasionally significantly, lower in the preparations incubated with antagonist, and it can be observed from Figure 4 that the increase in isoprenaline pD₂ values was related to these changes in contractile tone (cf. gallamine experiments); a similar explanation applies to the increases in maximum relaxability observed in case of 1.0 µM methacholine (relationship not shown). Similarly, the significant change in isoprenaline pD₂ values in the presence of hexahydrosiladiphenidol with histamine as the contractile agonist appears to be related to the higher contraction level reached in those incubations (Figure 3, Table 2).

A role for muscarinic M₂ receptors in functional antagonism?

Summarizing the experiments with the selective muscarinic antagonists, pirenzepine, AF-DX 116, gallamine, and hexahydrosiladiphenidol, it must be concluded that the data can be explained without introducing a specific role for muscarinic M₂ receptors in the functional antagonism between methacholine and isoprenaline in bovine trachea. Furthermore, these experiments clearly showed the importance of taking into account the starting level of contraction, since rather small variation in this level, i.e. only a few percent, can critically determine isoprenaline relaxation, both regarding pD₂ and E_{max} values. The question remains why, in contrast to bovine trachea, muscarinic M₂ receptors inhibiting β-adrenoceptor-stimulated adenylyl cyclase or potassium channel activity appear to contribute, although only moderately, to functional antagonism in canine, rabbit and guinea-pig trachea, provided that these studies have indeed taken into account any small differences in starting contractions. The first and rather interesting possibility is that a role for these M₂ receptors can only be observed using isometric tension measurement, as was applied in all studies concerned (Fernandes *et al.*, 1992; Arjona *et al.*, 1993; Mitchell *et al.*, 1993; Watson & Eglén, 1994). This is indirectly supported by our own observation, using isotonic measurement, that such a role is absent in guinea-pig trachea (Roffel *et al.*, 1993). Alternatively, the relative population of tracheal smooth muscle M₂ receptors in these species and/or their ability to inhibit adenylyl cyclase may be involved. The first of these possibilities does not seem to be valid, since this proportion has been reported to be smaller in guinea-pig than in the other species (Haddad *et al.*, 1991), and rather similar in dog, cow, and rabbit (Roffel *et al.*, 1988; Lucchesi *et al.*, 1990; Schaefer *et al.*, 1992; Fernandes *et al.*, 1992; Mahesh *et al.*, 1992). Concerning the inhibition of adenylyl cyclase by these M₂ receptors, it appears that this response is less efficacious in guinea-pig compared to canine and bovine trachea (cf. Jones *et al.*, 1987; Meurs *et al.*, 1992; Pyne *et al.*, 1992; Challiss *et al.*, 1993; Ethier *et al.*, 1993; see Roffel & Zaagsma, 1995 for review), which therefore does not readily explain the reported differences in contraction/relaxation experiments. As a final explanation, it may be suggested that the other mechanism that has been implicated in functional antagonism, i.e. cross-talk between receptor-mediated phospholipase C activation and β-adrenoceptor function through protein kinase C (see Introduction), is more pronounced in bovine trachea compared to the other species. However, information on this subject is not available, and further experiments should resolve this intriguing possibility.

We gratefully acknowledge the kind gifts of pirenzepine and AF-DX 116 by Thomae GmbH (Biberach an der Riss, Germany) and of hexahydrosiladiphenidol by Dr G. Lambrecht (Frankfurt am Main, Germany). Dr B.H. Hoiting is acknowledged for kindly providing computer drawings.

References

- AL-HASSANI, M.H., GARCIA, J.G.N. & GUNST, S.J. (1993). Differences in Ca²⁺ mobilization by muscarinic agonists in tracheal smooth muscle. *Am. J. Physiol.*, **264**, L53-L59.
- AMSTERDAM, R.G.M. VAN, MEURS, H., BERGE, R.E.J., TEN, VENINGA, C.C.M., BROUWER, F. & ZAAGSMA, J. (1990). Role of phosphoinositide metabolism in human bronchial smooth muscle contraction and in functional antagonism by β -adrenoceptor agonists. *Am. Rev. Respir. Dis.*, **142**, 1124-1128.
- AMSTERDAM, R.G.M. VAN, MEURS, H., BROUWER, F., POSTEMA, J.-B. TIMMERMANS, A. & ZAAGSMA, J. (1989). Role of phosphoinositide metabolism in functional antagonism of airway smooth muscle contraction by β -adrenoceptor agonists. *Eur. J. Pharmacol.*, **172**, 175-183.
- ARJONA, N.C., SCHRAMM, C.M. & GRUNSTEIN, M.M. (1993). Maturation of muscarinic M₂ receptor modulation of beta-adrenoceptor responsiveness in rabbit airway smooth muscle. *Am. Rev. Respir. Dis.*, **147**, A177.
- CHALLISS, R.A.J., ADAMS, D., MISTRY, R. & BOYLE, J.P. (1993). Second messenger and ionic modulation of agonist-stimulated phosphoinositide turnover in airway smooth muscle. *Biochem. Soc. Trans.*, **21**, 1138-1145.
- ETHIER, M.F., SCHAEFER, O.P. & MADISON, J.M. (1993). Muscarinic receptor reserve for inhibition of cAMP accumulation in bovine trachealis cells. *Am. Rev. Respir. Dis.*, **147**, A172.
- FERNANDES, L.B., FRYER, A.D. & HIRSHMAN, C.A. (1992). M₂ Muscarinic receptors inhibit isoproterenol-induced relaxation of canine airway smooth muscle. *J. Pharmacol. Exp. Ther.*, **262**, 119-126.
- FRYER, A.D. & EL-FAKAHANY, E.E. (1990). Identification of three muscarinic receptor subtypes in rat lung using binding studies with selective antagonists. *Life Sci.*, **47**, 611-618.
- GARDIER, R.W., BLAXALL, H.S., KILLIAN, L.N. & CUNNINGHAM, J. (1991). Reserpine-induced post-receptor reduction in muscarinic-mediated airway smooth muscle contraction. *Life Sci.*, **48**, 1705-1713.
- GRANDORDY, B.M. & BARNES, P.J. (1987). Phosphoinositide turnover. *Am. Rev. Respir. Dis.*, **136**, S17-S20.
- GRANDORDY, B.M., MAK, J.C.W. & BARNES, P.J. (1993). Modulation of airway smooth muscle β -adrenoceptor function by a muscarinic agonist. *Life Sci.*, **54**, 185-191.
- GUNST, S.J., STROPP, J.Q. & FLAVAHAN, N.A. (1987). Analysis of receptor reserves in canine tracheal smooth muscle. *J. Appl. Physiol.*, **62**, 1755-1758.
- GUNST, S.J., STROPP, J.Q. & FLAVAHAN, N.A. (1989). Muscarinic receptor reserve and β -adrenergic sensitivity in tracheal smooth muscle. *J. Appl. Physiol.*, **67**, 1294-1298.
- HADDAD, E.-B., LANDRY, Y. & GIES, J.-P. (1991). Muscarinic receptor subtypes in guinea-pig airways. *Am. J. Physiol.*, **261**, L327-L333.
- JONES, C.A., MADISON, J.M., TOM-MOY, M. & BROWN, J.K. (1987). Muscarinic cholinergic inhibition of adenylate cyclase in airway smooth muscle. *Am. J. Physiol.*, **253**, C97-C104.
- KOTLIKOFF, M.I., FLEISCHMANN, B., KUME, H. & TOMASIC, M. (1994). Signal transduction and calcium homeostasis in airway smooth muscle. In *Bronchitis V*. ed. Postma, D.S. & Gerritsen, J. pp. 97-105. Assen, The Netherlands: Van Gorcum.
- LEE, N.H. & EL-FAKAHANY, E.E. (1991). Allosteric interactions at the m1, m2 and m3 muscarinic receptor subtypes. *J. Pharmacol. Exp. Ther.*, **256**, 468-479.
- LUCCHESI, P.A., SCHEID, C.R., ROMANO, F.D., KARGACIN, M.E., MULLIKIN-KILPATRICK, D., YAMAGUNCHI, H. & HONEYMAN, T.W. (1990). Ligand binding and G protein coupling of muscarinic receptors in airway smooth muscle. *Am. J. Physiol.*, **258**, C730-C738.
- MAHESH, V.K., NUNAN, L.M., HALONEN, M., YAMAMURA, H.I., PALMER, J.D. & BLOOM, J.W. (1992). A minority of muscarinic receptors mediate rabbit tracheal smooth muscle contraction. *Am. J. Respir. Cell Mol. Biol.*, **6**, 279-286.
- MEURS, H., ELZINGA, C.R.S., BOER, R.E.P. DE, AMSTERDAM, R.G.M. VAN, BROUWER, F. & ZAAGSMA, J. (1992). Muscarinic receptor mediated inhibition of adenylate cyclase and its role in the functional antagonism of cholinergic airway smooth muscle contraction by β -agonists. *Am. Rev. Respir. Dis.*, **145**, A438.
- MEURS, H., KAUFFMAN, H.F., KOËTER, G.H., TIMMERMANS, A. & VRIES, K. DE (1987). Regulation of the beta-receptor-adenylate cyclase system in lymphocytes of allergic patients with asthma: possible role for protein kinase C in allergen-induced nonspecific refractoriness of adenylate cyclase. *J. Allergy Clin. Immunol.*, **80**, 326-339.
- MEURS, H., ROFFEL, A.F., ELZINGA, C.R.S., BOER, R.E.P. DE & ZAAGSMA, J. (1993). Muscarinic receptor-mediated inhibition of adenylate cyclase and its role in the functional antagonism of isoprenaline in airway smooth muscle. *Br. J. Pharmacol.*, **108**, 208P.
- MITCHELL, R.W., KOENIG, S.M., POPOVICH, K.J., KELLY, E., TALLEY, J. & LEFF, A.R. (1993). Pertussis toxin augments β -adrenergic relaxation of muscarinic contraction in canine trachealis. *Am. Rev. Respir. Dis.*, **147**, 327-331.
- MUTSCHLER, E., MOSER, U., WESS, J. & LAMBRECHT, G. (1988). New approaches to the subclassification of muscarinic receptors. In *Recent Advances in Receptor Chemistry*. ed. Melchiorre, C. & Gianella, M. pp. 195-217. Amsterdam, The Netherlands: Elsevier.
- PYNE, N.J., GRADY, M.W., SHEHNAZ, D., STEVENS, P.A., PYNE, S. & RODGER, I.W. (1992). Muscarinic blockade of β -adrenoceptor-stimulated adenylate cyclase: the role of stimulatory and inhibitory guanine-nucleotide binding regulatory proteins (G_s and G_i). *Br. J. Pharmacol.*, **107**, 881-887.
- ROFFEL, A.F., ELZINGA, C.R.S., AMSTERDAM, R.G.M. VAN, ZEEUW, R.A. DE & ZAAGSMA, J. (1988). Muscarinic M₂ receptors in bovine tracheal smooth muscle: discrepancies between binding and function. *Eur. J. Pharmacol.*, **153**, 73-82.
- ROFFEL, A.F., ELZINGA, C.R.S. & ZAAGSMA, J. (1990a). Muscarinic M₃ receptors mediate contraction of human central and peripheral airway smooth muscle. *Pulm. Pharmacol.*, **3**, 47-51.
- ROFFEL, A.F., MEURS, H., ELZINGA, C.R.S. & ZAAGSMA, J. (1990b). Characterization of the muscarinic receptor subtype involved in phosphoinositide metabolism in bovine tracheal smooth muscle. *Br. J. Pharmacol.*, **99**, 293-296.
- ROFFEL, A.F., MEURS, H., ELZINGA, C.R.S. & ZAAGSMA, J. (1993). Muscarinic M₂ receptors do not participate in the functional antagonism between methacholine and isoprenaline in guinea-pig tracheal smooth muscle. *Eur. J. Pharmacol.*, **249**, 235-238.
- ROFFEL, A.F. & ZAAGSMA, J. (1995). Muscarinic cholinergic receptors. In *Airways Smooth Muscle; a Reference Source*. Volume III. ed. Giembycz, M.A. & Raeburn, D. Basel, Switzerland: Birkhäuser, (in press).
- RUSSELL, J.A. (1984). Differential inhibitory effect of isoproterenol on contractions of canine airways. *J. Appl. Physiol.*, **57**, 801-807.
- SANKARY, R.M., JONES, C.A., MADISON, J.M. & BROWN, J.K. (1988). Muscarinic cholinergic inhibition of cyclic AMP accumulation in airway smooth muscle. *Am. Rev. Respir. Dis.*, **138**, 145-150.
- SCHAEFER, O.P., ETHER, M.F. & MADISON, J.M. (1992). Characterization of the muscarinic receptor subtype mediating inhibition of cyclic AMP accumulation in bovine trachealis cells. *Am. Rev. Respir. Dis.*, **145**, A438.
- THOMAS, E.A., BAKER, S.A. & EHLERT, F.J. (1993). Functional role for the M₂ muscarinic receptor in smooth muscle of guinea-pig ileum. *Mol. Pharmacol.*, **44**, 102-110.
- TORPHY, T.J. (1984). Differential relaxant effects of isoproterenol on methacholine-versus leukotriene D₄-induced contraction in the guinea-pig trachea. *Eur. J. Pharmacol.*, **102**, 549-553.
- TORPHY, T.J., RINARD, G.A., RIETOW, M.G. & MAYER, S.E. (1983). Functional antagonism in canine tracheal smooth muscle: inhibition by methacholine of the mechanical and biochemical response to isoproterenol. *J. Pharmacol. Exp. Ther.*, **227**, 694-699.
- WATSON, N. & EGLIN, R.M. (1994). Effects of muscarinic M₂ and M₃ receptor stimulation and antagonism on responses to isoprenaline of guinea-pig trachea *in vitro*. *Br. J. Pharmacol.*, **112**, 179-187.
- WIDDOP, S., DAYKIN, K. & HALL, I.P. (1993). Expression of muscarinic M₂ receptors in cultured human airway smooth muscle cells. *Am. J. Respir. Cell Mol. Biol.*, **9**, 541-546.
- YANG, C.M., CHOU, S.-P. & SUNG, T.-C. (1991). Muscarinic receptor subtypes coupled to generation of different second messengers in isolated tracheal smooth muscle cells. *Br. J. Pharmacol.*, **104**, 613-618.

(Received September 5, 1994

Revised February 23, 1995

Accepted February 27, 1995)