British Journal of Pharmacology (2001) 132, 1261–1270

www.nature.com/bjp

Chronic systemic administration of salmeterol to rats promotes pulmonary β_2-adrenoceptor desensitization and down-regulation of $G_{s\alpha}$

[1]Paul A. Finney, [1]Louise E. Donnelly, [2]Maria G. Belvisi, [3]Tsu-Tshen Chuang, [2]Mark Birrell, [4]Andrew Harris, [1]Judith C.W. Mak, [3]Carol Scorer, [1]Peter J. Barnes, [1]Ian M. Adcock & *,[1]Mark A. Giembycz

[1]Thoracic Medicine, National Heart & Lung Institute, Imperial College School of Medicine, London; [2]Cardiothoracic Surgery, National Heart & Lung Institute, Imperial College School of Medicine, London; [3]GlaxoWellcome Research and Development, Medicines Research Centre, Stevenage, Hertfordshire and [4]GlaxoWellcome Research and Development, UK Research BioMet, Ware, Hertfordshire

1 The aim of the present study was to examine the effects of chronic infusion of the long-acting agonist salmeterol on pulmonary β_2-adrenoceptor function in Sprague-Dawley rats *in vivo* and to elucidate the molecular basis of any altered state.

2 Systemic administration of rats with salmeterol for 7 days compromised the ability of salmeterol and prostaglandin E_2 (PGE_2), given acutely by the intravenous route, to protect against ACh-induced bronchoconstriction when compared to rats treated identically with vehicle.

3 β_1- and β_2-adrenoceptor density was significantly reduced in lung membranes harvested from salmeterol-treated animals, which was associated with impaired salmeterol- and PGE_2-induced cyclic AMP accumulation *ex vivo*.

4 Three variants of $G_{s\alpha}$ that migrated as 42, 44 and 52 kDa peptides on SDS polyacrylamide gels were detected in lung membranes prepared from both groups of rats but the intensity of each isoform was markedly reduced in rats that received salmeterol.

5 The activity of cytosolic, but not membrane-associated, G-protein receptor-coupled kinase was elevated in the lung of salmeterol-treated rats when compared to vehicle-treated animals.

6 The ability of salmeterol, administered systemically, to protect the airways of untreated rats against ACh-induced bronchoconstriction was short-acting (t_{off} ~45 min), which contrasts with its long-acting nature when given to asthmatic subjects by inhalation.

7 These results indicate that chronic treatment of rats with salmeterol results in heterologous desensitization of pulmonary G_s-coupled receptors. In light of previous data obtained in rats treated chronically with salbutamol, we propose that a primary mechanism responsible for this effect is a reduction in membrane-associated $G_{s\alpha}$. The short-acting nature of salmeterol, when administered systemically, and the reduction in β-adrenoceptor number may be due to metabolism to a biologically-active, short-acting and non-selective β-adrenoceptor agonist.

British Journal of Pharmacology (2001) **132,** 1261–1270

Keywords: β_2-adrenoceptor desensitization *in vivo*; salmeterol; down-regulation of $G_{s\alpha}$

Abbreviations: FEF_{25-75}, forced expiratory flow at 25% and 75% of vital capacity; FEV_1, forced expiratory volume in 1 s; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GRK, G-protein receptor-coupled kinase; ICYP, iodocyanopindolol; KH, Krebs-Henseleit; PGE_2, prostaglandin E_2; PKA, cyclic AMP-dependent protein kinase; ROS, rod outer segments; SSC, standard sodium citrate; IBMX, 3-isobutyl-1-methylxanthine

Introduction

Repeated administration of high-dose β_2-adrenoceptor agonists renders susceptible individuals tolerant to their beneficial effects in asthma. With regard to long-acting agonists, there is evidence that tolerance develops both to their dilator and protective effects in the airways. Lipworth and colleagues (Newnham *et al*., 1994; 1995) have reported that 4 weeks treatment of asthmatic subjects with inhaled eformoterol and salmeterol produced a rightwards shift in the bronchodilator dose-response curve for both agonists. This state of tolerance was reflected in terms of a decrease in forced expiratory volume in 1 s (FEV_1) and forced expiratory flow at 25% and 75% of vital capacity (FEF_{25-75}), indicating that subsensitivity of β_2-adrenoceptors to salmeterol and eformoterol had occurred in the large and small airways. Equally, tolerance to the protective effect of salmeterol against methacholine- and exercise-induced bronchoconstriction has been documented in mild to moderate asthmatic subjects after chronic administration (Boulet *et al*., 1998; Cheung *et al*., 1992; Drotar *et al*., 1998; Simons *et al*., 1997).

*Author for correspondence at: Thoracic Medicine, National Heart & Lung Institute, Imperial College School of Medicine, Dovehouse Street, London SW3 6LY, U.K. E-mail: m.giembycz@ ic.ac.uk

The molecular aetiology of β_2-adrenoceptor sub-sensitivity in asthma is unclear. Airways resected from asthmatic patients fail to relax normally to isoprenaline, supporting a possible defect in β-adrenoceptor function (Bai *et al*., 1992; Cerrina *et al*., 1986; Goldie *et al*., 1986), although it is equivocal whether this is the result of treatment or a consequence of the disease process itself. Nevertheless, down-regulation of β-adrenoceptor number in lung and airways smooth muscle has been reported in animals given isoprenaline and noradrenaline chronically by infusion (Nerme *et al*., 1990; Nishikawa *et al*., 1993; 1994) and this is associated with a reduced functional responsiveness towards β-adrenoceptor agonists *ex vivo* (Nerme *et al*., 1990; Nishikawa *et al*., 1994). Thus, it is possible that compromised bronchodilatation and loss of protection against various bronchoconstrictor challenges in humans is due, at least in part, to pulmonary β_2-adrenoceptor desensitization.

Two major molecular mechanisms have been delineated in isolated cells that result in short-term β_2-adrenoceptor desensitization. One of these promotes homologous refractoriness and involves the uncoupling of the agonist-occupied form of the receptor from the stimulatory guanine nucleotide binding protein, G_s, by mechanisms that require phosphorylation of serine and threonine residues at the carboxy-terminus of the agonist-occupied receptor (Fredericks *et al*., 1996; Premont *et al*., 1995). This reaction can be catalysed by at least three members of the G-protein receptor-coupled kinase (GRK) superfamily family, including GRK2 and GRK3 (Krupnick & Benovic, 1998; Premont *et al*., 1995). The subsequent binding of β-arrestin, a soluble protein that prevents further coupling to G_s (Lohse *et al*., 1990), then halts signalling through the receptor. Short-term desensitization is also effected by cyclic AMP-dependent protein kinase (PKA) following phosphorylation of serine and threonine residues present within the third intracellular loop of the protein in response to an increase in intracellular cyclic AMP (Lohse *et al*., 1990). In contrast, prolonged periods of desensitization can involve physical internalization and subsequent degradation of receptors (Lohse, 1993) due to an inhibition of transcription and/or increased post-transcriptional processing of β_2-adrenoceptor mRNA (Lohse, 1993; Mayor *et al*., 1998). In addition, another less-well characterized process is a reduction of membrane-associated $G_{s\alpha}$ (Milligan, 1993), although the functional relevance of this process has not been rigorously explored *in vitro* or *in vivo*.

Although desensitization of G-protein-coupled receptors (GPCR) has been studied extensively, most of the information to date has been gathered from cultured cell systems and the extent to which this applies to the *in vivo* situation is little investigated. Here we describe pulmonary β_2-adrenoceptor desensitization in rats treated chronically with the long-acting β_2-adrenoceptor agonist, salmeterol and have investigated the molecular aetiology of this altered state. Although little is known of the regulation of the β_2-adrenoceptor in the rat, it was selected for two reasons. First, the rat is relatively more steroid-sensitive than other small laboratory animals such as the guinea-pig (Hirshman & Downes, 1985). Second, we have shown previously that glucocorticoids, such as dexamethasone, protect against pulmonary β_2-adrenoceptor desensitization in the rat *in vivo* (Mak *et al*., 1995), which mimics the effect of steroids in humans (Barnes, 1995).

Methods

Animals and surgery

Male Sprague-Dawley rats (Charles River Ltd, Kent) of 275–300 g body weight were housed in a temperature-controlled (21°C) environment with food and water available *ad libitum*. Animals were sedated with 2% v v^{-1} Hypnorm (0.3 ml kg^{-1}) and implanted sub-cutaneously with osmotic mini pumps (Alzet model 2001) delivering salmeterol (10 μg kg^{-1} h^{-1}) or vehicle (0.1% glacial acetic acid in phosphate-buffered saline) for 7 days as described previously (Finney *et al*., 2000). The dose of salmeterol is approximately 25% of the dose of salbutamol used in patients hospitalized with acute severe asthma (Bohn *et al*., 1984; Cheong *et al*., 1988; Cluzel *et al*., 1990; O'Driscoll *et al*., 1988) and is based on equivalence studies in human asthmatic subjects, where a weight-for-weight dose ratio for salmeterol:salbutamol of 1 : 4 was found (Higham *et al*., 1997). Salmeterol was given as an infusion to allow a comparison with a previous study where desensitization of pulmonary β_2-adrenoceptors was induced in rats treated systemically with salbutamol (Finney *et al*., 2000).

Instrumentation of rats for measurement of airway mechanics

Each rat was anaesthetized with urethane and the left carotid artery and left jugular vein were cannulated for measuring changes in blood pressure and for the injection of drugs respectively. The trachea was cannulated and the animal ventilated at constant volume with a pump operating at 75 strokes per minute. Changes in respiratory insufflation pressure were measured using a modification of the method described by Konzett & Rössler (1940) as described in Finney *et al*. (2000).

Assessment of pulmonary β_2-adrenoceptor desensitization in vivo

Vehicle- and salmeterol-treated rats were given ACh (500 μg kg^{-1} i.v, bolus) every 5 min to establish a constant degree of bronchoconstriction. When airway function had normalized (15 min after last dose of ACh), a submaximal concentration of salmeterol (100 μg kg^{-1} i.v. bolus) was administered and the magnitude of ACh-induced bronchoconstriction was reassessed 5 min later. An identical protocol was used to assess the potential bronchoprotective effect of PGE_2 (300 μg kg^{-1} i.v. bolus).

Quantification of β-adrenoceptor number in lung membranes

Lung membranes were prepared according to Mak *et al*. (1995) and Nishikawa *et al*. (1993), and total, β_1- and β_2-adrenoceptor density (B_{max}) and the affinity (K_D) of [^{125}I]-iodocyanopindolol ([^{125}I]-ICYP) was determined as described previously (Finney *et al*., 2000).

Measurement of steady-state β_1- and β_2-adrenoceptor mRNA levels

Total RNA was isolated from lung as described by Chomczynski & Sacchi (1987) and the abundance of β_1- and β_2-adrenoceptor mRNA transcripts estimated by Northern blot analysis. Samples of denatured mRNA (20 μg lane^{-1}) were size fractionated on 1% agarose/formaldehyde gels containing 20 mM MOPS (pH 7), 5 mM sodium acetate and 1 mM EDTA, and transferred to Hybond-N$^+$ membranes (Amersham) by capillary action using 20× standard sodium citrate (SSC; 1× SCC, 150 mM NaCl, 15 mM sodium citrate, pH 7). The RNA was permanently fixed to the membranes using a UV Stratalinker 2400 (Stratagene, Cambridge).

Random primer labelling was carried out with the 851 bp *Sma*I/*Pvu*II and 439 bp I fragments from the human β_1- and β_2-adrenoceptor cDNAs respectively using [α-^{32}P]-dCTP. A 1272 bp *Pst*I fragment from the rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was used as a 'house-keeping' gene. Pre-hybridization and hybridization were carried out at 42°C with the probes labelled to approximately 1.5×10^6 c.p.m. ml^{-1} in buffer A (50 mM Tris-HCl (pH 7.5), 50% formamide, 2× SSC, 5× Denhardt's solution, 0.1% SDS, 5 mM EDTA, 250 μg ml^{-1} denatured salmon sperm DNA). After hybridization, the blots were washed to a stringency of 0.1× SCC/0.1% SDS at 65°C and exposed to Kodak X-OMAT film at −80°C for 1 to 4 days. The autoradiograms were quantified by laser-scanning densitometry and expressed as a ratio to GAPDH.

Ex vivo *cyclic AMP accumulation studies*

Lung was chopped (~5 mm^3) and equilibrated for 30 min at 37°C in oxygenating (95% O_2/5% CO_2) Krebs-Henseleit (KS) solution (in mM: 118 NaCl, 5.9 KCl, 1.2 $MgSO_4.7H_2O$, 1.2 $NaH_2PO_4.2H_2O$, 2.5 $CaCl_2.6H_2O$, 10 glucose, 25 $NaHCO_3$) containing 10 μM indomethacin. Tissue was incubated for 30 min in the presence of IBMX (100 μM), and salmeterol (1 μM) or PGE_2 (1 μM) was added for 5 min. Tissue was removed from the KH solution, blotted on absorbent paper, immersed in liquid N_2 and stored at −80°C. When required the cyclic AMP content was extracted from the lung and measured by radioimmunoassay as described previously (Finney *et al*., 2000; Seybold *et al*., 1998).

Measurement of GRK activity

Cytosolic and particulate GRK was prepared from rat lung according to Benovic *et al*. (1987) and GRK activity was determined immediately by the method of Mayor *et al*. (1987).

Western immunoblot analyses

Frozen lung was homogenized, denatured and subjected to electrophoresis on 10% SDS/Tris polyacrylamide gels as described previously (Finney *et al*., 2000). Proteins were transferred onto Hybond nitrocellulose paper and blocked overnight in 10 mM Tris-base/0.05% Tween 20 containing 5% skimmed milk. Membranes were then incubated at 25°C for 1 h with a rabbit polyclonal antibody specific to either $G_{s\alpha}$, Gβ or the β_2-adrenoceptor diluted 1 : 1000, 1 : 1000 and 1 : 750 respectively. Membranes were washed, incubated with a donkey, anti-rabbit horseradish peroxidase-conjugated antibody and treated with ECL reagent according to the manufacturer's instructions. Proteins were visualized by exposure of the membranes to Kodak X-OMAT film and quantified by laser-scanning densitometry.

Protein estimation

Protein was measured using a BioRad kit according to the manufacturer's instructions.

Drugs and analytical reagents

Salmeterol was provided by GlaxoWellcome (Stevenage, Hertfordshire, U.K.). ECL reagent, horseradish peroxidase-conjugated secondary antibody and [α^{32}P]-dCTP and [^{125}I]-ICYP (specific activities >3000 Ci mmol^{-1} and 2000 Ci mmol^{-1} respectively) were supplied by Amersham International (Amersham, Buckinghamshire, U.K.). Antibodies against $G_{s\alpha}$ and Gβ were purchased from NEN/Dupont (code NEI 805 and NEI 808 respectively) and the β_2-adrenoceptor antibody was from Santa Cruz (*sc* # 569). All other reagents were from Sigma (Poole, Dorset, U.K.).

Statistical analysis

Data points and bars represent the mean ± s.e.mean of '*n*' independent observations. When appropriate, data were analysed non-parametrically using Mann–Whitney *U*-test. The null hypothesis was rejected when $P<0.05$.

Results

Repeated administration of ACh (500 μg kg^{-1} i.v. ~ED_{50}) to saline- and salmeterol-treated rats provoked bronchoconstriction that was highly reproducible over 60 min (data not shown). The resting mean arterial blood pressure was not significantly different ($P>0.05$) between vehicle (76.1 ± 3.7 mmHg, $n=28$)- and salmeterol (77.9 ± 8.2 mmHg, $n=31$)-treated animals. Similarly, intravenous administration of ACh (500 μg kg^{-1}) evoked a depressor response in both groups of rats of equivalent magnitude (vehicle: 34.4 ± 1.5 mmHg, $n=28$; salmeterol: 29.1 ± 3.1 mmHg, $n=28$, $P>0.05$).

Effect of chronic treatment of rats with salmeterol on the ability of salmeterol and PGE_2 to protect against ACh-induced bronchoconstriction

Intravenous administration of salmeterol (100 μg kg^{-1} bolus) or PGE_2 (300 μg kg^{-1} bolus) to vehicle-treated rats significantly reduced (by 44 and 32% respectively) the magnitude of ACh-induced bronchoconstriction whereas in the salmeterol-treated group of animals no significant protection was observed with either agonist indicating that the desensitization was heterologous (Figure 1a,b; Table 1). In none of the experiments did salmeterol or PGE_2 affect resting airways tone.

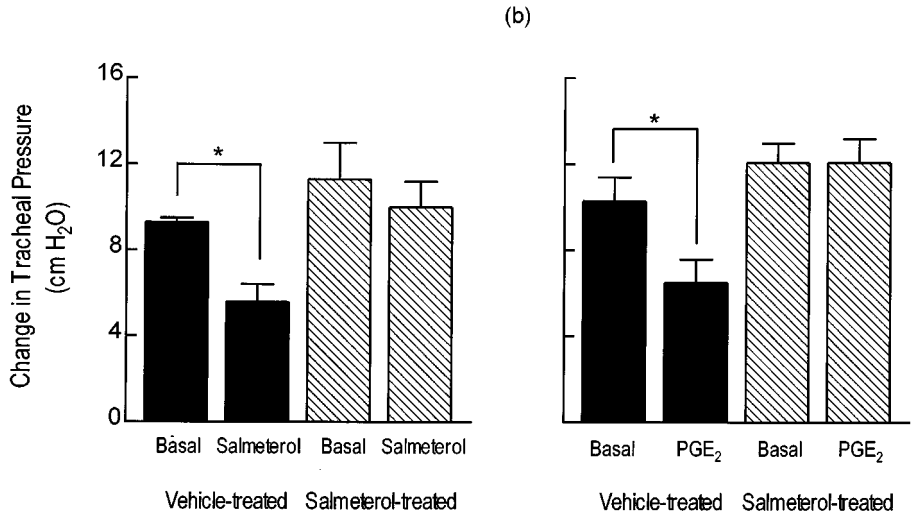

Figure 1 Effect of chronic systemic treatment of rats with salmeterol on the ability of salmeterol and PGE_2, administered acutely, to protect against ACh-induced bronchoconstriction. Rats were treated with vehicle or salmeterol (10 μg kg^{-1} h^{-1}). After 7 days, each animal was instrumented for the measurement of lung function. ACh (500 μg kg^{-1} i.v.) was administered and the maximum increase in overflow pressure was measured. When baseline lung function was re-established, salmeterol (100 μg kg^{-1} i.v. bolus) or PGE_2 (300 μg kg^{-1} i.v. bolus) was given and 5 min later ACh was administered again and any change in overflow pressure was noted. Each bar represents the mean$\pm$s.e.mean of four determinations made in different rats. *$P<0.05$, significant protection of ACh-induced bronchoconstriction.

Table 1 Effect of chronic systemic treatment of rats with salmeterol on the ability of salmeterol and PGE_2, given acutely, to protect against ACh-induced bronchoconstriction

	Tracheal pressure (cmH_2O)					
	Vehicle-treated rats			*Salmeterol-treated rats*		
	Pre-	*Post-*	*% Protection (mean)*	*Pre-*	*Post-*	*% Protection (mean)*
Salmeterol (100 μg kg^{-1} i.v.)	9.3$\pm$0.2	5.6$\pm$0.8*	43.5$\pm$8.6	11.3$\pm$1.7	10.0$\pm$1.2	11.2$\pm$3.6
PGE_2 (300 μg kg^{-1} i.v.)	10.3$\pm$1.1	7.0$\pm$1.1*	32.3$\pm$3.8	12.1$\pm$0.9	12.1$\pm$1.1	0.4$\pm$3.7

Rats were treated with vehicle or salmeterol (100 μg kg^{-1} h^{-1}). After 7 days, each animal was instrumented for the measurement of lung function as described in the Methods section. ACh (500 μg kg^{-1} i.v.) was administered and the maximum increase in overflow pressure was measured. When baseline lung fuction was re-established, salmeterol (100 μg kg^{-1} i.v., bolus) or PGE_2 (300 μg kg^{-1} i.v., bolus) was given and 5 min later ACh was administered again and any change in overflow pressure was noted. Data represent the mean+s.e.mean of four determinations made in different rats. *$P<0.05$, significant protection of ACh-induced bronchoconstriction.

Effect of chronic treatment of rats with salmeterol on β_1- and β_2-adrenoceptor density and mRNA expression in lung

Chronic treatment of rats with salmeterol (10 μg kg^{-1} h^{-1}) produced a significant ($\sim$70%) reduction in β-adrenoceptor density when compared to naïve animals, without affecting the affinity of the non-selective ligand, [^{125}I]-ICYP (Table 2). In the presence of selective antagonists it was established that salmeterol reduced β_1- and β_2-adrenoceptor number by 50 and 70% respectively but did not alter the K_D of [^{125}I]-ICYP for either receptor subtype (Table 2). To determine whether the *in vivo* binding of salmeterol to an 'exosite' within the β_2-adrenoceptor reduced, artificially, the total number of binding sites available to [^{125}I]-ICYP *ex vivo*, β_2-adrenoceptor protein in the lung from both groups of animals was measured by Western blotting. Consistent with the radioligand binding data, chronic treatment of rats with salmeterol reduced β_2-adrenoceptor protein in lung membranes by $\sim$50% (Figure 2). Despite the reduction in receptor expression at day 7, the steady state level of β_1- and β_2-adrenoceptor mRNA transcripts was unchanged in lung harvested from salmeterol-treated rats when compared to animals that received vehicle (Figure 3).

Effect of chronic treatment of rats with salmeterol on the ability of salmeterol and PGE_2 to increase cyclic AMP mass in lung ex vivo

To determine if down-regulation of β-adrenoceptor number was accompanied by compromised signal transduction, the ability of salmeterol to increase cyclic AMP mass in lung parenchyma was assessed *ex vivo*. No significant difference in the basal cyclic AMP content was detected between lung excised from either group of animals (Figure 4). However, salmeterol (1 μM)-induced cyclic AMP accumulation was significantly attenuated in lung taken from salmeterol-treated rats (1.56 fold increase over basal level) when compared to

Table 2 Effect of chronic systemic treatment of rats with salmeterol on the density of cell-surface β_2-adrenoceptors and on the affinity of [^{125}I]-ICYP

	Total β-adrenoceptors		*β_1-adrenoceptors**		*β_2-adrenoceptors**	
	K_d (*pM*)	B_{max} (fmol mg^{-1} protein)	K_d (*pM*)	B_{max} (fmol mg^{-1} protein)	K_d (*pM*)	B_{max} (fmol mg^{-1} protein)
Vehicle	5.5±2.5	119±14.0	24.2±4.3	61±7	4.9±0.7	94±10
Salmeterol	5.8±0.7	36±8†	39.2±11.3	31±7†	5.9±0.8	29±6†

Lung membranes were prepared from vehicle- and salmeterol (10 μg kg^{-1} h^{-1})-treated rats and total, β_1- and β_2-adrenoceptor density and the affinity of [I^{125}]-ICYP were determined as described in the Methods section. Data represent the mean±s.e.mean of eight independent observations from lung taken from different animals. †$P<0.05$, significant reduction in B_{max} compared to vehicle-treated rats. *β_2-Subtype defined with the selective β_1-adrenoceptor antagonist CGP 20712A (100 nM). *β_1-Subtype defined with the selective β_2-adrenoceptor antagonist ICI 118551 (100 nM).

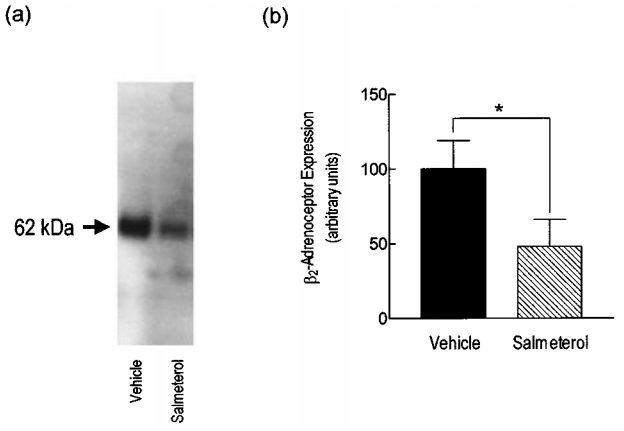

Figure 2 Effect of chronic systemic treatment of rats with salmeterol on β_2-adrenoceptor protein expression in lung membranes. Rats were treated with salmeterol (10 μg kg^{-1} h^{-1}) or vehicle for 7 days and the expression of β_2-adrenoceptor protein was measured. (a,b) show a representative Western blot and bar chart of the mean±s.e.mean of six determinations respectively. Equal loading was confirmed by staining of the gels with Coomassie Blue (not shown). *$P<0.05$, significant reduction in β_2-adrenoceptor protein in salmeterol-treated rats when compared to vehicle-treated animals.

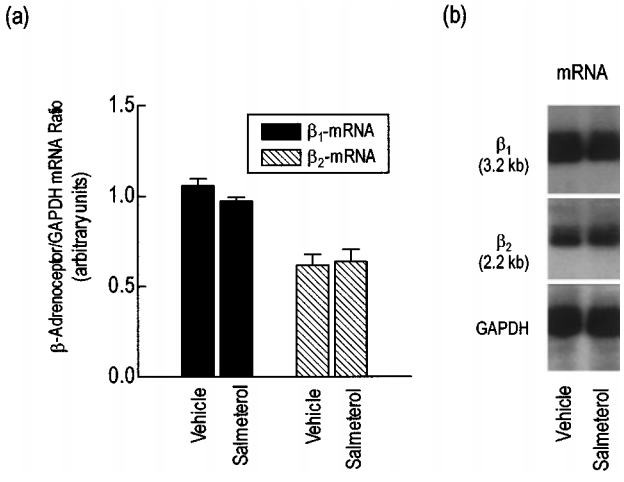

Figure 3 Effect of chronic systemic treatment of rats with salmeterol on the steady-state level of β_1- and β_2-adrenoceptor mRNA transcripts. Rats were treated with salmeterol (10 μg kg^{-1} h^{-1}) or vehicle for 7 days. Lungs were excised and β_1- and β_2-adrenoceptor mRNA was extracted and estimated by Northern analysis. (a) shows the mean±s.e.mean of six independent determinations that are expressed relative to the 'house-keeping' gene GAPDH. A representative autoradiogram is shown in (b).

animals that received vehicle (2.74 fold increase over basal level; Figure 4a). The desensitization of β-adrenoceptor-mediated cyclic AMP accumulation by salmeterol was heterologous in that the ability of PGE_2 to increase the cyclic AMP content in lung was profoundly impaired (Figure 4b).

Effect of chronic treatment of rats with salmeterol on the expression of $G_{s\alpha}$ and G_β subunits in lung

Western blotting was performed to determine if the compromised ability of salmeterol to elevate the cyclic AMP content in lung taken from salmeterol-treated rats was associated with a reduction in the expression of $G_{s\alpha}$ (Figure 5). A primary antibody raised against an epitope at the carboxy-terminus of the α-subunit of G_s, identified three bands in lung membranes prepared from vehicle-treated rats that migrated as 42, 44 and 52 kDa peptides on SDS polyacrylamide gels. Treatment of rats for 7 days with salmeterol significantly reduced the intensity of each of these bands by between 45 and 70% (Figure 5a,b). A primary antibody raised against an epitope at the carboxy-terminus of the common β-subunit of heterotrimeric GTP-binding proteins, detected a single 35 kDa band in lung membranes prepared from vehicle-treated rats. However, unlike $G_{s\alpha}$ variants, G_β was not altered in lung taken from salmeterol-treated animals (data not shown). Western blotting failed to detect $G_{s\alpha}$ or G_β subunits in the cytosolic fraction of lung tissue taken from either group of animals at day 7.

Effect of chronic treatment of rats with salmeterol on GRK activity in lung

Chronic administration of rats with salmeterol produced a significant (~2 fold over basal level) increase in cytosolic GRK activity in lung parenchyma when compared to vehicle-treated animals (from 164±25 to 341±87 fmol min^{-1} mg protein^{-1}; $n=12$, $P<0.05$). No change in GRK activity was detected in the particulate fraction of salmeterol-treated rats at day 7.

Duration of action of intravenous salmeterol

The duration of action of salmeterol was assessed by monitoring the magnitude of ACh (500 μg kg^{-1} i.v.)-induced bronchoconstriction repeatedly in naïve rats over a period of 60 min. The anti-spasmogenic activity of salmeterol (100 μg kg^{-1}; bolus) was short-lasting with an offset halftime

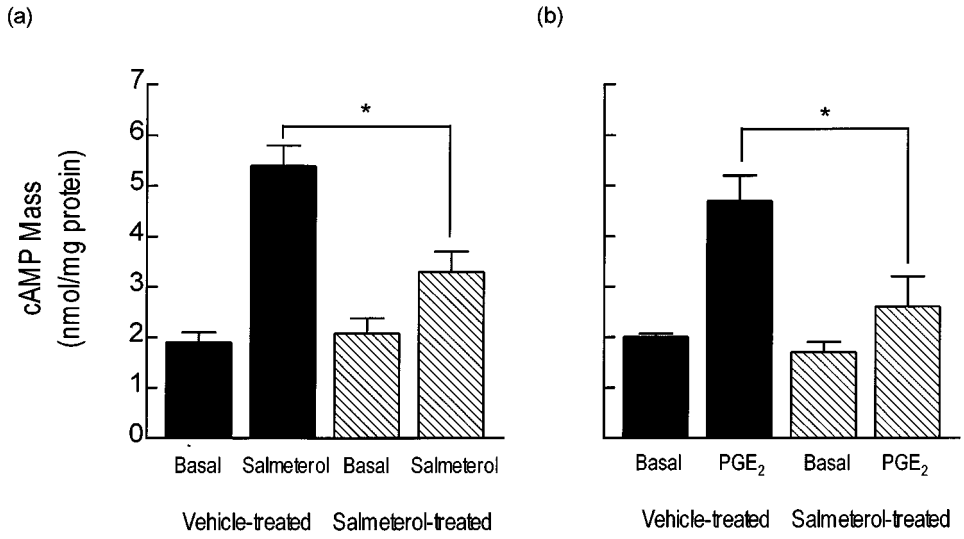

Figure 4 Effect of chronic systemic treatment of rats with salmeterol on salmeterol- and PGE_2-induced cyclic AMP accumulation in lung *ex vivo*. Rats were treated with salmeterol (10 μg kg^{-1} h^{-1}; filled bars) or vehicle (hatched bars) for 7 days and the ability of salmeterol (a) and PGE_2 (b) to increase cyclic AMP mass in lung was determined *ex vivo* in the presence of IBMX (100 μM). Each bar represents the mean$\pm$s.e.mean of six independent determinations using tissue from different animals. *$P<0.05$, significant reduction in salmeterol- and PGE_2-induced cyclic AMP formation.

(defined as the time required for the peak of ACh-bronchoconstriction to recover by 50%) of $\sim$45 min (Figure 6). Protection against ACh-induced bronchoconstriction was re-established when salmeterol was re-administered 65 min after the first dose, indicating that the short duration of action was not due to β_2-adrenoceptor desensitization.

Discussion

Systemic infusion of salmeterol to rats for 7 days promoted pulmonary β_2-adrenoceptor desensitization that was seen both at the functional and molecular level. This finding is consistent with a number of previous studies where desensitization of β_2-adrenoceptor-mediated relaxation has been demonstrated *ex vivo* in tracheae and lung taken from rats treated chronically with isoprenaline and noradrenaline (Avner & Noland, 1978; Nishikawa *et al*., 1994), and *in vivo* in rats given salbutamol (Finney *et al*., 2000). Significantly, the anti-spasmogenic activity of PGE_2, which relaxes rat tracheae through EP_4-like prostanoid receptors (Lydford & McKechnie, 1994), was also impaired indicating that the desensitization was heterologous. Although this mode of desensitization is poorly documented *in vivo*, it has been observed in the lung and heart of rats given salbutamol (Finney *et al*., 2000) and isoprenaline (Zeiders *et al*., 1997) respectively.

Many processes can effect desensitization of β_2-adrenoceptors and, as the molecular aetiology of long-term subsensitivity *in vivo* is largely unexplored, several mechanisms were investigated.

Down-regulation of β_2-adrenoceptor number

Systemic treatment of rats with salmeterol produced a marked (70%) reduction in pulmonary β_2-adrenoceptor density relative to animals that received vehicle. These results agree with a previous study where rats were treated chronically with salbutamol (Finney *et al*., 2000), but the degree of receptor down-regulation effected by salmeterol was much greater. The reason for this disparity is currently unclear. One explanation is that the decrease in B_{max} may have been over-estimated due to pseudo-irreversible *in vivo* binding of salmeterol to the 'exosite' within the β_2-adrenoceptor, which would reduce the total number of binding sites available to [^{125}I]-ICYP *ex vivo*. However, this possibility seems unlikely as a comparable (52%) reduction in β_2-adrenoceptor protein was found in lung membranes by Western analysis.

The results of the present and a previous study (Finney *et al*., 2000) are consistent with the concept that chronic exposure of animals to β_2-adrenoceptor agonists effects tolerance by stimulating the internalization and degradation of the cognate receptors (Lohse, 1993). Although destabilization of β_2-adrenoceptor mRNA can account for this effect, there was no evidence for a reduction in mRNA transcripts in the lung of salmeterol-treated rats at day 7. This was unexpected and contrary to previous *in vivo* experiments performed in rats and guinea-pigs given isoprenaline (Mak *et al*., 1995; Nishikawa *et al*., 1993) and noradrenaline (Nishikawa *et al*., 1994) respectively. At least three explanations could account for this discrepancy. First, the efficacy and selectivity of salmeterol, noradrenaline and isoprenaline differ between β-adrenoceptor subtypes, which may influence the magnitude and kinetics of mRNA degradation. Second, β-adrenoceptor expression and function vary between strains of rat (Van Liefde *et al*., 1994). Thus, mRNA in the lung of Sprague-Dawley rats treated with salmeterol (this study) may be degraded relatively rapidly and recover to steady-state levels by day 7 when compared to Wistar rats treated identically with isoprenaline

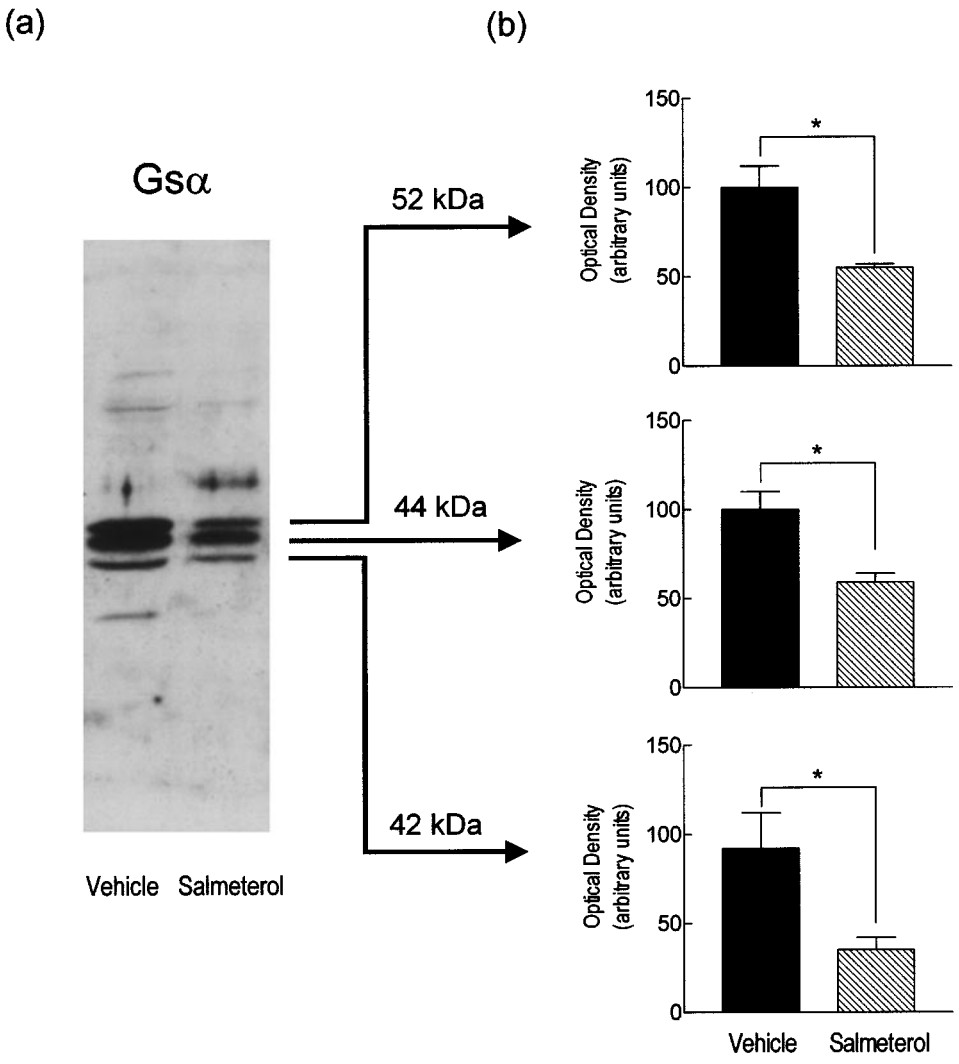

Figure 5 Effect of chronic systemic treatment of rats with salmeterol on $G_{s\alpha}$ expression in lung membranes. Rats were treated with salmeterol (10 μg kg^{-1} h^{-1}) or vehicle for 7 days and the expression of membrane-associated $G_{s\alpha}$ variants was measured by Western blotting. (a,b) show representative blot and a bar charts of the mean data ($\pm$s.e.mean of six determinations) respectively. Equal loading was confirmed by staining of the gels with Coomassie Blue (not shown). *$P<0.05$, significant reduction in $G_{s\alpha}$ variant expression.

(Mak *et al*., 1995; Nishikawa *et al*., 1993). Third, salmeterol may down-regulate pulmonary β_2-adrenoceptors in Sprague-Dawley rats by a process unrelated to mRNA destabilization.

While tolerance to salmeterol could be due to the reduction in pulmonary β_2-adrenoceptor density, other processes *must* be involved, as PGE_2-induced responses were also impaired. Indeed, the desensitization of salmeterol-induced cyclic AMP accumulation in lung *ex vivo* was very similar in magnitude to that produced in the lung of salbutamol-treated rats (Finney *et al*., 2000), which would not be expected if β_2-adrenoceptor down-regulation was the only determinant of tolerance. In addition, heterologous desensitization may have resulted, at least in part, from down-regulation/uncoupling of the EP_4-like receptor. Although the mechanism by which this could occur is unclear, PKA is an unlikely effector as activation of this enzyme by salbutamol was significantly impaired in lung taken from salbutamol-treated rats (Finney *et al*., 2000).

Activation of GRKs

Phosphorylation of GPCRs by GRKs can promote desensitization. However, GRK activity was not elevated in lung membranes prepared from salmeterol- (this study) or salbutamol-treated rats (Finney *et al*., 2000), which is consistent with GRK-induced receptor phosphorylation being a short-term process. Moreover, as GRKs effect *homologous* desensitization it is difficult to envisage how this process could also inhibit PGE_2-induced responses. In contrast, cytosolic GRK activity was significantly increased in the lung of salmeterol-treated rats, in agreement with a previous study where salbutamol was used (Finney *et al*., 2000), which may be due to increased GRK2 gene transcription and/or mRNA stability (Iaccarino *et al*., 1998).

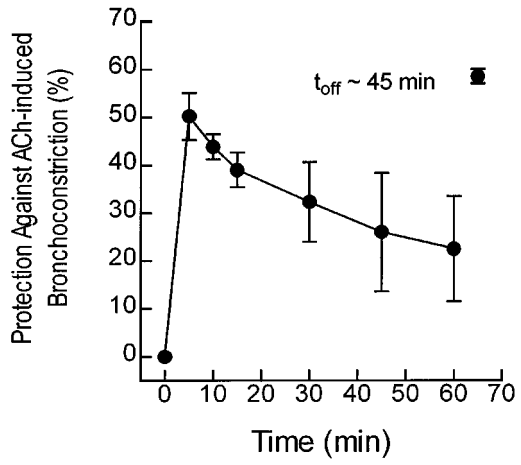

Figure 6 Duration of action of intravenous salmeterol in rats. Naïve rats were given ACh (500 μg kg^{-1}, i.v.) and changes in overflow pressure were measured. When basal lung function was re-established salmeterol (100 μg kg^{-1} i.v.; bolus) was given and 5, 10, 15, 30, 45 and 60 min later the magnitude of ACh-induced bronchoconstriction was measured. At 65 min salmeterol (100 μg kg^{-1} i.v.; bolus) was given again and the protection against the original bronchoconstrictor response was measured. Data points represent the mean$\pm$s.e.mean of six independent determinations.

Down-regulation of $G_{s\alpha}$

Heterologous desensitization GPCRs can be accounted for by a reduction in the abundance of membrane-associated $G_{s\alpha}$ (Milligan, 1993). Indeed, three molecular weight species of $G_{s\alpha}$ were detected in rat lung membranes as reported previously (Finney *et al*., 2000) but the expression was significantly reduced in tissue taken from those animals that received salmeterol. Further support for this mechanism derives from a previous study where chronic treatment of rats with salbutamol reduced the ability of cholera toxin to increase the cyclic AMP content in lung *ex vivo* (Finney *et al*., 2000). Moreover, the bronchoprotective effect of forskolin and the phosphodiesterase inhibitor, 3-isobutyl-1-methyl-xanthine, was preserved in rats treated chronically with salbutamol providing further evidence that the site of desensitization is upstream of adenylyl cyclase (Finney *et al*., 2000). Mechanistically, down-regulation of $G_{s\alpha}$ would blunt the ability of salmeterol, and other agonists (e.g. PGE_2) that utilize the same pool of $G_{s\alpha}$, to activate adenylyl cyclase and protect the airways against ACh-induced bronchoconstriction. A similar explanation is suggested for the impaired activation of adenylyl cyclase by isoprenaline and glucagon *ex vivo* in cardiac membranes purified from rats treated chronically with isoprenaline (Zeiders *et al*., 1997).

Down-regulation of $G_{s\alpha}$ could be effected by at least three mechanisms (discussed in Finney *et al*., 2000) including decreased $G_{s\alpha}$ gene transcription, destabilization of $G_{s\alpha}$ mRNA transcripts or redistribution/degradation of membrane-associated $G_{s\alpha}$. In cell-based experiments there is strong evidence that β-adrenoceptor agonists promote the translocation of $G_{s\alpha}$ from the membrane to a cytosolic pool (Ransnas *et al*., 1989; Wedegaertner *et al*., 1996) by a process that involves depalmitoylation of the protein (Wedegaertner & Bourne, 1994; Loisel *et al*., 1999). This is followed, after chronic exposure, by degradation of $G_{s\alpha}$ (Milligan, 1993). The results presented herein are consistent with the degradation theory as the reduction in membrane-associated $G_{s\alpha}$ was not associated with a commensurate increase of $G_{s\alpha}$ in the cytosol at day 7.

A critical question that arises from the present study is whether a 45 to 70% reduction in $G_{s\alpha}$ is sufficient to effect tolerance to salmeterol. As discussed previously (Finney *et al*., 2000) evidence provided by Paulssen *et al*. (1992) suggests that changes in $G_{s\alpha}$ expression can, indeed, affect signalling through GPCRs in an agonist-dependent manner.

Duration of action of intravenous salmeterol

An unexpected observation of the present investigation was the short-acting behaviour of salmeterol when given to rats by the intravenous route that was not the result of acute β_2-adrenoceptor desensitization. If the duration of action of salmeterol is to be explained by binding to an exosite (Green *et al*., 1996; Rong *et al*., 1999) then this should be independent of the route of administration. Thus, other mechanisms must account for this difference. One possibility is that salmeterol was rapidly metabolized to a shorter-acting molecule that retained affinity and efficacy at the β_2-adrenoceptor. This situation would be in contrast to administration by inhalation where salmeterol's high lipophilicity would ensure that it is rapidly absorbed and retained in the airways; indeed, bronchoprotection effected by inhaled salmeterol in asthmatic subjects persists for more than 12 h (Rabe *et al*., 1993). Previous studies in rats have established that salmeterol is cleared predominantly by metabolism and at least two, albeit minor, products can be formed by *O*-dealkylation of the phenalkyloxalkyl 'tail' that, theoretically could be short-acting (Manchee *et al*., 1993). In our own preliminary studies we have found that following bolus intravenous administration of salmeterol to Sprague-Dawley rats, the circulating level fell rapidly due to the combined effects of a very high volume of distribution (previously estimated at 42 ml min^{-1} kg^{-1}) and metabolism with products resulting from *O*-dealkylation detected (Authors' unpublished observations). Metabolism of salmeterol could also account for the unanticipated reduction in pulmonary β_1-adrenoceptor density after chronic treatment if these *O*-dealkylated metabolites are agonists at both β_1- and β_2-adrenoceptor subtypes. Alternatively, the down-regulation of β_1-adrenoceptors might simply reflect the weak agonist activity of salmeterol at this subtype (Ball *et al*., 1991; Roux *et al*., 1996).

Conclusion

Chronic systemic treatment of rats with salmeterol produced pulmonary β_2-adrenoceptor desensitization. Although several processes could contribute to this effect, the heterologous nature of the desensitization and the results of a previous investigation with salbutamol (Finney *et al*., 2000) where the bronchoprotective effect of forskolin and IBMX were preserved, suggest that a primary molecular aetiology is a reduction in the abundance of membrane-associated $G_{s\alpha}$. As salmeterol is a partial agonist on many tissues the marked reduction in β_2-adrenoceptor density may also have contributed to desensitization. It is important to appreciate that

these experiments were performed with healthy rats and the relevance of the findings to patients with asthma is unknown. However, evidence that asthma, itself, does not predispose to β_2-adrenoceptor desensitization has been presented (Penn *et al*., 1996). Thus, down-regulation of $G_{s\alpha}$ may be pertinent to the treatment of asthma where susceptible individuals become tolerant to the beneficial effects of salmeterol or high doses of short-acting β_2-adrenoceptor agonists such as salbutamol.

Note added in proof

Since submission of this manuscript the short duration of action of salmeterol at pulmonary β_2-adrenoceptors has been confirmed in the guinea-pig when given by the intravenous route.

The authors gratefully acknowledge GlaxoWellcome, Monsanto/Searle and the Royal Brompton Hospital Clinical Research Committee for financial support.

References

AVNER, B.P. & NOLAND, B. (1978). *In vivo* desensitization to β-receptor-mediated bronchodilator drugs in the rat: decreased beta receptor affinity. *J. Pharmacol. Exp. Ther.,* **207,** 23 – 33.

BAI, T.R., MAK, J.C. & BARNES, P.J. (1992). A comparison of β-adrenergic receptors and *in vitro* relaxant responses to isoproterenol in asthmatic airway smooth muscle. *Am. J. Respir. Cell Mol. Biol.,* **6,** 647 – 651.

BALL, D.I., BRITTAIN, R.T., COLEMAN, R.A., DENYER, L.H., JACK, D., JOHNSON, M., LUNTS, L.H., NIALS, A.T., SHELDRICK, K.E. & SKIDMORE, I.F. (1991). Salmeterol, a novel, long-acting β_2-adrenoceptor agonist: characterization of pharmacological activity *in vitro* and *in vivo*. *Br. J. Pharmacol.,* **104,** 665 – 671.

BARNES, P.J. (1995). β-Adrenergic receptors and their regulation. *Am. J. Respir. Crit. Care Med.,* **152,** 838 – 860.

BENOVIC, J.L., MAYOR, F., STANISZEWSKI, C., LEFKOWITZ, R.J. & CARON, M.G. (1987). Purification and characterization of the β-adrenergic receptor kinase. *J. Biol. Chem.,* **262,** 9026 – 9032.

BOHN, D., KALLOGHLIAN, A., JENKINS, J., EDMONDS, J. & BARKER, G. (1984). Intravenous salbutamol in the treatment of status asthmaticus in children. *Crit. Care Med.,* **12,** 892 – 896.

BOULET, L.P., CARTIER, A., MILOT, J., COTE, J., MALO, J.L. & LAVIOLETTE, M. (1998). Tolerance to the protective effects of salmeterol on methacholine-induced bronchoconstriction: influence of inhaled corticosteroids. *Eur. Respir. J.,* **11,** 1091 – 1097.

CERRINA, J., LE ROY LADURIE, M., LABAT, C., RAFFESTIN, B., BAYOL, A. & BRINK, C. (1986). Comparison of human bronchial muscle responses to histamine *in vivo* with histamine and isoproterenol agonists *in vitro*. *Am. Rev. Respir. Dis.,* **134,** 57 – 61.

CHEONG, B., REYNOLDS, S.R., RAJAN, G. & WARD, M.J. (1988). Intravenous β-agonist in severe acute asthma. *Br. Med. J.,* **297,** 448 – 450.

CHEUNG, D., TIMMERS, M.C., ZWINDERMAN, A.H., BEL, E.H., DIJKMAN, J.H. & STERK, P.J. (1992). Long-term effects of a long-acting beta$_2$-adrenoceptor agonist, salmeterol, on airway hyperresponsiveness in patients with mild asthma. *New Engl. J. Med.,* **327,** 1198 – 1203.

CHOMCZYNSKI, P. & SACCHI, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.,* **162,** 156 – 159.

CLUZEL, M., BOUSQUET, J., DAURES, J.P., RENON, D., CLAUZEL, A.M., GODARD, P.H. & MICHEL, F.B. (1990). Ambulatory long-term subcutaneous salbutamol infusion in chronic severe asthma. *J. Allergy Clin. Immunol.,* **85,** 599 – 606.

DROTAR, D.E., DAVIS, E.E. & COCKCROFT, D.W. (1998). Tolerance to the bronchoprotective effect of salmeterol 12 hours after starting twice daily treatment. *Ann. Allergy Asthma Immunol.,* **80,** 31 – 34.

FINNEY, P.A., BELVISI, M.G., DONNELLY, L.E., CHUANG, T.T., MAK, J.C., SCORER, C., BARNES, P.J., ADCOCK, I.M. & GIEMBYCZ, M.A. (2000). Albuterol-induced downregulation of $G_{s\alpha}$ accounts for pulmonary β_2-adrenoceptor desensitization *in vivo*. *J. Clin. Invest.,* **106,** 125 – 135.

FREDERICKS, Z.L., PITCHER, J.A. & LEFKOWITZ, R.J. (1996). Identification of the G protein-coupled receptor kinase phosphorylation sites in the human β_2-adrenergic receptor. *J. Biol. Chem.,* **271,** 13796 – 13803.

GOLDIE, R.G., SPINA, D., HENRY, P.J., LULICH, K.M. & PATERSON, J.W. (1986). *In vitro* responsiveness of human asthmatic bronchus to carbachol, histamine, β-adrenoceptor agonists and theophylline. *Br. J. Clin. Pharmacol.,* **22,** 669 – 676.

GREEN, S.A., SPASOFF, A.P., COLEMAN, R.A., JOHNSON, M. & LIGGETT, S.B. (1996). Sustained activation of a G protein-coupled receptor via 'anchored' agonist binding. Molecular localization of the salmeterol exosite within the β_2-adrenergic receptor. *J. Biol. Chem.,* **271,** 24029 – 24035.

HIRSHAM, C.A. & DOWNES, H. (1985). Experimental asthma in animals. In: *Bronchial Asthma – Mechanisms and Therapeutics*. 2nd edn. (eds.) Weiss, E.B., Segal, M.S. & Stein, M. Little, Brown & Co: Boston, pp 280 – 299.

HIGHAM, M.A., SHARARA, A.M., WILSON, P., JENKINS, R.J., GLENDENNING, G.A. & IND, P.W. (1997). Dose equivalence and bronchoprotective effects of salmeterol and salbutamol in asthma. *Thorax,* **52,** 975 – 980.

IACCARINO, G., TOMHAVE, E.D., LEFKOWITZ, R.J. & KOCH, W.J. (1998). Reciprocal *in vivo* regulation of myocardial G protein-coupled receptor kinase expression by β-adrenergic receptor stimulation and blockade. *Circulation,* **98,** 1783 – 1789.

KONZETT, H. & RÖSSLER, R. (1940). Versuchsanordnung zu untersuchungen an der bronchialmuskulatur. *Naunyn Schmiedeberg's Arch. Exp. Path. Pharmacol.,* **195,** 71 – 74.

KRUPNICK, J.G. & BENOVIC, J.L. (1998). The role of receptor kinases and arrestins in G protein-coupled receptor regulation. *Ann. Rev. Pharmacol. Toxicol.,* **38,** 289 – 319.

LOHSE, M.J. (1993). Molecular mechanisms of membrane receptor desensitization. *Biochim. Biophys. Acta.,* **1179,** 171 – 188.

LOHSE, M.J., BENOVIC, J.L., CODINA, J., CARON, M.G. & LEFKOWITZ, R.J. (1990). β-Arrestin: a protein that regulates β-adrenergic receptor function. *Science,* **248,** 1547 – 1550.

LOISEL, T.P., ANSANAY, H., ADAM, L., MARULLO, S., SEIFERT, R., LAGACE, M. & BOUVIER, M. (1999). Activation of the β_2-adrenergic receptor-$G\alpha_s$ complex leads to rapid depalmitoylation and inhibition of repalmitoylation of both the receptor and $G\alpha_s$. *J. Biol. Chem.,* **274,** 31014 – 31019.

LYDFORD, S.J. & MCKECHNIE, K. (1994). Characterization of the prostaglandin E_2 sensitive (EP)-receptor in the rat isolated trachea. *Br. J. Pharmacol.,* **112,** 133 – 136.

MAK, J. C., NISHIKAWA, M., SHIRASAKI, H., MIYAYASU, K. & BARNES, P.J. (1995). Protective effects of a glucocorticoid on downregulation of pulmonary β_2-adrenergic receptors *in vivo*. *J. Clin. Invest.,* **96,** 99 – 106.

MANCHEE, G.R., BARROW, A., KULKARNI, S., PALMER, E., OXFORD, J., COLTHUP, P. V., MACONACHIE, J.G. & TARBIT, M.H. (1993). Disposition of salmeterol xinafoate in laboratory animals and humans. *Drug Met. Disp.,* **21,** 1022 – 1028.

MAYOR, F., BENOVIC, J.L., CARON, M.G. & LEFKOWITZ, R.J. (1987). Somatostatin induces translocation of the β-adrenergic receptor kinase and desensitizes somatostatin receptors in S49 lymphoma cells. *J. Biol. Chem.,* **262,** 6468 – 6471.

MAYOR, F., PENELA, P. & RUIZ-GOMEZ, A. (1998). Role of G-protein-coupled receptor kinase 2 and arrestins in β-adrenergic receptor internalization. *Trends Cardiovasc. Med.,* **8,** 234 – 240.

MILLIGAN, G. (1993). Agonist regulation of cellular G protein levels and distribution: mechanisms and functional implications. *Trends Pharmacol. Sci.,* **14,** 413 – 418.

MOHAMMED, S.P., TAYLOR, C.V., WEYMAN-JONES, C.B., MATHER, M.E. VEND, Y.K., DOUGALL, I.G. & YOUNG, A. (2000). Duration of action of inhaled *vs.* intravenous β_2-adrenoceptor agonists in an anaesthetized guinea-pig model. *Pulm. Pharmacol. Ther.,* **13,** 287 – 292.

NERME, V., ABRAHAMSSON, T. & VAUQUELIN, G. (1990). Chronic isoproterenol administration causes altered β-adrenoceptor-Gs-coupling in guinea pig lung. *J. Pharmacol. Exp. Ther.,* **252,** 1341 – 1346.

NEWNHAM, D.M., GROVE, A., MCDEVITT, D.G. & LIPWORTH, B.J. (1995). Subsensitivity of bronchodilator and systemic β_2-adrenoceptor responses after regular twice daily treatment with eformoterol dry powder in asthmatic patients. *Thorax,* **50,** 497 – 504.

NEWNHAM, D.M., MCDEVITT, D.G. & LIPWORTH, B.J. (1994). Bronchodilator subsensitivity after chronic dosing with eformoterol in patients with asthma. *Am. J. Med.,* **97,** 29 – 37.

NISHIKAWA, M., MAK, J.C., SHIRASAKI, H. & BARNES, P.J. (1993). Differential down-regulation of pulmonary β_1- and β_2- adrenoceptor messenger RNA with prolonged *in vivo* infusion of isoprenaline. *Eur. J. Pharmacol.,* **247,** 131 – 138.

NISHIKAWA, M., MAK, J.C., SHIRASAKI, H., HARDING, S.E. & BARNES, P.J. (1994). Long-term exposure to norepinephrine results in down-regulation and reduced mRNA expression of pulmonary β-adrenergic receptors in guinea pigs. *Am. J. Respir. Cell Mol. Biol.,* **10,** 91 – 99.

O'DRISCOLL, B.R., RUFFLES, S.P., AYRES, J.G. & COCHRANE, G.M. (1988). Long-term treatment of severe asthma with subcutaneous terbutaline. *Br. J. Dis. Chest.,* **82,** 360 – 367.

PAULSSEN, R.H., PAULSSEN, E.J., GAUTVIK, K.M. & GORDE-LADZE, J.O. (1992). The thyroliberin receptor interacts directly with a stimulatory guanine nucleotide-binding protein in the activation of adenylyl cyclase in GH_3 rat pituitary tumour cells. Evidence obtained by the use of antisense RNA inhibition and immunoblocking of the stimulatory guanine nucleotide-binding protein. *Eur. J. Biochem.,* **204,** 413 – 418.

PENN, R.B., SHAVER, J.R., ZANGRILLI, J.G., POLLICE, M., FISH, J.E., PETERS, S.P. & BENOVIC, J.L. (1996). Effect of inflammation and acute β-agonist inhalation on β_2-AR signaling in human airways. *Am. J. Physiol.,* **271,** L601 – L608.

PREMONT, R.T., INGLESE, J. & LEFKOWITZ, R.J. (1995). Protein kinases that phosphorylate activated G protein-coupled receptors. *FASEB J.,* **9,** 175 – 182.

RABE, K.F., JORRES, R., NOWAK, D., BEHR, N. & MAGNUSSEN, H. (1993). Comparison of the effects of salmeterol and formoterol on airway tone and responsiveness over 24 hours in bronchial asthma. *Am. Rev. Respir. Dis.,* **147,** 1436 – 1441.

RANSNAS, L.A., SVOBODA, P., JASPER, J.R. & INSEL, P.A. (1989). Stimulation of β-adrenergic receptors of S49 lymphoma cells redistributes the α-subunit of the stimulatory G protein between cytosol and membranes. *Proc. Natl. Acad. Sci. U.S.A.,* **86,** 7900 – 7903.

RONG, Y., ARBABIAN, M., THIRIOT, D.S., SEIBOLD, A., CLARK, R.B. & RUOHO, A.E. (1999). Probing the salmeterol binding site on the β_2-adrenergic receptor using a novel photoaffinity ligand, [^{125}I]iodoazidosalmeterol. *Biochemistry,* **38,** 11278 – 11286.

ROUX, F.J., GRANDORDY, B. & DOUGLAS, J.S. (1996). Functional and binding characteristics of long-acting β_2-agonists in lung and heart. *Am. J. Respir. Crit. Care Med.,* **153,** 1489 – 1495.

SEYBOLD, J., NEWTON, R., WRIGHT, L., FINNEY, P.A., SUTTORP, N., BARNES, P.J., ADCOCK, I.M. & GIEMBYCZ, M.A. (1998). Induction of phosphodiesterases 3B, 4A4, 4D1, 4D2, and 4D3 in Jurkat T-cells and in human peripheral blood T-lymphocytes by 8-bromo-cAMP and Gs-coupled receptor agonists. Potential role in β_2-adrenoreceptor desensitization. *J. Biol. Chem.,* **273,** 20575 – 20588.

SIMONS, F.E., GERSTNER, T.V. & CHEANG, M.S. (1997). Tolerance to the bronchoprotective effect of salmeterol in adolescents with exercise-induced asthma using concurrent inhaled glucocorticoid treatment. *Pediatrics,* **99,** 655 – 659.

VAN LIEFDE, I., VAN ERMEN, A., VAN WITZENBURG, A., FRAEYMAN, N. & VAUQUELIN, G. (1994). Species- and strain-related differences in the expression and functionality of β-adrenoceptor subtypes in adipose tissue. *Arch. Int. Pharmacodyn. Ther.,* **327,** 69 – 86.

WEDEGAERTNER, P.B. & BOURNE, H.R. (1994). Activation and depalmitoylation of Gsα. *Cell.,* **77,** 1063 – 1070.

WEDEGAERTNER, P.B., BOURNE, H.R. & VON ZASTROW, M. (1996). Activation-induced subcellular redistribution of Gsα. *Mol. Biol. Cell.,* **7,** 1225 – 1233.

ZEIDERS, J.L., SEIDLER, F.J. & SLOTKI, T.A. (1997). Ontogeny of regulatory mechanisms for β-adrenoceptor control of rat cardiac adenylyl cyclase: targeting of a G-protein and cyclase catalytic subunit. *J. Mol. Cell Cardiol.,* **29,** 603 – 615.

(Received June 19, 2000
Revised November 13, 2000
Accepted January 15, 2001)