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Effects of β_2-agonist- and dexamethasone-treatment on relaxation and regulation of β-adrenoceptors in human bronchi and lung tissue

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1 Long-term treatment with β_2-adrenoceptor agonists can lead to a decreased therapeutic efficacy of bronchodilatation in patients with obstructive pulmonary disease. In order to examine whether or not this is due to β-adrenoceptor desensitization, human bronchial muscle relaxation was studied in isolated bronchial rings after pretreatment with β_2-adrenoceptor agonists. Additionally, the influence of pretreatment with dexamethasone on desensitization was studied.

2 The effect of β_2-agonist incubation alone and after coincubation with dexamethasone on density and affinity of β-adrenoceptors was investigated by radioligand binding experiments.

3 In human isolated bronchi, isoprenaline induces a time- and concentration-dependent β-adrenoceptor desensitization as judged from maximal reduction in potency by a factor of 7 and reduction of $73 \pm 4\%$ in efficacy of isoprenaline to relax human bronchial smooth muscle.

4 After an incubation period of 60 min with 100 μmol l^{-1} terbutaline, a significant decline in its relaxing efficacy ($81 \pm 8\%$) and potency (by a factor 5.5) occurred.

5 Incubation with 30 μmol l^{-1} isoprenaline for 60 min did not impair the maximal effect of a subsequent aminophylline response but led to an increase in potency (factor 4.4).

6 Coincubation of dexamethasone with isoprenaline (120 min; 30 μmol l^{-1}) preserved the effect of isoprenaline on relaxation ($129 \pm 15\%$).

7 In radioligand binding experiments, pretreatment of lung tissue for 60 min with isoprenaline (30 μmol l^{-1}) resulted in a decrease in β-adrenoceptor binding sites (B_{max}) to $64 \pm 1.6\%$ ($P < 0.05$), while the antagonist affinity (K_D) for [^{3}H]-CGP-12177 remained unchanged.

8 In contrast, radioligand binding studies on lung tissue pretreated with either dexamethasone (30 μmol l^{-1}) or isoprenaline (30 μmol l^{-1}) plus dexamethasone (30 μmol l^{-1}) for 120 min did not lead to a significant change of B_{max} ($160 \pm 22.1\%$ vs $142.3 \pm 28.7\%$) or K_D (5.0 nmol l^{-1} vs 3.5 nmol l^{-1}) compared to the controls.

9 In conclusion, pretreatment of human bronchi with β-adrenoceptor agonists leads to functional desensitization and, in lung tissue, to down-regulation of β-adrenoceptors. This effect can be counteracted by additional administration of dexamethasone. Our model of desensitization has proved useful for the identification of mechanisms of β-adrenoceptor desensitization and could be relevant for the evaluation of therapeutic strategies to counteract undesirable effects of long-term β-adrenoceptor stimulation.

Keywords: Human bronchi; human lung parenchyma; β-adrenoceptor agonist; corticosteroid; isoprenaline, dexamethasone; aminophylline; desensitization; down-regulation; asthma

Introduction

β-Adrenoceptor agonists are the most potent compounds known to reduce increased airway resistance. However, these drugs are reputed to induce desensitization and down-regulation of β_2-adrenoceptors during prolonged or high-dose treatment (Martinsson *et al*., 1987).

Clinical studies on the relevance of these effects have shown conflicting results. Diminished β-adrenoceptor responsiveness became obvious in normal subjects during repeated drug administration after prior inhalation of salbutamol. However, this was not the case in asthmatic and atopic subjects (Harvey & Tattersfield, 1982). Moreover, an increased risk of death due to asthma has been associated with the inhalation of β_2-agonists like fenoterol in studies from New Zealand (Grainger *et al*., 1991). The latter authors discussed the potential causative role of high dosage application of the drug for the development of desensitization and subsequent adverse clinical outcome observed in asthmatic patients. In contrast, other groups did not observe any tachyphylaxis or β-adrenoceptor down-regulation after inhalation of formoterol (Arvidsson *et al*., 1989) or other β-adrenoceptor agonist treatments (Tashkin *et al*., 1983; Hauck *et al*., 1990).

In asthmatics, changes in β-agonist action are difficult to characterize because each *in vivo* reduction in bronchodilator activity can be due to a variety of factors which are often directly associated with the severity of the disease (Svedmyr, 1990). This implies that an experimental model of studying human lung tissues is needed in order to investigate objectively the mechanisms responsible for the development of tachyphylaxis and identify potential strategies for counteraction.

Previously, it was shown in an *in vitro* model of lung tissue that down-regulation of β-adrenoceptors (Böhm *et al*., 1991) and decrease in receptor-mRNA (Collins *et al*., 1990) could represent the underlying mechanisms for a decline in responsiveness to β-adrenoceptor stimulants. Additionally, a loss in β_2-adrenoceptor responsiveness due to prolonged maximal relaxation by β-agonists has also been discussed as a potential consequence of such decline (Herepath & Broadley, 1991). In

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some studies, there was evidence that even short-term exposure to β-adrenoceptor agonists can lead to phosphorylation of β-adrenoceptors and to alterations in the β-receptor-G-protein complex (Roth *et al*., 1991) with uncoupling of β-adrenoceptors from the stimulant G-protein (Pitcher *et al*., 1992).

This study sought to develop a desensitization model of human bronchi in an organ bath which allows for studies on the role of β-agonists on changes observed in the functional response of human bronchi after short- and long-term pretreatment. Additionally, the purpose of this project was to analyse whether the resulting functional effects could be counteracted by concomitant application of corticosteroids. Finally, we sought to study the biochemical processes associated with desensitization in human lung membranes by conducting radioligand binding experiments to examine the density and binding characteristics of β-adrenoceptors of the lung.

Methods

Patients

Thirty six patients who had undergone pneumonectomy or lobectomy for treatment of bronchial carcinoma were investigated. The female to male ratio was 0.44; the smoker to non-smoker ratio was 1.4. Mean age was calculated to be 59.9 ± 1.7 (range: 34–76 years). Only patients with confirmed normal preoperative lung function were included in the study. None was taking any medication which interfered with the sympathetic or parasympathetic nervous system. All patients underwent anaesthesia according to the same regimen. Informed consent was obtained from all patients before lung surgery.

Human isolated bronchi

For functional investigations, bronchi between 5–8 mm in diameter were dissected from lung and connective tissue and cut into 2–3 mm thick rings. Tissues were then stored overnight at 4°C in Tyrode solution (composition given below) and aerated with carbogen (95% O_2 and 5% CO_2) to rinse off blood compounds and narcotics. Subsequently, the bronchial rings were placed in 20 ml organ baths filled with a warmed (37°C) and carbogen-gassed Tyrode solution at a pH of 7.4, containing indomethacin (3 μmol l^{-1}). Isometric tension was measured with an inductive force transducer, F 30 type 372, attached to an analogous digital converter, type 663 (Hugo Sachs, Freiburg; Germany). During an equilibration period of 90 min, the bathing solution was changed every 30 min and the bronchi were mounted under an isometric load of 2 g.

After precontraction induced by carbachol EC_{50} (0.4 μmol l^{-1}), the bronchi were relaxed by the administration of 0.001–30 μmol l^{-1} isoprenaline or 0.01–100 μmol l^{-1} terbutaline. In order to study the influence of the incubation time on the relaxant activity of isoprenaline, the agonist was added to the organ bath for 30, 60, 120 and 240 min. The bathing solution was replaced in each experiment after 60 min. As a control, experiments were repeated with Tyrode solution (composition stated below). In order to investigate whether the development of desensitization is dependent upon the concentration, bronchi were incubated with 0.001 to 30 μmol l^{-1} isoprenaline for 60 min. For comparison, bronchi were incubated with the selective β_2-adrenoceptor agonist terbutaline at 100 μmol l^{-1} for 60 min, a concentration known from previous experiments to provoke maximal reduction in the number of β-adrenoceptors (Böhm *et al*., 1991). Agonist incubation was followed by a 2 h washout period to reinstitute the basal tone of the bronchial rings. Following the return to baseline, bronchi were contracted for a second time for carbachol EC_{50} (0.4 μmol l^{-1}), and were relaxed afterwards by isoprenaline (0.001–30 μmol l^{-1}) or terbutaline (0.01–100 μmol l^{-1}). The results obtained from these experiments were compared with the preincubation data.

In a further set of experiments it was determined whether isoprenaline-induced desensitization is a result of impaired β-adrenoceptor function. Therefore, the non-selective phosphodiesterase inhibitor aminophylline was used. Before and after 60 min incubation of 30 μmol l^{-1} isoprenaline, bronchi were relaxed with aminophylline in cumulative concentrations ranging from 0.3–3000 μmol l^{-1} and the pre- and post-incubation data obtained were compared.

The impact of corticosteroids on agonist-induced desensitization was studied by adding dexamethasone (30 μmol l^{-1}) to isoprenaline (30 μmol l^{-1}) during an incubation period of 120 min. The isoprenaline-stimulated response in carbachol-induced precontracted bronchi was compared to the data obtained from testing without dexamethasone. In order to exclude an effect on relaxation activity by the steroid itself, control experiments were performed in which bronchi were incubated with dexamethasone (30 μmol l^{-1}) in the absence of β-adrenoceptor agonists.

Human lung tissue

Approximately 50 g (wet weight) of tumour-free lung parenchyma were collected during lung surgery and stored in pre-aerated ice cold Tyrode solution. After separation of pleura, small blood vessels and bronchi, the lung tissue was cut into strips and samples were incubated at 37°C for 60 min with indomethacin (3 μmol l^{-1}) plus isoprenaline (30 μmol l^{-1}). The other samples were used as controls and incubated in Tyrode solution with indomethacin (3 μmol l^{-1}). The influence of corticosteroids on these preparations was investigated by incubation of the lung tissue specimens with dexamethasone (30 μmol l^{-1}), dexamethasone (30 μmol l^{-1}) plus isoprenaline (30 μmol l^{-1}) or with Tyrode solution for control samples. Since not enough lung parenchyma was generally available to investigate the four conditions in parallel, experiments were performed with tissue from different patients.

Lung parenchyma was frozen in liquid nitrogen and stored at −80°C until radioligand binding experiments could be performed. Frozen tissue was allowed to thaw at 4°C over approximately 2 h. For all subsequent steps, the temperature was kept at 4°C. Storage did not alter β-adrenoceptor density (data not shown). Then, the tissue was minced and homogenized in TE-buffer (composition given below) twice for 1 min with an Ultra Turrax (Jahnke and Kunkel, Staufen, Germany). The homogenate was centrifuged at 2000 *g* for 15 min, and the supernatant was recentrifuged in a Beckman Ultra-Centrifuge I-128 (Beckman, Palo Alto, U.S.A.) at 100,000 *g* for 30 min. The pellet was resuspended in TE-buffer, homogenized by hand with a glass-glass homogenizer and again centrifuged at 100,000 *g* for 30 min. The final pellet was resuspended in incubation buffer and the protein concentration was determined according to the methods of Bradford (Bradford, 1976).

Radioligand binding experiments

For binding experiments, the radiolabelled ligand [^{3}H]-CGP-12177 with a specific activity of 46 Ci $mmol^{-1}$ was used. Specific binding was defined as the difference in binding in the absence and presence of (−)-propranolol (1 μmol l^{-1}). Lung membrane studies were performed with 100 μg of protein/tube in a final assay volume of 250 μl. Incubation was carried out at 37°C for 90 min. These conditions allowed complete equilibration of receptor and radioligand. The reaction was terminated by rapid vacuum filtration through Whatman GF/C filters and the sample was washed three times with 10 ml of ice cold incubation buffer. Radioactivity was determined by a β-scintillation counter (Rackbeta 1219, LKB Wallac, U.S.A.). The maximal density (B_{max}) and apparent affinity (K_D) of binding sites was obtained in individual experiments from Scatchard plots, determined by linear regression analysis (Scatchard, 1949).

Substances and buffers

Indomethacin, carbachol, isoprenaline-HCl, terbutaline, aminophylline and dexamethasone were produced by Sigma; the radiolabelled ligand [^{3}H]-CGP-12177 (4-[3-*t*-butylamino-2-hydroxypropoxy]benzimidazol-2-one) was acquired from Amersham-Buchler (Braunschweig, Germany). Tyrode solution consisted of (mmol l^{-1}): NaCl 136.9, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.05, NaH_2PO_4 0.42, $NaHCO_3$ 16.6, Na_2EDTA 0.05, ascorbic acid 0.28 and glucose 5.0. The TE-buffer consisted of (mmol l^{-1}): Tris/HCl (pH 8.0) 5.5 and EDTA 2.0. Incubation- and washing-buffer consisted of (mmol l^{-1}): $MgCl_2$ 10.0 and Tris/HCl (pH 7.4) 50.0.

Statistics

In functional experiments, EC_{50} values were graphically estimated as the concentration producing half the maximal contractile and relaxant effect. To compare the results obtained in the same bronchi before and after incubation, or in treated or untreated lung tissue from the same patient, statistical significance was estimated by using Student's *t* test for paired observations. For the remaining cases, Student's *t* test for independent observations and a Bonferroni correction was applied. A *P* value of less than 0.05 was considered significant.

Results

Time-dependence of isoprenaline desensitization

Bronchi were incubated with isoprenaline (30 μmol l^{-1}) for a period of 30, 60, 120 and 240 min. After 30 min, no reduction in the maximal relaxant effect of isoprenaline was observed. Starting with the 60 min incubation, a significant reduction in efficacy was observed ($83 \pm 6\%$), which further decreased to $81 \pm 4\%$ at 120 min and $73 \pm 4\%$ after 240 min ($P < 0.05$; Table 1; Figure 1a). Incubation with bathing solution (Tyrode) did not induce a reduction in the maximal effect of isoprenaline, even after the longest incubation time.

With regard to the EC_{50} values obtained in isoprenaline-treated bronchi after as little as 30 min of incubation, a significant increase (by a factor of 3.7, $P < 0.05$) could be demonstrated compared to the respective drug concentration in the preincubation period. For longer incubation times, a further rightward shift of the dose-response curves was achieved, with a maximal (and significant) 7.4 fold increase in EC_{50} after 120 min (Table 1). Incubation with control solution did not reveal a significant change in EC_{50} at any time investigated.

Concentration-dependence of isoprenaline desensitization

During an incubation period of 60 min, isoprenaline was added to the organ bath in concentrations of 0.003, 3 and 30 μmol l^{-1}. At the lowest drug concentration (0.003 μmol l^{-1}), no alteration in the maximal relaxant effect could be measured, whereas maximal loss in efficacy occurred at 3 and 30 μmol l^{-1} of isoprenaline and was found to be $87 \pm 4\%$ and $83 \pm 6\%$ ($P < 0.05$ vs control), respectively (Table 2, Figure 1b).

In contrast, only 30 μmol l^{-1} isoprenaline, the highest agonist concentration used, led to a significant increase in EC_{50} (by a factor of 3) between the pre- and post-incubation periods. Specifically, the concentrations of EC_{50} were calculated to be 0.026 μmol l^{-1} (0.017 – 0.04 μmol l^{-1}) and 0.078 μmol l^{-1} (0.056 – 0.107 μmol l^{-1}; $P < 0.05$), respectively (Table 2).

Effect of terbutaline on desensitization

During an incubation period of 60 min, terbutaline was added to the organ bath in a concentration of 100 μmol l^{-1}. It was shown that terbutaline induced a significant reduction in maximal effect (to $81 \pm 8\%$) and an increase in EC_{50} by a factor of 5.5 for subsequent terbutaline relaxation (Table 2, Figure 1b).

Effect of isoprenaline on aminophylline response

Bronchi were incubated for 60 min with isoprenaline at a concentration (30 μmol l^{-1}) known from the previous experiments to induce significant reduction of relaxation response to $83 \pm 6\%$. Subsequent stimulation of the carbachol EC_{50}-contracted bronchi with aminophylline (0.3 – 3000 μmol l^{-1}), revealed no reduction in its maximal bronchodilator effect, being $103 \pm 11\%$ of the preincubation response (16.8 ± 1.5 mN vs 16.7 ± 1.6 mN). However, a significant leftward shift of the dose-response curve occurred with a reduction in EC_{50} from 182.0 μmol l^{-1} (139.7 – 231.1 μmol l^{-1}) to 41.7 μmol l^{-1} (32.6 – 53.2 μmol l^{-1}; $P < 0.05$) (Figure 1c).

Effect of dexamethasone on isoprenaline-induced desensitization

Human bronchial preparations were incubated with dexamethasone (30 μmol l^{-1}) and isoprenaline (30 μmol l^{-1}) for 120 min. In comparison to incubation with the β-agonist alone, coincubation with dexamethasone completely abolished the reduction in the maximal relaxant effect of isoprenaline (81% vs 129%) and, consequently, it did not differ significantly from the control tests (106%) (Table 3 and Figure 1d). Furthermore, there was only a minor shift in the dose-response curve, as the resultant EC_{50} of 0.204 μmol l^{-1} (0.059 ± 0.703 μmol l^{-1}) did no differ significantly from the corresponding preincubation value of 0.043 μmol l^{-1} (0.012 – 0.0148 μmol l^{-1}). When incubated alone, dexamethasone (30 μmol l^{-1}) led to a marked (NS) increased relaxant response to a subsequent isoprenaline stimulation with no concomitant change in EC_{50}. This effect held true when compared

Table 1 Time-dependence of functional desensitization of human bronchial rings after incubation with isoprenaline (30 μmol l^{-1})

Time (min)	n	*Maximal isoprenaline-effect before incubation* (mN)	*Maximal isoprenaline-effect after incubation* (mN)	*Isoprenaline-effect* (% of preincubation)	*Isoprenaline-EC_{50} before incubation* (μmol l^{-1})	*Isoprenaline-EC_{50} after incubation* (μmol l^{-1})
30	5	13.4 ± 0.8	13.5 ± 0.7	101 ± 4	0.015 (0.01 – 0.021)	0.055* (0.038 – 0.079)
60	5	14.5 ± 1.6	12.0 ± 1.5	83 ± 6*	0.026 (0.017 – 0.04)	0.078* (0.056 – 0.107)
120	6	16.5 ± 1.5	13.5 ± 1.9	81 ± 4*	0.016 (0.01 – 0.025)	0.118* (0.056 – 0.214)
240	7	14.3 ± 2.3	10.2 ± 1.1	73 ± 4*	0.019 (0.009 – 0.040)	0.098* (0.063 – 0.151)

Values are mean $\pm$ s.e.mean and mean with 95% confidence limits, respectively. *$P < 0.05$.

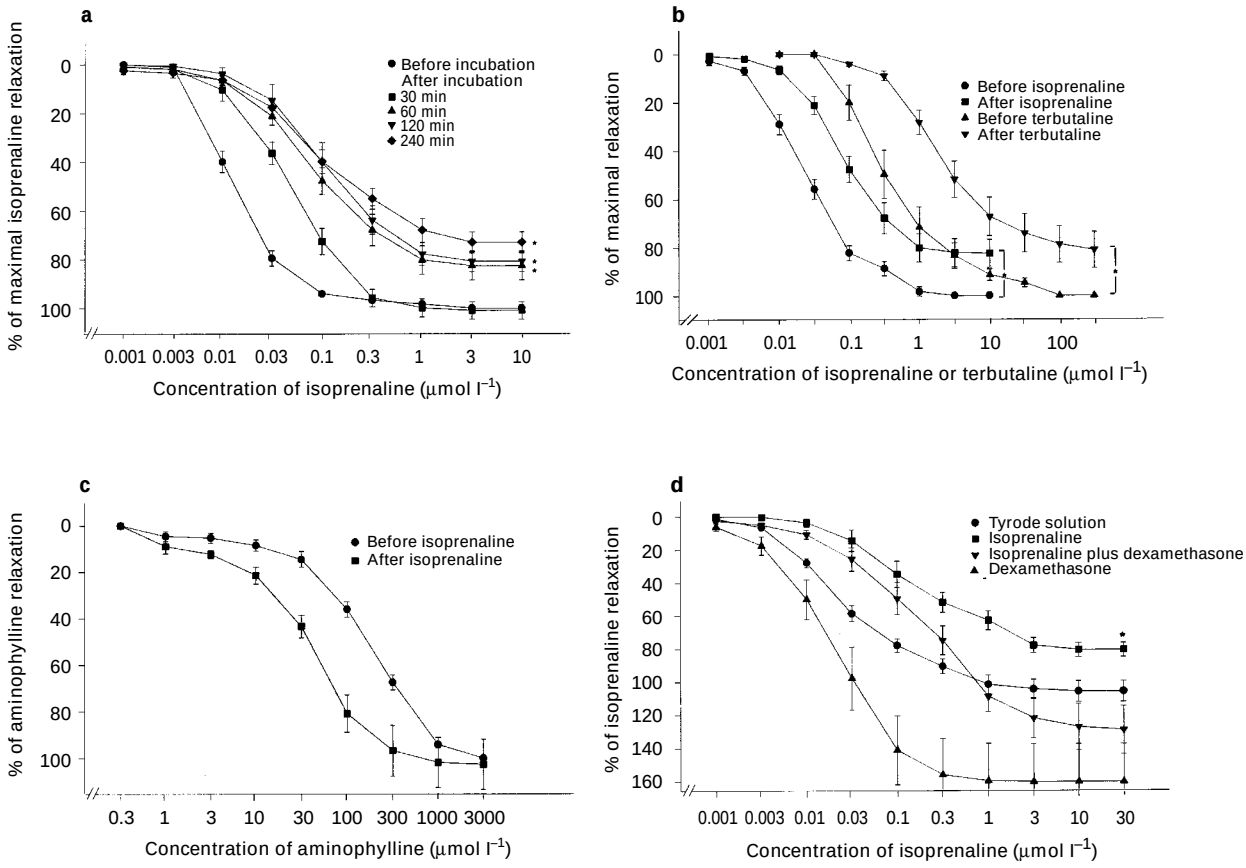

Figure 1 (a) Isoprenaline induced relaxation of carbachol EC_{50} (0.4 μmol l^{-1}) precontracted bronchi shown as % of the maximal relaxation obtained before and after incubation with 30 μmol l^{-1} isoprenaline. Ordinate scale: isoprenaline-induced relaxation as %. Abscissa scale: concentration of isoprenaline in μmol l^{-1} (*$P<0.05$ vs preincubation data). Note that after 60 min of preincubation with isoprenaline a significant decrease in relaxation activity occurred. (b) Isoprenaline and terbutaline induced relaxation of carbachol EC_{50} (0.4 μmol l^{-1}) precontracted bronchi shown as % of the maximal relaxation obtained before and after 60 min incubation with 30 μmol l^{-1} isoprenaline, and 100 μmol l^{-1} terbutaline. Ordinate scale: isoprenaline and terbutaline-induced relaxation as %. Abscissa scale: concentration of isoprenaline or terbutaline in μmol l^{-1} (*$P<0.05$). Note that after 60 min of incubation with either isoprenaline or terbutaline a significant decrease in relaxation activity occurred. (c) Maximal aminophylline-induced relaxation of carbachol EC_{50} (0.4 μmol l^{-1}) precontracted human bronchi before and after 60 min incubation with 30 μmol l^{-1} isoprenaline. Ordinate scale: % of maximal aminophylline relaxation. Abscissa scale: concentration of aminophylline in μmol l^{-1}. Note that after 60 min of isoprenaline incubation no decrease in relaxation activity for aminophylline occurred. (d) Maximal isoprenaline-induced relaxation of carbachol EC_{50} (0.4 μmol l^{-1}) contracted human bronchi after a 120 min incubation with either Tyrode solution, 30 μmol l^{-1} isoprenaline, 30 μmol l^{-1} isoprenaline plus 30 μmol l^{-1} dexamethasone or 30 μmol l^{-1} dexamethasone. Ordinate scale: % of maximal relaxation obtained after 120 min incubation. Abscissa scale: concentration of isoprenaline in μmol l^{-1} (*$P<0.05$ vs preincubation data). Note that the isoprenaline-induced reduction of maximal relaxation was completely abolished by coincubation of isoprenaline with dexamethasone.

Table 2 Effect of a 60 min pretreatment of isolated bronchi with either isoprenaline or terbutaline on the subsequent relaxation activity of each agonist

Incubation concentration (μmol l^{-1})	n	*Maximal effect before incubation* (mN)	*Maximal effect after incubation* (mN)	*Maximal effect* (% of preincubation)	*EC_{50} before incubation* (μmol l^{-1})	*EC_{50} after incubation* (μmol l^{-1})
Isoprenaline						
0.003	4	19.3±2.2	18.9±2.4	98±3	0.024 (0.015–0.039)	0.0229 (0.015–0.035)
3	6	19.4±2.2	16.8±2.0	87±4*	0.038 (0.012–0.126)	0.054 (0.019–0.156)
30	5	14.5±1.6	12.0±1.5	83±6*	0.026 (0.017–0.04)	0.078* (0.056–0.107)
Terbutaline						
100	7	14.9±2.0	12.5±2.5	81±8*	0.398 (0.155–1.02)	2.19* (1.48–3.24)

Values are mean±s.e.mean and mean with 95% confidence limits, respectively. *$P<0.05$.

with control as well as when the results obtained after isoprenaline and dexamethasone treatment were compared with control (Table 3).

β-Adrenoceptors after incubation with dexamethasone, isoprenaline or isoprenaline plus dexamethasone

Lung parenchyma from five patients was incubated with either Tyrode, dexamethasone (30 μmol l^{-1}), isoprenaline (30 μmol l^{-1}), or isoprenaline (30 μmol l^{-1}) plus dexamethasone (30 μmol l^{-1}) for 60 or 120 min. After 60 min incubation with 30 μmol l^{-1} isoprenaline the maximal number of measurable binding sites (B_{max}) was significantly ($P<0.01$) reduced by 36%, without any relevant change in the apparent affinity (K_D) of the radioligand for the β-adrenoceptor, as calculated by Scatchard analysis (Table 4, Figure 2a).

In a second set of experiments, tissues were incubated with dexamethasone alone for 120 min. After this preincubation, B_{max} was not significantly different from the value obtained after incubation with Tyrode solution. When isoprenaline was incubated together with dexamethasone (2 h), any agonist-induced down-regulation of β-adrenoceptors was prevented by the presence of the corticosteroid. Thus, dexamethasone induced a significant restoration of β-adrenoceptors ($P<0.05$) in comparison to B_{max} after isoprenaline incubation (Table 4). With regard to the binding affinity, dexamethasone exerted no effect (Table 4; Figure 2b).

Discussion

In the present study, it has been shown by using a desensitization model of human bronchi that preincubation with β_2-adrenoceptor agonists promotes a reduction in the efficacy and potency of a subsequent β-agonist-induced bronchial relaxation. This loss of relaxation ability occurs in parallel with a reduction in the number of β-adrenoceptors. However, dexamethasone was able to counterbalance these unwanted β-agonist effects.

β-Adrenoceptor agonists are the most established and most effective substances used in the treatment of obstructive lung disease. However, it is widely debated whether a loss in their bronchodilator activity occurs during long-term and/or high-dose treatment, or as a consequence of an active state of disease (Ellul-Micallef & Fenech, 1975; Harvey & Tattersfield, 1982). Occurrence of desensitization is of particular clinical interest for the treatment of bronchial obstruction because it can lead to a failure of the β-adrenoceptors on bronchial smooth muscles to respond to exogenous and endogenous stimuli with relaxation, and consequently, to a progression and worsening of disease symptoms.

Therefore, in a model of human bronchi we focused, first, on the impact of duration of the non-selective β-adrenoceptor agonist isoprenaline on the development of resistance and found that short-term treatment even at a highest agonist dose did not affect maximal relaxation activity, whereas 'chronic' incubation, for one to four hours, evoked a significant reduction of maximal effect, by about one quarter. Besides regular administration, tachyphylaxis with progressive bronchodilator decline can be enhanced both by increasing plasma concentrations of β-agonists or by accumulation of the drug within the lung tissue (van Metre, 1969; Reisman, 1970; Holgate *et al*., 1977; Galant *et al*., 1978; Harvey & Tattersfield, 1982). Therefore, we also tested the effect of drug concentration after constant 60 min incubations to ensure a significant reduction in bronchodilatation, as shown by the above experiments. Since isoprenaline is no longer used clinically, terbutaline, a selective β_2-adrenoceptor agonist, was investigated for com-

Table 3 Subsequent relaxation activity of isoprenaline after a 120 min incubation with either Tyrode solution, isoprenaline, isoprenaline plus dexamethasone or dexamethasone

Incubation	n	*Maximal isoprenaline-effect before incubation* (mN)	*Maximal isoprenaline-effect after incubation* (mN)	*Isoprenaline-effect* (% of preincubation)	*Isoprenaline-EC_{50} before incubation* (μmol l^{-1})	*Isoprenaline-EC_{50} after incubation* (μmol l^{-1})
Tyrode	7	17.1±3.2	17.7±3.0	106±6	0.018 (0.014–0.023)	0.028 (0.02–0.041)
Isoprenaline (30 μmol l^{-1})	6	16.5±1.5	13.5±1.9	81±4*	0.016 (0.01–0.025)	0.118* (0.056–0.214)
Isoprenaline plus dexamethasone (30 μmol l^{-1})	5	7.7±1.0	9.5±1.0	129±15	0.043 (0.012–0.148)	0.204 (0.059–0.703)
Dexamethasone (30 μmol l^{-1})	6	11.2±2.0	16.0±2.0	160±23	0.008 (0.003–0.022)	0.023 (0.017–0.031)

Values are mean±s.e.mean or mean with 95% confidence limits. *$P<0.05$ vs preincubation data.

Table 4 B_{max} and K_D values in memebranes from human peripheral lung after incubation with either Tyrode solution, isoprenaline, dexamethasone or isoprenaline plus dexamethasone

Incubation	*Time* (min)	n	B_{max} (fmol mg^{-1} protein)	B_{max} (% of control)	K_D (nmol l^{-1})
Tyrode	60	5	211.2±17.7		3.2 (2.6–4.1)
Isoprenaline (30 μmol l^{-1})	60	5	134.0±11.5**	64±1.6	2.5 (1.8–3.3)
Tyrode	120	5	162.8±27.5		4.3 (3.0–6.1)
Dexamethasone (30 μmol l^{-1})	120	5	160.0±22.1	102±7	5.0 (3.2–8.0)
Isoprenaline plus dexamethasone (30 μmol l^{-1})	120	5	142.3±28.7	88±9*	3.5 (2.4–5.0)

Values are mean±s.e.mean or mean with 95% confidence limits. *$P<0.05$ vs isoprenaline; **$P<0.01$.

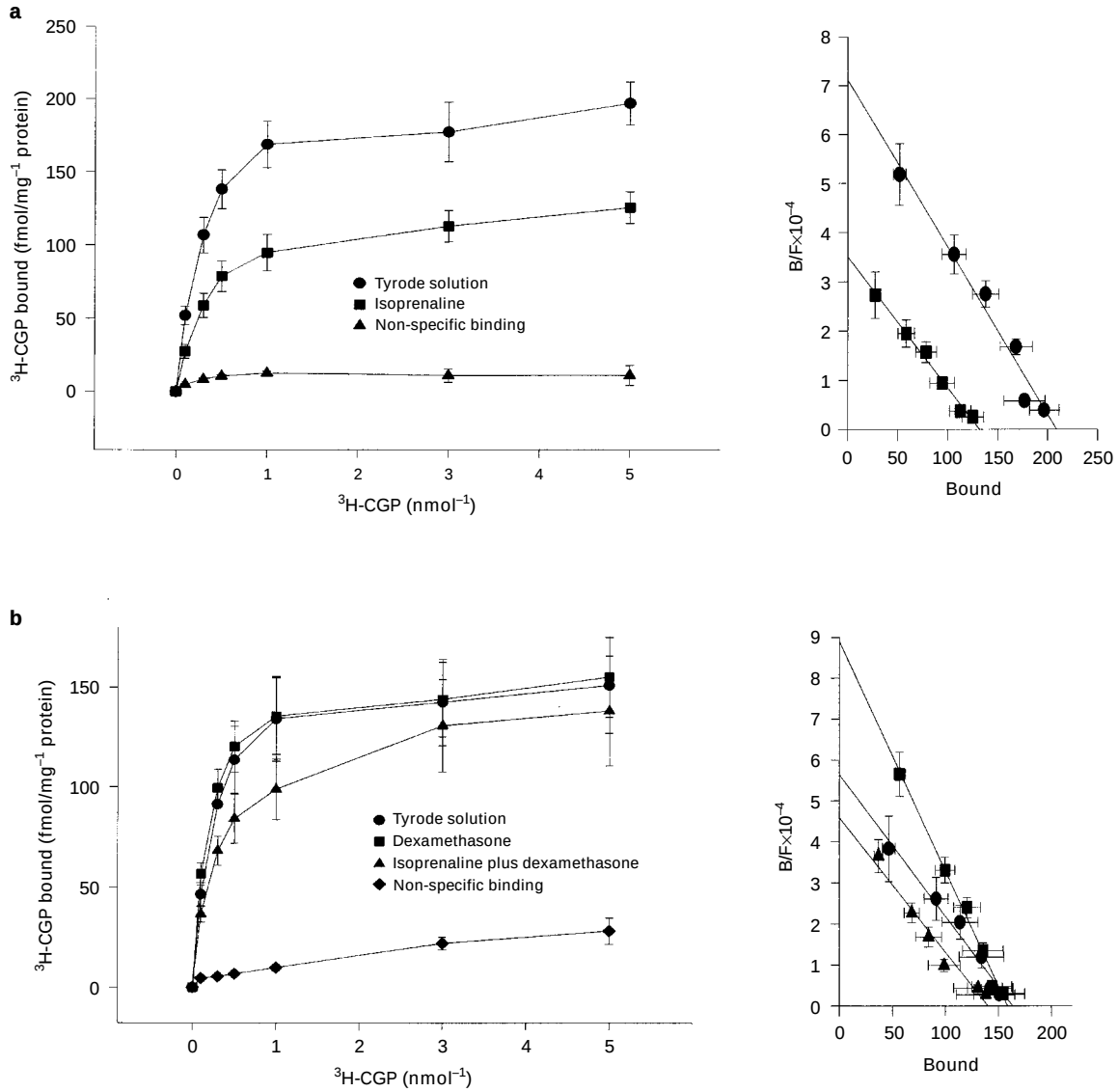

Figure 2 Mean binding isotherms of specific and non-specific binding of [^{3}H]-CGP 12177 in membranes of human lung tissue after a 60 min incubation with Tyrode solution and isoprenaline (a) and in comparison, with Tyrode solution, dexamethasone and isoprenaline plus dexamethasone at 37°C (b). Ordinate scales: bound [^{3}H]-CGP 12177 in fmol mg^{-1} protein. Abscissa scales: concentration of [^{3}H]-CGP 12177 in nmol l^{-1}. Inset: [^{3}H]-CGP 12177 bound mg^{-1} protein is plotted as a function of the ratio ($B/F \times 10^{-4}$) of bound [^{3}H]-CGP 12177 to free [^{3}H]-CGP 12177. The intercept with the abscissa scale gives the maximal number of binding sites (B_{max}); the slope represents the apparent affinity (K_D).

parison at a concentration of 100 μmol l^{-1} (Böhm *et al*., 1991). As has been suggested from animal models (Fernandes *et al*., 1988), at moderate to high concentrations both the selective and non-selective β-agonist elicited a statistically similar reduction of about 20% in maximal bronchodilatation and in EC_{50}. Similar results have been obtained in guinea-pig and in pig lung, in which isoprenaline administered at concentrations of 1–5 μmol l^{-1} over 1–3 h evoked a significant reduction in responsiveness to subsequent relaxant stimuli (Goldie *et al*., 1986; Fernandes *et al*., 1988; Herepath & Broadley, 1992).

If β-agonist desensitization is due to dysfunctional β-adrenoceptors, the response of bronchi to inhibitors of phosphodiesterase (PDE) will remain unchanged after β-agonist treatment (Goldie *et al*., 1986). Indeed, we did not observe tachyphylaxis, in the experiments performed, with the non-selective phosphodiesterase inhibitor aminophylline. As it has been shown that a short-term increase of intracellular adenosine 3′:5′-cyclic monophosphate (cyclic AMP) can even enhance sensitivity of distinct isoenzymes of PDE 4 to inhibition by highly selective PDE-inhibitors (Alvarez *et al*., 1995), this could possibly provide an explanation for the enhanced aminophylline potency which we observed after isoprenaline stimulation. On the other hand, an enhanced intracellular concentration of cyclic AMP has been shown to induce an increase in hydrolytic activity of distinct PDE isoenzymes, especially during long term regulation (Torphy *et al*., 1992; Manning *et al*., 1995). Hence, whether changes in the state of PDE activity are responsible for the increase in aminophylline sensitivity observed remains unproven.

In addition, desensitization could even take place as a consequence of influence on adenylate cyclase activity. However, in experiments performed in an animal model with the direct adenylate cyclase stimulator forskolin, no impairment in the function of adenylyl cyclase was observed. On the contrary, an increase in potency of forskolin was detected after isoprenaline incubation (Goldie *et al*., 1986), and a synergistic effect between both compounds has been discussed leading to an increase in adenylate cyclase activity (Goldie *et al*., 1986; Seamon & Daly, 1989). From our experimental approach, experiments with forskolin could not be performed because this compound cannot be washed out from the preparation. Therefore, no reliable condition could be established under which to perform a subsequent isoprenaline incubation and a second forskolin stimulation for comparison.

Interestingly, in our experiments long-term stimulation with a low or moderate concentration of β-agonists did not affect the half-maximal relaxation response. This provides some explanation for the lack of tachyphylaxis which has been observed in many clinical studies of patients with mild to moderate asthma, who consequently are treated with mild or moderately high doses of β-agonists (Larsson *et al*., 1977). In those studies, the near-maximal to maximal bronchodilator effect of compounds was not investigated as regards clinical outcome and the development of tachyphylaxis at low concentrations was studied and shown not to occur even *in vitro*. This was further supported by experiments on human lung parenchyma recently performed in our laboratory in which we did not observe down-regulation of β-adrenoceptors at drug concentrations usually used in the treatment of bronchial obstruction (Böhm *et al*., 1991). On the other hand, the observed reduction in the maximal relaxation effect on human bronchi after agonist incubation at higher concentrations over a prolonged period may have major implications for patients with excessive drug usage or requiring maximal relaxation response, i.e. during severe asthma. Although concomitant pathological alterations, such as mucus plugging, mucosal oedema and inflammatory changes in airway walls (McFadden & Gilbert, 1992), cannot be taken into consideration in our model, they represent additional factors leading to compromised disease control and bronchodilatation, in which full β-agonist bronchodilator action is crucial. Representative studies in a group of severe asthma patients are rare, but those performed have shown that the effects of adrenergic stimulation are reduced and response to corticosteroid treatment is delayed in patients during an active state of the disease (Inoue, 1967; Ellul-Micallef & Fenech, 1975).

As desensitization occurred within the first hour of the interaction between agonist and β-adrenoceptor, this time interval was used for radioligand binding experiments with membranes from the lung tissue to investigate whether β-adrenoceptor down-regulation plays a role in the development of tachyphylaxis. Such preparations necessarily contain β-adrenoceptors of several cell types and about 3 times the quantity found in bronchial preparations, but without differences in the binding affinity (Zöllinger *et al*., 1996). Thus, those differences in B_{max} must be kept in mind for interpretations which focus on the absolute number of binding sites.

The results obtained from lung parenchyma after one hour of agonist incubation revealed significant down-regulation and therefore, desensitization is largely due to β-adrenoceptor dysregulation. Firstly, with isoprenaline and terbutaline, significant down-regulation of β-adrenoceptors was measurable (Böhm *et al*., 1991). Secondly, studies with the less potent and effective (+)-isomer of isoprenaline show that exposure consequently leads to less desensitization. Thirdly, desensitization could be completely abolished if bronchi were pretreated with the β-adrenoceptor antagonist propranolol (Goldie *et al*., 1986; Fernandes *et al*., 1988), and fourthly, after 1 h incubation with high-dose isoprenaline, the PDE-inhibitor aminophylline showed no impairment of its relaxant activity on human bronchi, which indicates that desensitization affects signalling compounds upstream from cyclic AMP-generation. However, tachyphylaxis could also be due to phosphorylation of β-adrenoceptors by the cyclic AMP-dependent protein kinase A (PKA) and/or PDE, triggered by increased levels of cyclic AMP (Torphy *et al*., 1992). This can represent an additional desensitization effect and could gain relevance during long term administration of β-agonistic compounds (Barnes & Chung, 1992). From the above data, adenylate cyclase does not seem to be affected by tachyphylaxis. Nevertheless, tachyphylaxis could be due to changes in β-adrenoceptor affinity. Our experiments revealed that after 60 min incubation with the agonist, the binding affinity of β-adrenoceptors, as judged from the K_D value, did not show any change. In addition to this finding, alterations in coupling of the β-adrenoceptor to G protein and/or adenylate cyclase could have taken place. Thus, there is evidence that uncoupling from G_S-proteins can occur and could lead to a decrease in the proportion of high-affinity binding sites with a concomitant reduction in sensitivity to guanosine 5′-triphosphate (GTP) stimulation and change in efficacy of coupling to adenylate cyclase (Nerme *et al*., 1990).

In recent years many groups have investigated the effect of corticosteroids, generally after 6 or more hours of preincubation (Davis & Conolly, 1980; Foster & Harden, 1980; Hall *et al*., 1993). However, whether or not steroids exert a direct effect on β-adrenoceptor function and β-adrenoceptor regulation in human bronchi has not been established. In our experiments, dexamethasone was incubated for 120 min at a concentration of 30 μmol l^{-1}. This somewhat higher concentration was used to compensate for the relatively short duration of dexamethasone pretreatment. Whether this led to equivalent tissue levels as those achieved in severe asthma or chronic obstructive pulmonary disease is difficult to ascertain, but it is plausible because of the high perfusion rate which exists within inflamed airways. Moreover, this concentration ensured that upregulation of β-adrenoceptors could occur, as could an increase in β-adrenoceptor mRNA (Foster & Harden, 1980; Malbon & Hadcock, 1988; Hadcock *et al*, 1989). In fact, with intact human bronchial rings, β-agonist-induced reduction in isoprenaline potency could be prevented by the administration of dexamethasone and in addition, reduction in the maximal relaxant effect of isoprenaline was completely abolished. Moreover, in comparison to control, dexamethasone induced a marked (NS) increase in relaxation response and abolished the isoprenaline-induced decrease in maximal relaxation and reduction in EC_{50}. This has even been shown for pig bronchi; cortisol prevented the reduction in responsiveness to isoprenaline at 3 and at 6 h (Goldie *et al*., 1986).

Since dexamethasone alone did not change the basal tone of bronchi in our experimental setup, a synergistic effect between both compounds must be postulated. This may be a consequence of the effect on coupling, preservation and recycling of preformed β-adrenoceptors (Djurup, 1981; Stiles & Lefkowitz, 1981), or on stability and transcription of β-adrenoceptor mRNA (Foster & Harden, 1980; Hadcock *et al*., 1989; Mak *et al*., 1995). Isoprenaline activity can be enhanced as a result of a corticosteroid-induced increase in adenylate cyclase activity, as has been shown in different preparations (Besse & Bass, 1976; Marone *et al*., 1980).

In the binding experiments we were able to show that after 2 h of coincubation with isoprenaline and dexamethasone, the β-agonist-induced down-regulation of β-adrenoceptors in lung tissue could be completely prevented. Unlike results shown by other groups (Johnson & Jaworski, 1983; Hadcock *et al*., 1989; Mano *et al*., 1979), this was not accompanied by a change in the antagonist binding affinity. Thus, these results provide strong evidence that down-regulation of β-adrenoceptors plays a decisive role in the desensitization process in human lung tissue and that steroids can counterbalance this unwanted effect by a direct action on β-adrenoceptor regulation, and/or an increase in coupling between β-adrenoceptors and adenylate cyclase (Davies & Lefkowitz, 1983).

In conclusion, it has been demonstrated in a model of β-agonist-induced desensitization in human bronchi that tachyphylaxis occurs, fairly late in the case of a half-maximal relaxation effect and earlier with regard to the maximal bronchodilator action. This change in function is paralleled by a down-regulation of lung β-adrenoceptors. Both adverse β-agonist effects can be counterbalanced by concomitant corticosteroid treatment, which restores β-agonist sensitivity and β-adrenoceptor quantity. This might be of particular importance in the treatment of obstructive lung disease, especially in the consideration of long-term and/or higher dose β-agonist treatment.

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