

Effect of fenoterol-induced constitutive β_2 -adrenoceptor activity on contractile receptor function in airway smooth muscle

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Abstract

In the present study, we investigated the effect of fenoterol-induced constitutive β_2 -adrenoceptor activity on muscarinic receptor agonist- and histamine-induced bovine tracheal smooth muscle contractions. Bovine tracheal smooth muscle strips were incubated with 10 μ M fenoterol or vehicle for various periods of time (5, 30 min, 18 h) at 37 °C. After extensive washout (3 h, 37 °C), isometric contractions were measured to the full muscarinic receptor agonist methacholine, the partial muscarinic receptor agonist 4-(*m*-chlorophenyl-carbamoyloxy)-2-butylnyltrimethylammonium (McN-A-343) and histamine. Fenoterol treatment significantly reduced the sensitivity (pEC_{50}) to methacholine in a time-dependent manner, without affecting maximal contraction (E_{max}). Fenoterol treatment similarly reduced the pEC_{50} of McN-A-343 and histamine; however, E_{max} values were also reduced, to approximately 70% of control after 18-h treatment. The inverse agonist timolol, having no effect on control preparations, consistently restored the reduced pEC_{50} and E_{max} values of the contractile agonists. Remarkably, in the presence of timolol the pEC_{50} values of McN-A-343 and histamine in fenoterol-treated airways were significantly enhanced compared to controls. In conclusion, fenoterol-induced constitutive β_2 -adrenoceptor activity reduces muscarinic receptor agonist- and histamine-induced contractions of bovine tracheal smooth muscle, which can be reversed by the inverse agonist timolol. Moreover, after β_2 -adrenoceptor agonist treatment, inverse agonism by β -adrenoceptor antagonists may cause enhanced airway reactivity to contractile mediators. © 2001 Published by Elsevier Science B.V.

Keywords: β_2 -Adrenoceptor activity, constitutive; Fenoterol; Smooth muscle, bovine, tracheal; Airway reactivity

1. Introduction

Recently, it has become clear that G-protein-coupled receptors, including the β_2 -adrenoceptor, not only transmit signals after stimulation by agonists but can also spontaneously couple to signaling pathways (Samama et al., 1993; Chidiac et al., 1994; Lefkowitz et al., 1993; Scheer and Cotecchia, 1997; Leurs et al., 1998). Experimental evidence obtained with a constitutively active mutant of the β_2 -adrenoceptor, with mutations in the C-terminal portion of the third intracellular loop of the receptor, have indicated that the β_2 -adrenoceptor isomerizes between an inactive conformation (R) and a constitutively active conformation (R^{*}), which can couple to the G_s-protein in the absence of an agonist (Samama et al., 1993). Under basal conditions, constitutive β_2 -adrenoceptor activity is low

because of structural constraints that keep the β_2 -adrenoceptor in the inactive R state by preventing an effective interaction between peptide sequences in the intracellular loops of the receptor and the G-protein. Agonist binding or mutations in the β_2 -adrenoceptor are believed to shift the equilibrium towards R^{*} by relieving this constraint. The activity of constitutively active β_2 -adrenoceptor can be inhibited by antagonists with negative intrinsic efficacy ('inverse agonists') such as timolol and propranolol, which shift the receptor from the active to the inactive conformation (Chidiac et al., 1994; Samama et al., 1994).

Until recently, basal constitutive activity of the wild type β_2 -adrenoceptor has only been visualized by overexpression of the receptor, which increases the absolute amount of R^{*} (Chidiac et al., 1994). However, using bovine tracheal smooth muscle, we have recently shown that agonist-independent constitutive β_2 -adrenoceptor activity may also be induced by β -adrenoceptor agonist treatment (De Vries et al., 2000). Thus, incubation of bovine tracheal smooth muscle with fenoterol caused a time- and concentration-dependent decrease of KCl-in-

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duced contraction after extensive washout of the β -adrenoceptor agonist. This effect could be acutely reversed by timolol, with even an increased KCl-induced contraction in the presence of this inverse agonist (De Vries et al., 2000).

Since β_2 -adrenoceptor agonists are the most frequently used bronchodilators in the treatment of asthma and chronic obstructive pulmonary disease (COPD) and β -adrenoceptor agonist-induced constitutive β_2 -adrenoceptor activity could diminish bronchoconstriction in response to endogenous neurotransmitters or mediators even after washout of the agonist, we investigated the effect of fenoterol-induced constitutive β_2 -adrenoceptor activity on bovine tracheal smooth muscle contractions to muscarinic receptor agonists and histamine. In addition, since it has been demonstrated previously that β_2 -adrenoceptor agonist therapy may cause enhanced airway reactivity to propranolol in patients with asthma after washout of the β -adrenoceptor agonist (Kraan et al., 1985; Koëter et al., 1983), we also sought evidence whether this hyperresponsiveness to β -adrenoceptor antagonists is related to inverse agonism of constitutive β_2 -adrenoceptor activity induced by β_2 -adrenoceptor agonist treatment.

2. Materials and methods

2.1. Tissue preparation and incubation

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 $\mu\text{g}/\text{ml}$ streptomycin and 10% fetal calf serum at 37 °C, in the absence or presence of 10 μM fenoterol. Short-term incubations (5 and 30 min) with fenoterol were performed by adding the agonist near the end of the total 18 h of incubation period, as appropriate.

2.2. Isometric contraction studies

After washing in 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 on Kipp BD41 flat bed recorders using Grass FT03 force displacement transducers connected to Grass low level DC amplifiers. During a 90-min equilibra-

tion 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 (three changes of bath volume). Under isoprenaline (0.1 μM)-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). Subsequently, the first concentration response curve was recorded by cumulative administration of methacholine (1 nM–0.1 mM) or histamine (10 nM–1 mM).

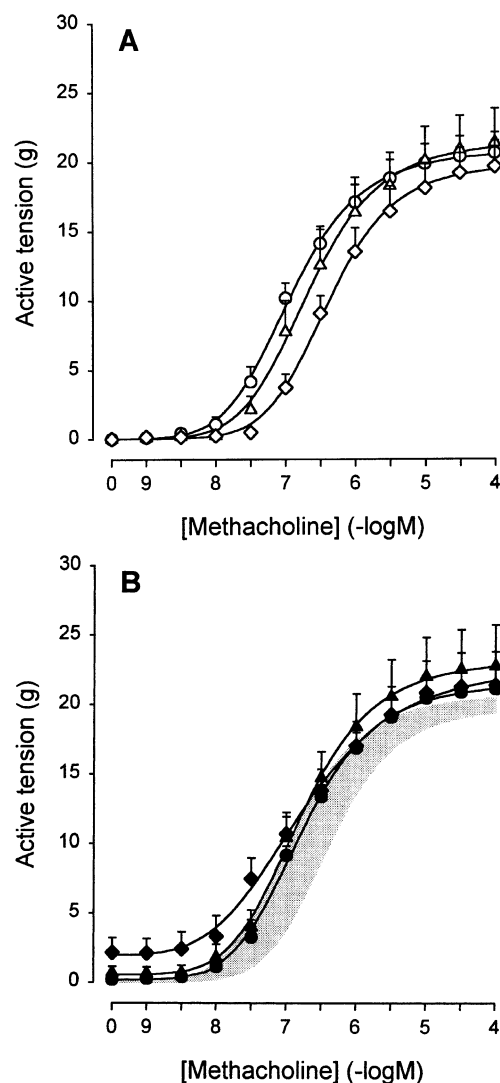


Fig. 1. Bovine tracheal smooth muscle contractions to the full muscarinic receptor agonist methacholine after preincubation of the tissue with 10 μM fenoterol for 30 min (triangles) and 18 h (diamonds) in the absence (open symbols, panel A) and subsequent presence (closed symbols, panel B) of 1 μM timolol. Strips receiving vehicle served as controls (circles). The shaded area in panel B represents the position of the bovine tracheal smooth muscle contractions in the absence of timolol as depicted in panel A. The results are means \pm S.E.M. of six experiments.

After washout for 45 min with three changes of bath volume, a second CRC to methacholine or histamine was recorded, following 30-min preincubation of 1 μ M timolol. Because of incomplete washout of the agonist, concentration response curves to 4-(*m*-chlorophenyl-carbamoyloxy)-2-butynyltrimethylammonium (McN-A-343; 10 nM–0.3 mM) in the absence and presence of 1 μ M timolol were recorded on separate strips.

2.3. Data analysis

Isometrically recorded contractile responses were expressed in grams of developed tension. Results are presented as means \pm S.E.M. of the indicated numbers of experiments. Statistical analysis was performed by the two-tailed Student's *t*-test for paired or unpaired observations, as appropriate. A value of $P < 0.05$ was considered statistically significant.

2.4. Materials

Tissue culture supplies were purchased from Gibco BRL Life Technologies (Paisley, UK). DMEM and methacholine were obtained from ICN Biomedicals (Costa Mesa, CA, USA). Fenoterol, (–)-isoprenaline, timolol and histamine were from Sigma (St. Louis, MO, USA); McN-A-343 was from RBI, Natick, MA.

3. Results

Incubation of bovine tracheal smooth muscle strips with 10 μ M fenoterol for 30 min and 18 h resulted, after

extensive washout of the β -adrenoceptor agonist, in a gradually and significantly decreased sensitivity (pEC_{50}) of the strips to the full muscarinic receptor agonist methacholine at both time points (shift in pEC_{50} of approximately 0.3 and 0.6 log units, respectively, $P < 0.05$), without significantly affecting the maximal effect (E_{max}) of the contractile agonist (Fig. 1A, Table 1). Incubation of the fenoterol-treated strips with the inverse agonist timolol (1 μ M) reversed the reduced pEC_{50} values of methacholine to control (Fig. 1B, Table 1). Timolol had no effect on pEC_{50} and E_{max} values of methacholine in the vehicle-treated control preparations (Table 1).

With the partial muscarinic receptor agonist McN-A-343, a significant shift in pEC_{50} was already present after 5 min of fenoterol incubation, which was slightly increased after 30 min and 18 h of incubation with the β -adrenoceptor agonist to approximately 0.4 log units ($P < 0.001$) (Fig. 2A, Table 1). In addition, a time-dependent decrease in E_{max} of McN-A-343 was observed, reaching 73% of control after 18-h incubation ($P < 0.05$). The reduced pEC_{50} values of McN-A-343 of the strips treated with fenoterol for 5 and 30 min, respectively, were restored to control in the presence of timolol. Remarkably, in the presence of the inverse agonist the pEC_{50} of McN-A-343 in the 18-h fenoterol-treated strips was significantly enhanced compared to vehicle-treated controls (Fig. 2B, Table 1). The E_{max} values of the β -adrenoceptor agonist-treated strips were normalized by timolol to levels not significantly different from control (Fig. 2B, Table 1). As with methacholine, timolol had no effect on pEC_{50} and E_{max} values in the control strips (Table 1).

β -Adrenoceptor agonist treatment of bovine tracheal smooth muscle also affected the contractile response to

Table 1

Effect of fenoterol (10 μ M) treatment on pEC_{50} and E_{max} -values of methacholine-, McN-A-343- and histamine-induced bovine tracheal smooth muscle contraction

Contractile agonist	Preincubation	pEC_{50} (–logM)		E_{max} (g)	
		– Timolol	+ Timolol	– Timolol	+ Timolol
Methacholine	vehicle	6.95 \pm 0.08	6.87 \pm 0.12	20.6 \pm 1.5	21.2 \pm 1.6
	30 min fenoterol	6.66 \pm 0.12 ^a	6.89 \pm 0.10	21.3 \pm 2.5	22.8 \pm 2.9
	18 h fenoterol	6.39 \pm 0.09 ^a	6.93 \pm 0.19 ^b	19.6 \pm 1.9	21.4 \pm 2.4 ^b
McN-A-343	vehicle	5.32 \pm 0.04	5.33 \pm 0.06	14.0 \pm 1.5	14.9 \pm 2.2
	5 min fenoterol	4.95 \pm 0.05 ^c	5.36 \pm 0.06 ^d	12.6 \pm 1.4	16.3 \pm 1.6 ^b
	30 min fenoterol	4.87 \pm 0.05 ^c	5.49 \pm 0.08 ^d	11.9 \pm 1.2	14.0 \pm 1.6
	18 h fenoterol	4.91 \pm 0.05 ^c	5.67 \pm 0.11 ^{a,d}	10.2 \pm 1.1 ^a	14.7 \pm 1.7 ^b
Histamine	vehicle	5.48 \pm 0.14	5.37 \pm 0.13	18.8 \pm 1.4	20.3 \pm 1.3
	5 min fenoterol	5.15 \pm 0.09 ^a	5.48 \pm 0.13 ^d	17.1 \pm 2.0	22.8 \pm 1.9 ^d
	30 min fenoterol	4.91 \pm 0.12 ^e	5.50 \pm 0.15 ^d	15.1 \pm 2.2 ^a	23.6 \pm 1.5 ^{a,d}
	18 h fenoterol	4.98 \pm 0.08 ^c	5.87 \pm 0.09 ^{a,d}	13.7 \pm 1.8 ^c	22.1 \pm 1.6 ^d

Values represent the mean \pm S.E.M. of 6 to 10 experiments.

^a $P < 0.05$, compared to vehicle.

^b $P < 0.05$, compared to absence of timolol.

^c $P < 0.001$, compared to vehicle.

^d $P < 0.001$, compared to absence of timolol.

^e $P < 0.01$, compared to vehicle.

histamine. In control preparations, the E_{\max} to histamine was slightly lower than that of methacholine, but higher than that of McN-A-343 (Figs. 1A, 2A and 3A; Table 1). As with McN-A-343, fenoterol incubation resulted in a time-dependent decrease of both the pEC_{50} and E_{\max} to histamine (Fig. 3A, Table 1). The quantitative changes in pEC_{50} (0.5 log unit, $P < 0.001$) and E_{\max} (to 73% of control, $P < 0.001$) after 18-h fenoterol treatment were similar to those observed with McN-A-343 (see above). In the presence of timolol, both the reduced pEC_{50} and E_{\max} values of histamine were reversed, with a significantly

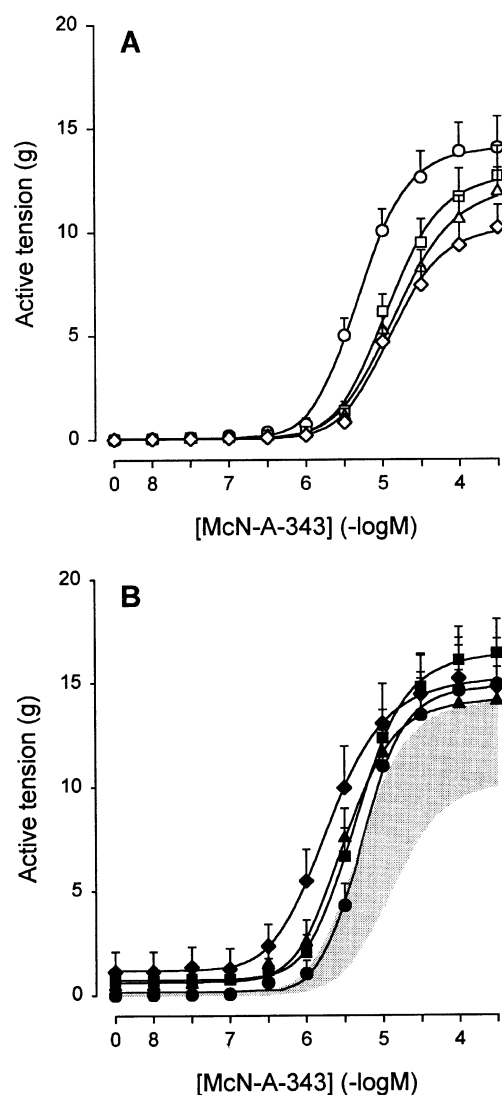


Fig. 2. Bovine tracheal smooth muscle contractions to the partial muscarinic receptor agonist McN-A-343 after preincubation of the tissue with 10 μ M fenoterol for 5 min (squares), 30 min (triangles) and 18 h (diamonds) in the absence (open symbols, panel A) and presence (closed symbols, panel B) of 1 μ M timolol. Strips receiving vehicle served as controls (circles). The shaded area in panel B represents the position of the bovine tracheal smooth muscle contractions in the absence of timolol as depicted in panel A. The results are means \pm S.E.M. of 7 to 10 experiments.

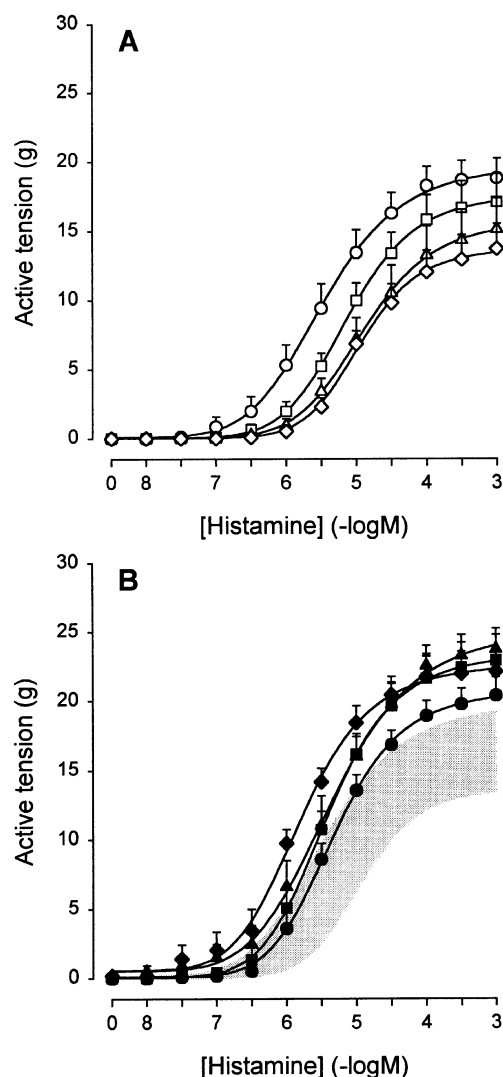


Fig. 3. Bovine tracheal smooth muscle contractions to histamine after preincubation of the tissue with 10 μ M fenoterol for 5 min (squares), 30 min (triangles) and 18 h (diamonds) in the absence (open symbols, panel A) and subsequent presence (closed symbols, panel B) of 1 μ M timolol. Strips receiving vehicle served as controls (circles). The shaded area in panel B represents the position of the bovine tracheal smooth muscle contractions in the absence of timolol as depicted in panel A. The results are means \pm S.E.M. of six experiments.

enhanced pEC_{50} value in the 18-h fenoterol-treated strips (Fig. 3B, Table 1).

4. Discussion

Recently, we have shown that constitutive activity of the wild type β_2 -adrenoceptor in bovine tracheal smooth muscle may be induced by incubation of this tissue with the β_2 -adrenergic agonist fenoterol. The agonist-independent β_2 -adrenoceptor activity caused a decrease of KCl-induced contraction, which was acutely reversed by various inverse agonists, including timolol (De Vries et al., 2000).

In the present study, we demonstrated that fenoterol-induced constitutive β_2 -adrenoceptor activity in bovine tracheal smooth muscle also reduces the contraction in response to muscarinic receptor agonists and histamine. Completely in line with theoretical models and previous experiments describing the pharmacology of functional antagonism between contractile and relaxing agonists in bovine tracheal smooth muscle (Van den Brink, 1973a,b), the reduced contractile response to the full muscarinic receptor agonist methacholine—displaying a considerable receptor and transduction reserve with respect to bovine tracheal smooth muscle contraction (Van den Brink, 1973a; Meurs et al., 1988)—was characterized by only a shift of the concentration response curve to the right, whereas the reduced response to the partial muscarinic receptor agonist McN-A-343—having no reserve (Meurs et al., 1988)—was additionally characterized by a reduced E_{\max} . The reduced response to histamine, which has a smaller receptor and transduction reserve than methacholine (Van den Brink, 1973b; Van Amsterdam et al., 1989), similarly displayed both a reduced pEC_{50} and E_{\max} .

Several observations from the present and a previous study (De Vries et al., 2000) strongly argue against the possibility that some residual fenoterol would have caused the reduced contractile responses. First, after incubation with this short-acting β -adrenoceptor agonist, the tissue was thoroughly washed, a procedure lasting 3 h and including at least 12 changes of bath volume, making the presence of residual β -adrenoceptor agonist in the tissue highly unlikely. Secondly, in the presence of timolol, 18-h fenoterol-treated tissue showed a significantly enhanced sensitivity to McN-A-343 and histamine as compared to control (Figs. 2B and 3B, Table 1). In a previous study, similarly enhanced contractions of fenoterol-treated bovine tracheal smooth muscle were observed in response to KCl in the presence of timolol (De Vries et al., 2000). Obviously, the enhanced sensitivity of fenoterol-treated bovine tracheal smooth muscle to the contractile agonists and KCl in the presence of timolol cannot be explained by displacement of residual β -adrenoceptor agonist. In addition, the inverse agonism of timolol in our previous study was competitively inhibited by the less efficacious inverse agonist labetalol (De Vries et al., 2000). This competition would not be expected if the response to timolol was brought about by displacement of residual β -adrenoceptor agonist from the receptor. Finally, the rank order of the inverse efficacies of six β -adrenoceptor antagonists on the constitutively active receptor did not correspond with the receptor occupancies on the inactive receptor (De Vries et al., 2000); in case of displacement of residual β -adrenoceptor agonist from the receptor, the effect of β -adrenoceptor antagonists would be related to their receptor occupancies.

Reduced contractile responses to muscarinic receptor agonists and histamine after β -adrenoceptor agonist treatment have been observed before, in vivo as well as in

vitro. Thus, in a dog model, repeated administration of isoprenaline aerosol for 60 min resulted in reduced methacholine-induced bronchoconstriction after washout of the β -adrenoceptor agonist (Stephan et al., 1980). A similar effect was observed for inhaled histamine after 7 days of subcutaneous administration of isoprenaline to guinea pigs (Douglas et al., 1977) and for the carbachol-induced contractility of isolated guinea pig trachea after 3-h incubation with isoprenaline (Fernandes et al., 1988). Interestingly, in all cases, the reduced airway reactivity to muscarinic receptor agonists and histamine after β -agonist treatment was accompanied by desensitization of the β -agonist-induced response (Stephan et al., 1980; Douglas et al., 1977; Fernandes et al., 1988). A possible association between constitutive β_2 -adrenoceptor activity and β_2 -adrenoceptor desensitization has also been indicated at the molecular level. Thus, a constitutively active mutant of the β_2 -adrenoceptor expressed in Chinese hamster ovary (CHO) cells showed agonist-independent activity but was also tonically down-regulated and partly uncoupled from the G-protein (Pei et al., 1994). This mutant receptor, reconstituted in phospholipid vesicles, was phosphorylated by β -adrenergic receptor kinase (β ARK) to a similar extent as the agonist-stimulated wild type receptor (Pei et al., 1994). Moreover, inverse agonists were found to decrease the basal phosphorylation of the mutant receptor (Samama et al., 1994). Hence, an active form of the desensitized receptor was proposed.

The mechanism of the induction of constitutive β_2 -adrenoceptor activity by fenoterol is presently unknown. Just like desensitization, it is a rapid process taking only 5–30 min to significantly depress contractile agonist- and KCl-induced contractions. Receptor phosphorylation, being a rapid regulatory mechanism, might be responsible for the induction of constitutive receptor activity. Receptor phosphorylation has recently been shown to play a role in morphine-induced constitutive μ -opioid receptor activation. In SH-SY neuroblastoma cells and in the guinea pig ileum, chronic activation of the μ -opioid receptor by morphine resulted in constitutive activation of the receptor and inverse agonistic behaviour of naloxone, as shown by a cAMP overshoot in the SH-SY cells and the ‘abstinence response’ of the ileum, respectively. In both cases, the protein kinase inhibitor 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7) prevented the inverse agonism of naloxone (Wang et al., 1994a,b; Cruz et al., 1996). In human embryonic kidney (HEK) 293 cells expressing the μ -opioid receptor, H7 was also shown to prevent both basal and morphine-stimulated phosphorylation of the receptor (Wang et al., 1996). Moreover, in acutely morphine-dependent mice naloxone induced withdrawal jumping, which again was inhibited by the protein kinase inhibitor H7 (Wang et al., 1994a,b; Bilsky et al., 1996).

Agonist-induced constitutive β_2 -adrenoceptor activity could be a protective mechanism against desensitization in reaction to short-term agonist treatment. However, it has

been demonstrated that long-term (2–6 weeks) treatment with regular β -adrenoceptor agonists may lead to enhanced bronchial reactivity after washout of the agonist, both in vivo and ex vivo, and may thus worsen asthma symptoms (Koëter et al., 1983; Kraan et al., 1985; Vathenen et al., 1988; Sears et al., 1990; Witt Enderby et al., 1993; Wang et al., 1994a,b).

Remarkably, McN-A-343- and histamine-induced contractions of 18-h fenoterol-treated tissue were significantly potentiated by timolol. In a previous study on constitutive β_2 -adrenoceptor activity in bovine tracheal smooth muscle, KCl-induced contractions were similarly potentiated by timolol after 5-, 30-min and 18-h incubation of the tissue with fenoterol (De Vries et al., 2000). Moreover, in Sf9 cells overexpressing the human β_2 -adrenoceptor, Chidiac et al. (1996) have shown that β -adrenoceptor agonist-induced desensitization of the cells is associated with increased inverse agonism of various antagonists with (partial) inverse efficacies. Collectively, these results indicate that enhanced inverse agonism by β -adrenoceptor antagonists after β -adrenoceptor agonist treatment may cause airway hyperreactivity to bronchoconstrictive stimuli. This mechanism could be of clinical relevance with regard to the observation that asthmatic patients showed enhanced airway hyperreactivity to propranolol—an antagonist with similar inverse efficacy compared to timolol (De Vries et al., 2000; Chidiac et al., 1996)—after 2–4 weeks of terbutaline treatment (Koëter et al., 1983; Kraan et al., 1985). In how far airway reactivity to propranolol in non- β -adrenoceptor agonist-treated patients is due to inverse agonism remains to be established.

In conclusion, the present study indicates that fenoterol-induced constitutive β_2 -adrenoceptor activity reduces muscarinic receptor agonist- and histamine-induced contractions of bovine tracheal smooth muscle, which can be reversed by the inverse agonist timolol. β -Adrenoceptor agonist-induced constitutive activity could contribute to the beneficial bronchodilating effect of these drugs. Conversely, inverse agonism by timolol after β -adrenoceptor-agonist treatment may cause enhanced airway reactivity to contractile mediators.

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