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Effects of fenoterol on β-adrenoceptor and muscarinic M_2 receptor function in bovine tracheal smooth muscle

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Abstract

Prolonged (18 h) incubation of isolated bovine tracheal smooth muscle with the β_2-adrenoceptor agonist fenoterol (10 μM) induced desensitization of isoprenaline-induced adenylyl cyclase activity in bovine tracheal smooth muscle membranes, characterized by a 25% decrease in maximal effect (E_{max}) ($P < 0.05$), while the sensitivity to the agonist (pEC_{50}) was unchanged. The E_{max} value of isoprenaline-induced smooth muscle relaxation of submaximal methacholine-induced contractile tones was similarly reduced by about 25% ($P < 0.001$), while the pEC_{50} value was diminished by 1.0 log unit ($P < 0.001$). As determined by 30 μM gallamine-induced muscarinic M_2 receptor antagonism and pertussis toxin-induced inactivation of $G_{i\alpha}$, muscarinic M_2 receptor-mediated functional antagonism did not play a role in isoprenaline-induced relaxation of bovine tracheal smooth muscle contracted by methacholine, both in control and in 18-h fenoterol-treated tissue. In line with these observations, we found no enhanced muscarinic M_2 receptor-mediated inhibition of 1 μM forskolin-stimulated adenylyl cyclase activity after 18-h fenoterol treatment. These data indicate that 18-h fenoterol treatment of bovine tracheal smooth muscle induces β_2-adrenoceptor desensitization and reduced functional antagonism of methacholine-induced contraction by β-adrenoceptor agonists, without a change of muscarinic M_2 receptor function. © 2001 Published by Elsevier Science B.V.

Keywords: β_2-Adrenoceptor; Muscarinic M_2 receptor; Smooth muscle, bovine, tracheal; Desensitization; Functional antagonism; Pertussis toxin

1. Introduction

In numerous cells and tissues, it has been established that stimulation of the β_2-adrenoceptor results in desensitization of the β_2-adrenoceptor-mediated response (Hausdorff et al., 1990; Lohse, 1993; Lefkowitz, 1998). Thus, upon activation, the Ser/Thr-rich carboxy tail of the β_2-adrenoceptor is rapidly phosphorylated by the β-adrenoceptor kinase (βARK), and subsequent β-arrestin binding to the phosphorylated receptor prevents its coupling to the G_s-protein. In addition, protein kinase A (PKA) can phosphorylate the β_2-adrenoceptor at Ser/Thr residues in the third intracellular loop, also causing reduced coupling of the receptor to the G_s-protein. The reduced coupling between receptor and G-protein may result in a diminished adenylyl cyclase response (Davis and Conolly, 1980; Penn et al., 1998) as well as a decreased activation of the calcium-activated potassium (K_{Ca}) channel (Kume and Takagi, 1999). Prolonged stimulation of the β_2-adrenoceptor leads to downregulation, thus contributing to the decreased responsiveness to β-adrenoceptor agonists (Hausdorff et al., 1990).

In airway smooth muscle of various species, it has been demonstrated that β-adrenoceptor desensitization may cause reduced β-adrenoceptor agonist-induced relaxation (Lohse, 1993; Barnes, 1995). In addition, it has been established that β-adrenoceptor agonist-induced relaxation decreases at increasing smooth muscle tone, with a relatively large loss of relaxation in the presence of increasing concentrations of full muscarinic agonists such as methacholine and carbachol compared to several other agonists, including histamine, leukotrienes, serotonin and partial muscarinc agonists (Meurs and Zaagsma, 1991).

In bovine tracheal smooth muscle, muscarinic M_2 and M_3 receptors coexist (Roffel et al., 1987, 1988; Eglen et al., 1996; Zaagsma et al., 1997). The M_3 receptor has been shown to mediate airway smooth muscle contraction via activation of phosphoinositide (PI) metabolism and subsequent mobilization of intra- and extracellular calcium (Meurs et al., 1988; Roffel et al., 1990; Hoiting et al., 1996). In bovine tracheal smooth muscle, the muscarinic M_2 receptor population is the most prevailing one, accounting for 70–80% of the muscarinic receptors in this

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tissue (Roffel et al., 1987, 1988) and similar high abundances have been found in other smooth muscle types and species (Eglen et al., 1996). Upon receptor activation, the muscarinic M_2 receptor couples to the G_i-protein and subsequently inhibits adenylyl cyclase (Sankary et al., 1988; Yang et al., 1991) as well as the K_{Ca} channel (Kume et al., 1992). In bovine tracheal smooth muscle, inhibition of adenylyl cyclase by muscarinic M_2 receptor activation has also been reported (Meurs et al., 1994; Schaefer et al., 1995; Ostrom and Ehlert, 1998), which could contribute to the phenomenon that cholinergic tone is relatively resistant to β-adrenoceptor agonist-induced relaxation. However, under basal conditions, muscarinic M_2 receptor stimulation does not attenuate isoprenaline-induced relaxation of methacholine-induced tone in bovine tracheal smooth muscle (Roffel et al., 1995). Only after inactivation of most of the M_3 receptor population by treatment with *N*-(2-chloroethyl)-4-piperidinyl diphenylacetate (4-DAMP mustard), muscarinic M_2 receptor-mediated functional antagonism was shown in forskolin-but not isoprenaline-induced relaxation of bovine tracheal smooth muscle contracted with histamine (Ostrom and Ehlert, 1998).

In addition to desensitization, prolonged β_2-adrenoceptor agonist treatment has been shown to cross-regulate the expression of the muscarinic M_2 receptor (Reithmann et al., 1992) and the G_i-protein (Eschenhagen et al., 1991; Reithmann et al., 1992) in myocardial tissues. Thus, by cross-regulation of heterologous receptor transduction mechanisms, prolonged β-adrenoceptor agonist treatment could increase the muscarinic M_2 receptor-mediated functional antagonism of β_2-adrenoceptor-induced relaxation, possibly providing an additional route for reduced β-adrenoceptor-induced relaxation of cholinergic contraction after β-adrenoceptor agonist treatment.

In bovine tracheal smooth muscle, β-adrenoceptor agonist-induced changes in the functional antagonism between cholinergic contraction and β-adrenoceptor agonist-induced relaxation have thus far not been established. In the present study, we investigated fenoterol-induced β_2-adrenoceptor desensitization in bovine tracheal smooth muscle, both at the level of adenylyl cyclase activation and at the level of smooth muscle relaxation. Secondly, we investigated the possibility that enhanced muscarinic M_2 receptor function could contribute to a reduced functional antagonism of cholinergic tone by isoprenaline.

2. Materials and methods

2.1. Isometric contraction studies

Fresh bovine tracheas were obtained from the slaughterhouse and transported rapidly to the laboratory in Krebs–Henseleit (KH) buffer (117.50 mM NaCl, 5.60 mM KCl, 1.18 mM $MgSO_4$, 1.28 mM NaH_2PO_4, 2.52 mM $CaCl_2$, 25.00 mM $NaHCO_3$ and 5.55 mM D-glucose), pregassed with 95% O_2 and 5% CO_2; pH 7.4. The tracheal smooth muscle was dissected carefully, prepared free of mucosa and connective tissue, and cut into strips with an average weight of 10 mg, while incubated in KH-buffer, gassed with 95% O_2 and 5% CO_2 at room temperature. Subsequently, all strips were incubated for 18 h in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 mM $NaHCO_3$, 20 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal calf serum at 37°C, in the absence or presence of 10 μM fenoterol and/or 200 ng/ml pertussis toxin.

After washing with several volumes of KH-buffer, gassed with 95% O_2 and 5% CO_2, pH 7.4 at 37°C, the bovine tracheal smooth muscle strips were mounted in 20-ml organ baths containing gassed KH-buffer (37°C) for isometric recording. During a 90-min equilibration period, with washings every 30 min, the resting tension was gradually adjusted to 3 g. Subsequently, the strips were precontracted with 20 and 40 mM isotonic KCl and, after two changes to fresh KH-buffer, precontracted by cumulative administration of methacholine (0.1, 1, 10 μM), followed by washout for 30 min with three changes of bath volume. Under 0.1 μM isoprenaline-induced basal tone, the strips were readjusted to 3 g resting tension, immediately followed by two changes of bath volume. The entire procedure resulted in a thorough washout of fenoterol (3 h, 37°C). To evaluate the effect of pertussis toxin treatment on functional antagonism, the strips were subsequently contracted with 3 μM methacholine and cumulative dose–relaxation curves were obtained with isoprenaline (0.1 nM–30 μM). When the maximal response to isoprenaline was obtained, the strips were washed twice and basal tone was established with 10 μM isoprenaline.

In a separate set of experiments, control and fenoterol-treated strips were contracted to methacholine (0.1, 1, 10 and 100 μM) to establish maximal contraction. After washout for 30 min with three changes of bath volume, 30 μM gallamine or vehicle was added to the tissue for a 30-min preincubation period. This concentration of gallamine was chosen to selectively block muscarinic M_2 receptors (fractional receptor occupancy 99%) compared with M_3-receptors (fractional receptor occupancy 28%) (Roffel et al., 1988). To correct for the (minor) effects of gallamine and fenoterol pretreatments on methacholine-induced contraction, all strips were contracted to an equal level of 80% of the maximal methacholine-induced contraction using agonist-concentrations ranging between 1 and 10 μM, and dose–relaxation curves with isoprenaline were obtained as described above.

2.2. Adenylyl cyclase responses

Isolated tracheal smooth muscle was chopped with a McIlwain tissue chopper, three times at a setting of 500 μM, followed by three times at a setting of 100 μM. The slices were washed three times in Krebs–Ringer–Henseleit

(KRH) buffer (125.0 mM NaCl, 6.0 mM KCl, 1.2 mM $CaCl_2$, 2.5 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 11.0 mM D-glucose and 25.0 mM HEPES) supplemented with 2.0 mM (±)-dithiothreitol and resuspended in a mixture of 1.5 U/ml collagenase P, 30 U/ml papain and 1 mg/ml trypsin inhibitor in KRH-buffer supplemented with (±)-dithiothreitol. After 20-min incubation in an incubator shaker (37°C, 55 rpm), the cells were gently dispersed with a wide-bored pipette and the suspension was incubated for another 10 min (37°C, 70 rpm). Subsequently, the suspension was filtered over a 50-μM gauze and the cells were collected by centrifugation ($200 \times g$, 10 min). The isolated bovine tracheal smooth muscle cells were washed three times in DMEM supplemented with 10 mM $NaHCO_3$, 20 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal calf serum and were incubated in a suspension of $0.5–1.0 \times 10^6$ cells/ml for 18 h in the absence or presence of 10 μM fenoterol. The cell viability was > 90% as assessed by trypan blue exclusion. Subsequently, the cells were washed six times in KRH-buffer, resuspended in 50 mM Tris, 1 mM EDTA and 2 mM (±)-dithiothreitol, pH 7.4 and kept on ice for 10 min. The cells were disrupted by 50 strokes in a Potter–Elvehjem (glass/teflon) homogenizer. The crude cell lysate was centrifuged at $36{,}000 \times g$ for 30 min at 4°C. The resulting membrane pellet was resuspended in 50 mM Tris, 10 mM $MgCl_2$ and 2 mM (±)-dithiothreitol, pH 7.4 using a Potter–Elvehjem (glass/teflon) homogenizer and stored on ice. Protein content of the membrane suspension was determined according to Bradford (1976).

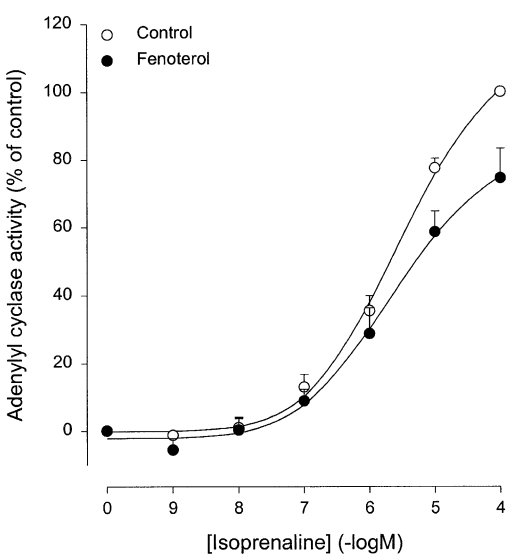

Fig. 1. Isoprenaline-induced adenylyl cyclase activity in membranes of control bovine tracheal smooth muscle cells and cells treated for 18 h with 10 μM fenoterol. Basal adenylyl cyclase activities were 128 ± 12 and 139 ± 17 pmol cAMP/mg protein/min for control and fenoterol-treated cells, respectively, and 100% adenylyl cyclase activity was 571 ± 103 pmol cAMP/mg protein/min. Results are means ± S.E.M. of seven experiments.

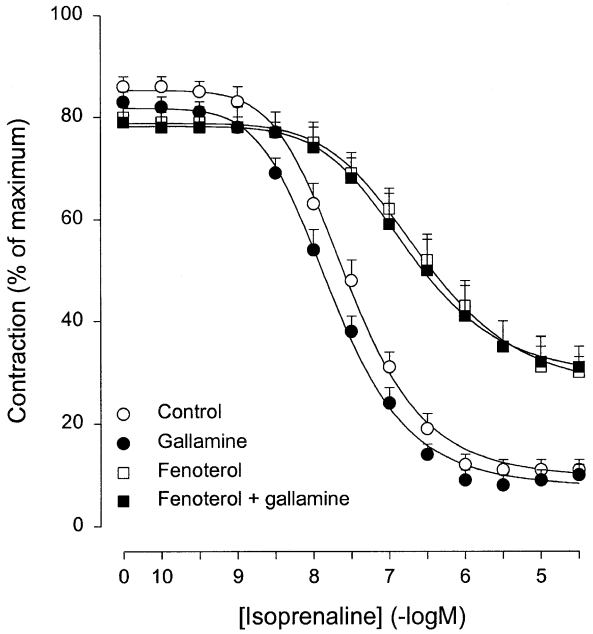

Fig. 2. Relaxation of a submaximal (80%) methacholine-induced bovine tracheal smooth muscle contraction by isoprenaline in the absence or presence of 30 μM gallamine. Bovine tracheal smooth muscle strips were pre-incubated for 18 h in the absence or presence of 10 μM fenoterol. The contraction is expressed as the percentage of maximal 100 μM methacholine-induced contraction. Results are means ± S.E.M. of five to six experiments.

Adenylyl cyclase activity was measured in triplicate samples by incubating the membrane suspension in a solution containing 50.0 mM Tris, 5.5 mM $MgCl_2$, 1.4 mM (±)-dithiothreitol, 0.1 mM ascorbic acid, 1.0 mM ATP, 0.1 mM GTP, 20 mM creatine phosphate, 50 U/ml creatine phosphokinase, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and the appropriate stimulus for 10 min at 30°C. Incubations were stopped by addition of an equal volume of 3.5% perchloric acid. Samples were neutralized with a 50% saturated $KHCO_3$ solution, centrifuged ($1000 \times g$, 10 min, 4°C) and cAMP was determined in the supernatant by a competitive protein-binding assay (Brown et al., 1971).

2.3. Data analysis

Results are expressed as means ± S.E.M. of the indicated numbers of experiments. Statistical analysis was performed by the two-tailed Student's *t*-test for paired observations. A value of $P < 0.05$ was considered statistically significant.

2.4. Materials

Tissue culture supplies were purchased form Gibco BRL Life Technologies (Paisley, UK). DMEM and methacholine were obtained from ICN Biomedicals (Costa Mesa, CA, USA). Trypsin inhibitor, fenoterol, (−)-isoprenaline,

Table 1
Relaxation of a submaximal (80%) methacholine-induced bovine tracheal smooth muscle contraction by isoprenaline in the absence and presence of 30 μM gallamine
Bovine tracheal smooth muscle strips were incubated for 18 h in the absence or presence of 10 μM fenoterol. The pEC_{50} is the negative logarithm of the concentration of isoprenaline causing half maximal relaxation. E_{max} is the maximal isoprenaline-induced relaxation expressed as the percentage of methacholine-induced tone. Data are shown as means ± S.E.M. of five to six experiments.

Incubation	Vehicle		Gallamine	
	pEC_{50} $(-\log M)$	E_{max} (%)	pEC_{50} $(-\log M)$	E_{max} (%)
Control	7.54 ± 0.09	88 ± 3	7.74 ± 0.09	91 ± 2
Fenoterol	6.56 ± 0.14[a]	65 ± 3[a]	6.76 ± 0.13[a]	62 ± 4[b]

[a] $P < 0.001$ compared to control.
[b] $P < 0.01$ compared to control.

Table 2
Relaxation of 3 μM methacholine-induced bovine tracheal smooth muscle contraction by isoprenaline after 18 h fenoterol (10 μM) and/or pertussis toxin (200 ng/ml) treatment
The pEC_{50} is the negative logarithm of the concentration of isoprenaline causing half-maximal relaxation. E_{max} is the maximal isoprenaline-induced relaxation expressed as the percentage of methacholine-induced tone. Data are shown as means ± S.E.M. of five experiments.

Incubation	Vehicle		Pertussis toxin	
	pEC_{50} $(-\log M)$	E_{max} (%)	pEC_{50} $(-\log M)$	E_{max} (%)
Control	7.09 ± 0.11	62 ± 3	7.25 ± 0.13	67 ± 2
Fenoterol	6.41 ± 0.09[a]	53 ± 6	6.52 ± 0.09[a]	61 ± 4

[a] $P < 0.001$ compared to control.

gallamine, forskolin, ATP, GTP, creatine phosphate, creatine phosphokinase and IBMX were from Sigma (St. Louis, MO, USA). Papain, collagenase and (±)-dithiothreitol were from Roche Diagnostics (Mannheim, Germany). Pertussis toxin was kindly provided by Dr. J. Westdijk (RIVM, Bilthoven, The Netherlands).

3. Results

Incubation of bovine tracheal smooth muscle cells for 18 h with 10 μM fenoterol resulted in a moderate desensitization of the isoprenaline-induced adenylyl cyclase response (Fig. 1), without a significant effect on basal adenylyl cyclase activity (128 ± 12 and 139 ± 17 pmol cAMP/mg protein/min for control cells and fenoterol-treated cells, respectively). After treatment, the maximal isoprenaline-induced adenylyl cyclase activity was significantly reduced to 75 ± 9% of control ($P < 0.05$), while the sensitivity to isoprenaline was unchanged (pEC_{50} values of 5.73 ± 0.07 and 5.82 ± 0.09 for control and fenoterol-treated cells, respectively).

Desensitization of the β-adrenoceptor response was also present at the smooth muscle level. Isoprenaline-induced relaxation of an established submaximal methacholine-induced tone (80% of maximal contraction using 1–10 μM methacholine) was reduced after 18-h incubation of the

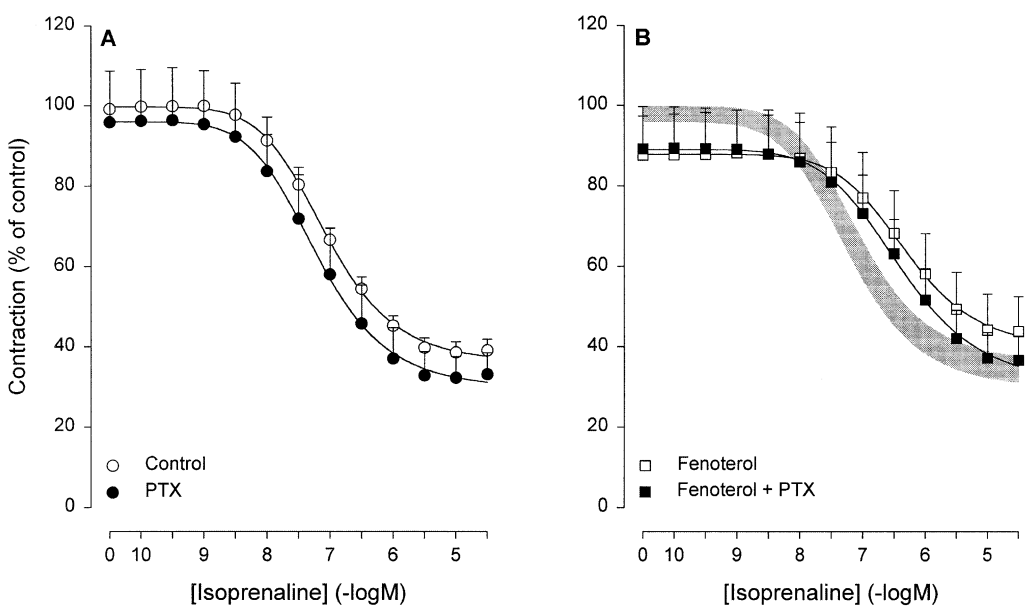

Fig. 3. Relaxation of 3 μM methacholine-induced bovine tracheal smooth muscle contraction by isoprenaline. Bovine tracheal smooth muscle strips were pre-incubated for 18 h with vehicle or 200 ng/ml pertussis toxin (panel A) and 10 μM fenoterol or 10 μM fenoterol plus 200 ng/ml pertussis toxin (panel B). The shaded area in panel B represents the position of the bovine tracheal smooth muscle contractions of vehicle- and pertussis toxin-treated strips as depicted in panel A. The contraction is expressed as the percentage of the active tension induced by 3 μM methacholine in vehicle-treated bovine tracheal smooth muscle strips (18.1 ± 3.7 g). Results are means ± S.E.M. of five experiments.

bovine tracheal smooth muscle strips with 10 μM fenoterol, as indicated not only by a reduced maximal relaxation (E_{max}) by approximately 25% ($P < 0.001$) to the agonist but also by a reduced sensitivity (pEC_{50} shift of 1.0 log unit, $P < 0.001$; Fig. 2, Table 1). The muscarinic M_2 receptor selective antagonist gallamine (30 μM) had no effect on isoprenaline-induced relaxation of both control and fenoterol-treated strips (Fig. 2, Table 1).

Desensitization of isoprenaline-induced relaxation was also apparent when using a fixed concentration of 3 μM methacholine (Fig. 3, Table 2). Treatment of bovine tracheal smooth muscle with fenoterol resulted in a significantly reduced sensitivity to isoprenaline (pEC_{50} shift of 0.7 log unit, $P < 0.001$) with a tendency to a reduced E_{max} by approximately 15% ($P = 0.06$; Fig. 3, Table 2). Treatment of the strips with 200 ng/ml pertussis toxin, to inactivate $G_{i\alpha}$, had no effect on the isoprenaline-induced relaxation of both control and fenoterol-treated strips (Fig. 3, Table 2).

Muscarinic M_2 receptor-mediated inhibition of adenylyl cyclase activity was determined on adenylyl cyclase stimulated by 1 μM forskolin. Maximal inhibition of forskolin-stimulated adenylyl cyclase activity reached by 100 μM methacholine amounted to $36 \pm 3\%$ of control activity ($P < 0.001$; Fig. 4). Fenoterol treatment of the cells for 18 h did not influence 1 μM forskolin-induced adenylyl cyclase activity (689 ± 69 and 728 ± 22 pmol cAMP/mg protein/min for control and fenoterol-treated cells, respectively) and did not affect the muscarinic M_2 receptor-mediated inhibition of adenylyl cylcase activity (Fig. 4).

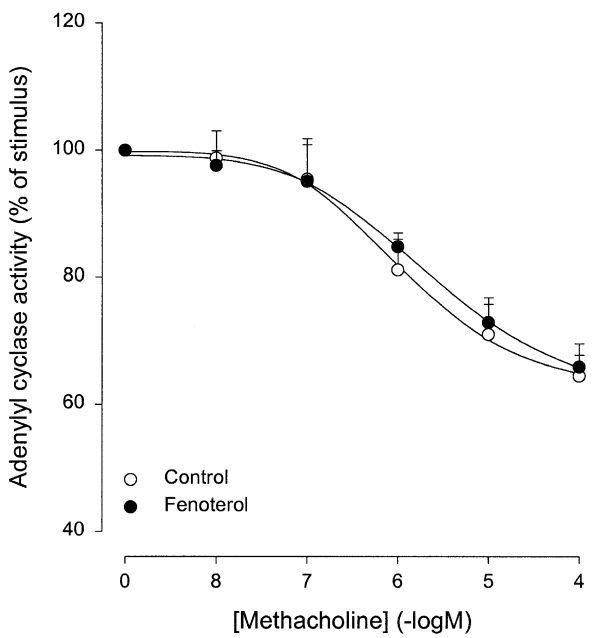

Fig. 4. Methacholine-induced inhibition of 1 μM forskolin-induced adenylyl cyclase activity. Bovine tracheal smooth muscle cells were pre-incubated for 18 h in the absence or presence of 10 μM fenoterol. Adenylyl cyclase activity was determined in the membranes of these cells. Results are means ± S.E.M. of four experiments.

4. Discussion

Treatment of freshly isolated bovine tracheal smooth muscle cells with 10 μM fenoterol for 18 h resulted in 25% desensitization of the 100 μM isoprenaline-induced adenylyl cyclase response, with no effect on the EC_{50} for isoprenaline and basal adenylyl cyclase activity. The observed desensitization was rather small when compared to, e.g. human airway smooth muscle. Thus, human airway smooth muscle cells showed already 60% desensitization of the maximal isoprenaline-induced cAMP response after 30 min of 1 μM isoprenaline treatment (Penn et al., 1998) and 85% desensitization after 24-h incubation with this agonist concentration (Hall et al., 1993). This is in line with the 72% desensitization of the isoprenaline-induced cAMP response found in human bronchial strips after 1 μM isoprenaline treatment for 1 h (Davis and Conolly, 1980).

In bovine tracheal smooth muscle strips treated with fenoterol for 18 h, the relaxation of a submaximal methacholine-induced contraction was considerably impaired; at a submaximal (80%) methacholine-induced tone the E_{max} was reduced by 25% similar to the adenylyl cyclase response of the fenoterol-pretreated cells, while the pEC_{50} for isoprenaline-induced relaxation was reduced by 1.0 log unit. Human bronchial strips treated with 1 or 400 μM isoprenaline for 1 h showed 21% and 78% desensitization, respectively, of relaxation of a histamine-induced contraction by isoprenaline with an estimated pEC_{50} shift of 0.4 and 1.5 log units, respectively. After the 400-μM isoprenaline treatment, the bronchial strips cAMP response showed 60% desensitization to isoprenaline (10 nM–100 μM) (Davis and Conolly, 1980).

In bovine tracheal smooth muscle, the observed functional desensitization appeared to be large when compared to the minor desensitization of the cAMP response to isoprenaline. Thus, an enhanced role for the muscarinic M_2 receptor in the reduced functional antagonism of methacholine-induced tone by β-adrenoceptor agonists could be envisaged. Especially from studies in the heart, there is some evidence that long-term β-adrenoceptor agonist treatment could induce upregulation of the muscarinic inhibitory pathway on adenylyl cyclase. Thus, 72-h treatment of embryonic chicken cardiomyocytes with 10 μM isoprenaline resulted in a significant increase of muscarinic M_2 receptor binding sites in membranes of these cells (Reithmann et al., 1992; Jackson and Nathanson, 1995). In addition, pertussis toxin-induced ribosylation of two membrane proteins—presumably $G_{i\alpha 2}$ and $G_{i\alpha 3}$—was doubled after β-adrenoceptor agonist treatment, indicating that these $G_{i\alpha}$ subunits were also upregulated (Reithmann et al., 1992). This was in line with the increased pertussis toxin-induced ribosylation and enhanced mRNA levels of $G_{i\alpha 2}$ and $G_{i\alpha 3}$ in ventricular homogenates of rats treated with a 4-day subcutaneous infusion of isoprenaline (Eschenhagen et al., 1991). In contrast to the above mentioned study, in

human embryonic lung cells a 40% decrease in muscarinic M_2 receptor binding was found after 24-h incubation with the β_2-adrenoceptor agonist procaterol and the inhibitory effect of carbachol on procaterol-induced cAMP accumulation was lost (Rousell et al., 1996). In the present study, 18-h fenoterol treatment of bovine tracheal smooth muscle cells had no effect at all on methacholine-induced inhibition of forskolin-stimulated adenylyl cyclase in isolated membranes of these cells.

Both by use of the selective muscarinic M_2 receptor antagonist gallamine and the G_i inactivator pertussis toxin, we could not demonstrate a functional role for the muscarinic M_2 receptor in antagonizing isoprenaline-induced relaxation of methacholine-induced contractions. This is in line with a previous study from our laboratory in which no muscarinic M_2 receptor-mediated functional antagonism of isoprenaline-induced relaxation of bovine tracheal smooth muscle contractions to methacholine was found (Roffel et al., 1995). Only after inactivation of the majority of the M_3 receptor population by treatment with 4-DAMP mustard, muscarinic M_2 receptor-mediated functional antagonism of forskolin-but not of isoprenaline-induced relaxation of bovine tracheal smooth muscle contracted by histamine could be observed, presumably because part of the relaxant response to isoprenaline is mediated through a non-cAMP-dependent mechanism, which is largely unopposed by the muscarinic M_2 receptor (Ostrom and Ehlert, 1998). As in bovine tracheal smooth muscle, a role for the muscarinic M_2 receptor in functional antagonism in human bronchial smooth muscle appears to be absent (Watson et al., 1995a). However, in canine, rabbit and guinea pig tracheal smooth muscle some investigators did find a role for muscarinic M_2 receptors or G_i in the functional antagonism of relaxation (Eglen et al., 1994; Roffel and Zaagsma, 1995). However, the effects were relatively small compared to the large changes in isoprenaline relaxant potency that can be obtained in these tissues as a result of varying cholinergic contraction levels (Roffel and Zaagsma, 1995). Moreover, in guinea pig trachea such a role for muscarinic M_2 receptors could not be observed by Roffel et al. (1993) and Watson et al. (1995b). Altogether, these observations indicate that the functional role for the muscarinic M_2 receptor in inhibiting smooth muscle relaxation is—at best—a minor one. In line with the adenylyl cyclase data mentioned above, 18-h fenoterol treatment of bovine tracheal smooth muscle did not expose effects of gallamine and pertussis toxin on the relaxation of methacholine-induced tone by isoprenaline. Thus, no functional evidence for β-adrenoceptor agonist-induced upregulation of $G_{i\alpha}$ or muscarinic M_2 receptor protein, as a possible contribution to the fenoterol-induced reduced functional antagonism of cholinergic tone by isoprenaline, was observed.

We previously demonstrated that fenoterol treatment of bovine tracheal smooth muscle induces constitutive β_2-adrenoceptor activity (De Vries et al., 2000). This agonist-independent receptor activity was shown to counteract methacholine-induced contraction, characterized by reduced sensitivity to methacholine with no effect on the maximal response (De Vries et al., 1999). Fenoterol-induced constitutive β-adrenoceptor activity may explain the slightly reduced submaximal methacholine-induced contraction levels observed after β-adrenoceptor agonist incubation compared to control preparations (see Fig. 3) and the need for slightly higher doses of methacholine to reach 80% of maximal contraction (on average 1.6 and 3.3 μM methacholine for control and fenoterol-treated tissue, respectively; Fig. 2). The observation of fenoterol-induced constitutive β-adrenoceptor activity in association with the reduced pEC_{50} and E_{max} of isoprenaline-induced relaxation under this condition fits well with the observation that constitutively active mutant β_2-adrenoceptors are constitutively phosphorylated and desensitized (Pei et al., 1994).

In conclusion, our observations indicate that 18-h fenoterol treatment of bovine tracheal smooth muscle desensitizes isoprenaline-induced adenylyl cyclase activity and relaxation of methacholine-induced contraction. Functional antagonism between the muscarinic M_2 receptor-mediated inhibition and the β_2-adrenoceptor-mediated stimulation of adenylyl cyclase is not involved in relaxation of methacholine-induced contractions by isoprenaline under basal condition; neither is there any evidence for a role of enhanced muscarinic M_2 receptor function in the reduced β-adrenoceptor agonist-induced relaxation of cholinergic tone after 18-h fenoterol treatment.

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