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Impaired activation of adenylyl cyclase in lung of the Basenji-greyhound model of airway hyperresponsiveness: decreased numbers of high affinity β-adrenoceptors

[1,*]C.W. Emala, †A. Aryana & *†C.A. Hirshman

*Department of Anesthesiology, The Johns Hopkins School of Medicine, and †Department of Environmental Health Sciences, The Johns Hopkins School of Hygiene & Public Health, Baltimore, Maryland 21205, U.S.A.

1 To evaluate mechanisms involved in the impaired β-adrenoceptor stimulation of adenylyl cyclase in tissues from the Basenji-greyhound (BG) dog model of airway hyperresponsiveness, we compared agonist and antagonist binding affinity of β-adrenoceptors, β-adrenoceptor subtypes, percentage of β-adrenoceptors sequestered, and coupling of the β-adrenoceptor to $G_s\alpha$ in lung membranes from BG and control mongrel dogs. We found that lung membranes from the BG dog had higher total numbers of β-adrenoceptors with a greater percentage of receptors of the β_2 subtype as compared to mongrel lung membranes.

2 Agonist and antagonist binding affinity and the percentage of β-adrenoceptors sequestered were not different in BG and mongrel dog lung membranes. However, the percentage of β-adrenoceptors in the high affinity state for agonist was decreased in BG lung membranes suggesting an uncoupling of the receptor from $G_s\alpha$.

3 Impaired coupling between the β-adrenoceptor and G protein documented by the decreased numbers of β-adrenoceptors in the high affinity state in BG lung membranes, is a plausible explanation for the reduced stimulation of adenylyl cyclase and the resultant reduction in airway smooth muscle relaxation in this model.

Keywords: Adenylyl cyclase; asthma; radioligand binding; dog lung; β-adrenoceptors

Introduction

Decreased relaxant responses to β-adrenoceptor agonists have been reported in airway smooth muscle from patients with fatal (Goldie *et al.*, 1986; Bai, 1990; 1991), and non-fatal (Goldie *et al.*, 1986; Cerrina *et al.*, 1986) asthma. This cannot be explained by downregulation of β-adrenoceptors as a result of prior therapy because some patients had not received β-adrenoceptor agonists (Goldie *et al.*, 1986), and the number of β-adrenoceptors in airway smooth muscle (Bai *et al.*, 1992) and lung (Spina *et al.*, 1989) were increased, not decreased. The β-adrenoceptor dysfunction appears to be related to the severity of the disease (Meurs *et al.*, 1984), rather than to an intrinsic defect in the gene endoding the β_2-adrenoceptors (Reihaus *et al.*, 1993). Taken together, these studies suggest that β-adrenoceptor dysfunction in the asthmatic lung is, at least in part, a consequence of the disease state.

To understand why and how β-adrenoceptor dysfunction develops in the lung in asthma, the mechanisms must be elucidated in the absence of confounding drug therapy. Possible mechanisms to account for decreased receptor function despite increased receptor number include β-adrenoceptors which have impaired agonist binding affinities, an altered ratio of β_2- to β_1-adrenoceptors (as β_1 receptors have been shown to couple less efficiently to G_s: (Levy *et al.*, 1993)), increased numbers of sequestered receptors, and an impaired ability of the receptor to convert from a low to high affinity state (Samma *et al.*, 1993; 1994; Bond *et al.*, 1995) with resultant decreased coupling of the β-adrenoceptor to $G_s\alpha$.

The Basenji-greyhound dog (BG) has been used as a model of human asthma. The BG dog is natively allergic, has non-specific airway hyperresponsiveness (Hirshman, 1985), and has increased numbers of inflammatory cells in the bronchial alveolar lavage (Hirshman *et al.*, 1986). The airway smooth muscle from this model shows decreased relaxant responses to β-adrenoceptor agonists (Downes *et al.*, 1989; Emala *et al.*, 1993) compared to both allergic and non-allergic control dogs. Moreover, the airway smooth muscle contains increased numbers of β-adrenoceptors but decreased stimulation of adenylyl cyclase in response to β-adrenoceptor agonists (Emala *et al.*, 1993), similar to that found in the airway smooth muscle of human subjects with fatal asthma (Bai *et al.*, 1992). These findings suggest that the BG dog is a reasonable model to use in order to gain insights into mechanisms accounting for β-adrenoceptor dysfunction in human asthma.

Previous studies of β-adrenoceptor function in the BG dog (Emala *et al.*, 1993) used tracheal and bronchial smooth muscle, but further characterization of the mechanisms involved in β-adrenoceptor dysfunction were not possible due to limited amounts of available airway smooth muscle. Cells of the lung parenchyma should be exposed to an environment similar to that which induced β-adrenoceptor dysfunction in airway smooth muscle. If the β-adrenoceptor impairment demonstrated in trachealis muscle of the BG dog (Emala *et al.*, 1993) is also present in lung parenchymal membranes, then lung parenchyma could serve as a model of β-adrenoceptor function of the airway smooth muscle. Thus, the larger amounts of lung parenchymal tissue available would allow further characterization of the mechanisms underlying the impaired β-adrenoceptor function. We first measured adenylyl cyclase activity in response to isoprenaline and prostaglandin E_1 in lung parenchymal membranes from BG and mongrel dogs. We then used radioligand binding techniques to determine whether the impaired β-adrenoceptor stimulation of adenylyl cyclase found was related to decreased β-adrenoceptor numbers, altered agonist binding affinities for the receptor, increased β_1-adrenoceptor numbers, increased sequestration of β-adrenoceptors or impaired β-adrenoceptor coupling to $G_s\alpha$.

[1] Author for correspondence at: Division of Physiology, Johns Hopkins School of Hygiene and Public Health, 615 N. Wolfe St./ Room 7006, Baltimore, Maryland 21205, U.S.A.

Methods

Study subjects

These studies were approved by the Animal Care and Use Committee of the Johns Hopkins University. The experimental animals used in these studies were adult Basenji-greyhound (BG) and control mongrel dogs (18–25 kg). The BG dogs were members of a long-term colony that have nonspecific airway hyperresponsiveness (Hirshman & Downes, 1986) and decreased sensitivity of airway smooth muscle to isoprenaline (Downes *et al.*, 1989; Emala *et al.*, 1993). Mongrel dogs were selected as control animals because previous *in vitro* studies have shown that airway smooth muscle from mongrel differed significantly from that of the BG with respect to β-adrenoceptor-mediated airway smooth muscle relaxation (Emala *et al.*, 1993) and had qualitatively and quantitatively similar responses to age-matched basenji and greyhound dogs which lacked airway hyperresponsiveness (Downes *et al.*, 1989).

Preparation of plasma membranes and separation of light membrane vesicles

Adult BG and mongrel dogs were killed by exsanguination during barbiturate anaesthesia. The lungs were removed *en bloc* following thoractomy and the most peripheral 2 cm of lung parenchyma was dissected from all lobes and suspended in cold 10% sucrose buffer (10% sucrose in 20 mM Tris, pH 7.4, 1 mM EDTA). These sections of lung parenchyma were finely minced with a razor blade and then disrupted with a Tissuemizer (Tekmar Co., Cincinnati, OH)(setting 10, 6 s). The tissue suspension was then filtered through one layer of gauze and homogenized with 30 strokes in a Potter-Elvejhem homogenizer (Wheaton Scientific, Millville, NJ, U.S.A.). The homogenate was diluted to 6 ml with cold 10% sucrose buffer and overlaid on a 5 ml cushion of 44.5% sucrose buffer (44.5% sucrose in 20 mM Tris, pH 7.4, 1 mM EDTA) and centrifuged at 90,000 *g* (r_{max}) in a SW41 rotor for 30 min at 4°C. The membrane fraction was recovered from the interface and resuspended in 10 ml cold 100 mM Tris, pH 7.4. The membranes were collected by centrifugation at 44,000 *g* (r_{max}) for 15 min at 4°C in the same rotor and resuspended in 100 mM Tris, pH 7.4 (2–8 mg ml^{-1}) and frozen at −70°C.

To quantitate numbers of sequestered β-adrenoceptors, light membrane vesicle fractions were isolated from plasma membrane fractions by the method of Lohse *et al.* (1990) with minor modifications. Briefly, dissected lung parenchyma was resuspended in cold TE buffer (20 mM Tris, pH 7.4, 1 mM EDTA) and minced and homogenixzed as described above. The homogenate was centrifuged at 400 *g* (r_{max}) for 10 min at 4°C to remove intact cells and debris; 6 ml of this supernatant was carefully layered over 4 ml of 30% sucrose in TE, which had first been layered over a 2 ml cushion of 60% sucrose in TE. Centrifugation was performed at 107,000 *g* (r_{max}) for 90 min at 4°C. Light membrane vesicles were recovered from the 0%/30% sucrose interface, and plasma membranes were recovered from the 30%/60% sucrose interface. Membranes and vesicles were resuspended in 10 ml cold TE and centrifuged at 198,000 *g* (r_{max}) for 60 min at 4°C. The resulting pellets were resuspended in cold TE at a concentration of 0.5–3.0 mg ml^{-1} and stored at −70°C, prior to assays for protein content, adenylyl cyclase activity and β-adrenoceptor binding.

Adenylyl cyclase assays

Adenylyl cyclase activity was determined as described by Salomon *et al.* (1974) with modifications (Emala *et al.*, 1993). Briefly, sucrose-purified plasma membrane fractions of lung membranes (40 μg) were incubated for 10 min at 30°C in 100 μl final volume containing 50 mM HEPES (pH 8.0), 50 mM NaCl, 0.4 mM EGTA, 0.25 mg ml^{-1} bovine serum albumin (BSA), 7 mM $MgCl_2$, 0.1 mM [^{32}P]-α-ATP (0.1–0.3 mCi μmol^{-1}), 1 mM cyclic AMP, 7 mM creatine phosphate, 10 mM Na azide, 125 units ml^{-1} myokinase (2000 units mg^{-1} protein) and 50 units ml^{-1} creatine phosphokinase (200 units mg^{-1} protein). Adenylyl cyclase activity was determined in the basal (unstimulated) state ($n=7$), in response to GTP (10 μM)($n=7$), GTP plus isoprenaline (100 μM–1 mM)($n=6$), and GTP plus prostaglandin E_1 (PGE_1)(100 μM–1 mM)($n=4$). PGE_1 stimulation was assayed since the prostaglandin E_1 receptor is thought to activate the same cellular pool of G_s proteins used by the β-adrenoceptor (McLellan *et al.*, 1992; Negishi *et al.*, 1995), and thus would help localize functional changes in the receptor-G protein-adenylyl cyclase cascade. Adenylyl cyclase activities in response to isoprenaline and PGE_1 are expressed as the percentage increase in activity compared to GTP alone. Preliminary experiments confirmed the linearity of adenylyl cyclase activity at the protein concentration and incubation time used. Cyclic AMP was isolated by chromatography (Salomon *et al.*, 1974) with column recoveries of 75–90%. Basal adenylyl cyclase activity is expressed as picomol of cyclic AMP synthesized per mg membrane protein for the 10 min incubation period.

In separate experiments adenylyl cyclase activity (using the same buffer, temperature and times as above) under basal and NaF-stimulated (10 mM) conditions was compared in 40 μg aliquots of sucrose-purifed plasma membrane fractions or light membrane vesicles to ensure that the light membrane fraction was free of contaminating plasma membranes and contained no adenylyl cyclase activity. This was to ensure that radioligand binding studies on light membrane vesicles would identify only sequestered β-adrenoceptors and not receptors from contaminating plasma membranes.

Saturation radioligand binding

Sucrose-purified plasma membranes (40 μg) prepared from 4 BG and 4 mongrel lungs were incubated with [^{125}I]-cyanopindolol (2200 Ci $mmol^{-1}$)(0–400 pM) in triplicate in the presence of absence of (−)-propranolol (1 μM) in binding buffer (12 mM Tris, pH 7.9, 0.5 mM ascorbic acid, 4 μg ml^{-1} BSA, 60 mM NaCl, 9 mM $MgCl_2$, 1.8 mM EDTA). All radioligand binding experiments were incubated in a final volume of 250 μl and performed for 45 min at 37°C. Preliminary experiments confirmed equilibrium binding had been achieved with this time and temperature of incubation. Preliminary experiments also confirmed that 10 μM propranolol did not improve the approximately 95% specific binding achieved with 1 μM propranolol. Binding assays were terminated by filtration through GF/C glass fibre filters and washed 3 times with 5 ml cold 0.9% NaCl. Filters were counted in a γ-emission counter (Packard 5000 series) with an efficiency of 73%. Specifically bound counts were analyzed by Rosenthal transformation of the data using the EBDA computer programme (Munson & Rodbard, 1980) to obtain the line of best fit. The equilibrium constant (K_d) was calculated as the negative reciprocal of the slope of the line and the maximum number of binding sites (B_{max}) was determined from the x-intercept.

In separate experiments, the percentage of total β-adrenoceptors was measured in plasma membranes and sequestered vesicles by saturation binding in membrance and vesicle fractions prepared from the lungs of 3 BG and 4 mongrel dogs. These experiments were performed as described above, except that single saturating doses of [^{125}I]-cyanopindolol (400 pM) were used to define total specific binding. The amount of specific binding measured in plasma membrane or light vesicle fractions was corrected for the total yield of each of these fractions from each sample of lung parenchyma. This allowed for a determination of the percentage of total receptors present in each fraction.

Isoprenaline competition radioligand binding

Sucrose-purified plasma membranes (40 μg) prepared from 9 mongrel and 6 BG lungs were incubated in triplicate in binding buffer in the presence of 80 pM [^{125}I]-cyanopindolol with 21

concentrations of isoprenaline (0.1 nm–1 mM). The competitive displacement of [^{125}I]-cyanopindolol by isoprenaline was analyzed by nonlinear regression and fit to a one site and two site equation for competition binding using the computer programme Inplot 4.0 (GraphPad Software, San Diego, CA, U.S.A.). An F test was performed using the sum of squares and degrees of freedom to determine whether the more complex fit (two-site model) fitted the data siginificantly better than the simpler model (one-site model). The data from all experiments were then fitted to a two site model to determine agonist affinities and the percentage of receptors in each affinity state. Affinity constants were calculated from EC_{50} values using the K_d value for [^{125}I]-cyanopindolol for each membrane preparation and the equation of Cheng & Prusoff (1973).

Competitive binding between isoprenaline and [^{125}I]-cyanopindolol was assayed under the same conditions as above, in the presence and absence of a non-hydrolyzable analogue of GTP (100 μM Gpp(NH)p) to distinguish between a low/high affinity state of a predominating β-adrenoceptor or two receptor subtypes with differing agonist affinities.

Determination of β-adrenoceptor subtypes

Although the agonist, isoprenaline (Green *et al.*, 1992; Kusayama *et al.*, 1994) and the antagonists, propranolol (Bilski *et al.*, 1983; Galitzky *et al.*, 1993) and [^{125}I]-cyanopindolol (Levy *et al.*, 1993; Neve *et al.*, 1996) do not discriminate between β_1- and β_2-adrenoceptors in radioligand binding experiments due to similar affinities for each receptor, the compound ICI 118.551 has been widely used (Bilski *et al.*, 1983; Galitzky *et al.*, 1993; Levy *et al.*, 1993; Nakada *et al.*, 1990) to distinguish β_2- from β_1-adrenoceptors due to its approximately 100 fold higher selectivity for β_2-adrenoceptors. Sucrose-purified plasma membranes (40 μg) prepared from 4 BG and 5 mongrel lungs were incubated in triplicate in the presence of 80 pM [^{125}I]-cyanopindolol and the β_2-selective antagonist ICI 118.551 (1 nM-0.1 mM) (Research Biochemicals International, Natick, MA, U.S.A.). The competitive displacement of [^{125}I]-cyanopindolol by ICI 118.551 was analyzed by nonlinear regression analysis and fitted to a two site equation for competition binding using the computer programme Inplot 4.0 (GraphPad Software, San Diego, CA, U.S.A). This allowed the determination of the percentage of receptors with high affinity for ICI 118.551 (β_2-adrenoceptors) and the percentage of receptors with low affinity for ICI 118.551 (β_1-adrenoceptors).

Statistics

All data are presented as the mean ±s.e.mean. Adenylyl cyclase activities under basal and GTP-stimulated conditions were analyzed by unpaired, two-tailed t test. Dose-responses to PGE_1 and isoprenaline were analyzed by a two-way ANOVA with Bonferroni post test comparisons. The following values derived from radioligand binding experiments were analyzed by unpaired, two-tailed t test: total receptor numbers (B_{max}), the log K_d of [^{125}I]-cyanopindolol, isoprenaline affinities for receptors in the low (K_L) and high (K_H) affinity states, the percentage of receptors in the low affinity state (R_L), the percentage of receptors of the β_2 subtype, and the percentage of total receptors that were sequestered. The null hypothesis was rejected when $P<0.05$.

Protein determination

Protein content of purified plasma membrane fractions and light membrane vesicles was assayed with the Pierce Chemical Co. (Rockford, IL) BCA protein assay reagent (Smith *et al.*, 1985). Bovine serum albumin was used as a standard.

Reagents

Except as noted, all reagents were purchased from Sigma. All radiosotopes were purchased from New England Nuclear.

Results

Adenylyl cyclase activity

Adenylyl cyclase activity in lung membranes from BG and mongrel dogs was not different under basal (70±31 and 50±20 pmol cyclicAMP mg^{-1} protein 10 min^{-1}, respectively, $n=7$) or GTP-stimulated (10 μM) (126±52 and 148±40 pmol cyclicAMP mg^{-1} protein 10 min^{-1} respectively, $n=7$) conditions (Table 1). However, isoprenaline (1 nM-100 μM) produced a significantly smaller increase (E_{max}) in adenylyl cyclase activity in lung membranes of BG than mongrel dogs ($n=6$) (Figure 1a and Table 1) with no significant change in the EC_{50} for isoprenaline (Table 1). In contrast, PGE_1 (1 nM-100 μM) produced a dose-dependent stimulation of adenylyl cyclase

Table 1 Adenylyl cyclase activities in mongrel and BG lung membranes

	Basal[1]	*GTP*[1]	*−log EC50 (Iso)*	*Emax (Iso)*[2]
Mongrel	50±20	148±40	6.96±0.22	86±20
BG	70±31	126±52	6.66±0.42	41±6.5*

(Iso)= isoprenaline; [1]Activity in pmol cyclicAMP mg^{-1} protein 10 min^{-1}; [2]Maximal percentage increase of isoprenaline-stimulated activity above GTP levels; *$P<0.05$.

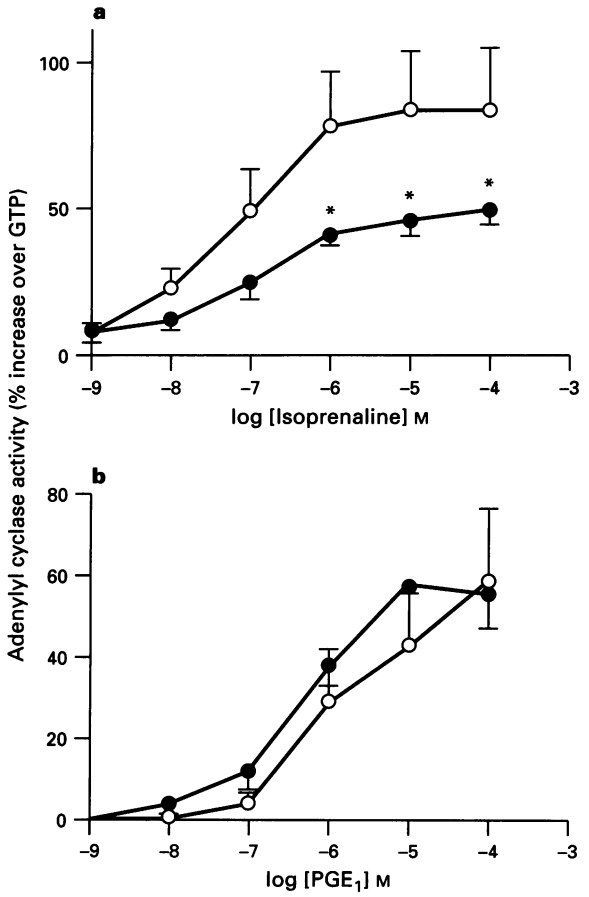


Figure 1 Adenylyl cyclase activity in lung membranes of BG (●) and mongrel (○) dogs. Adenylyl cyclase activity in response to (a) isoprenaline ($n=6$) or (b) PGE_1 ($n=4$). Activity is expressed as the percentage increase over the activity present with GTP alone. Adenylyl cyclase activity in response to isoprenaline (1 μM–100 μM) but not PGE_1 was significantly reduced in membranes from BG dogs. *$P<0.05$ between groups at indicated doses of isoprenaline.

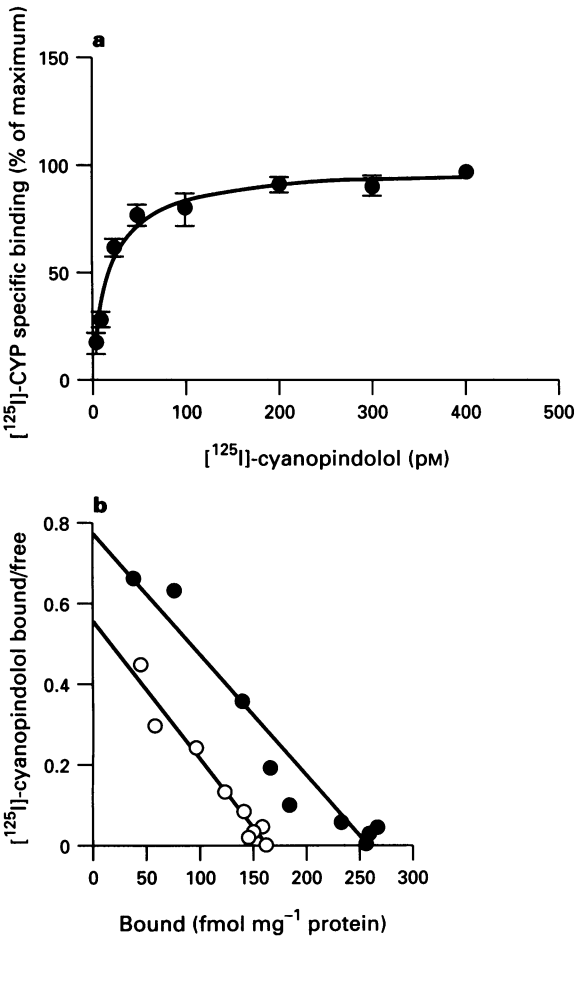
activity that was not different in membranes from BG and mongrels ($n=4$) (Figure 1b).

Saturation radioligand binding in lung membranes

Specific binding was saturable over the range of concentrations of [^{125}I]-cyanopindolol used (0–400 pM) (Figure 2a). Membranes prepared from lung parenchyma of 4 BG dogs contained greater numbers of total β-adrenoceptors (B_{max}) than membranes from 4 mongrel dogs (451±66 and 259±38 fmol mg^{-1} protein, respectively ($P<0.05$)) (Figure 2c). Antagonist affinities (K_d) for [^{125}I]-cyanopindolol were not different in lung membranes from BG and mongrels (−log K_d = 10.4±0.4 and 10.6±0.24, respectively, $P<0.75$) (Figure 2c). Saturating binding data fit a one site model best in all assays with average Hill coefficients of 0.98 and 0.99 for BG and mongrel membranes, respectively, suggesting a single class of binding sites consistent with the non-selectivity of [^{125}I]-cyanopindolol for β_1- and β_2-adrenoceptors described previously (Levy *et al.*, 1993; Neve *et al.*, 1996). Non-specific binding of [^{125}I]-cyanopindolol represented 2–6% of total binding in BG and mongrel membranes.

Isoprenaline competition radioligand binding

Statistical analysis of competition displacement curves of isoprenaline and [^{125}I]-cyanopindolol in lung membranes from BG and mongrel dogs revealed a best fit of the data to a two-site model (Figure 3). The addition of 100 μM Gpp(NH)p to isoprenaline competition binding assays in both BG and mongrel lung membranes resulted in a rightward shift of the displacement curve and a better statistical fit of the data to a one site rather than two site model (Figure 3). This is consistent with the conversion of the high and low affinity states of a predominating β-adrenoceptor to a single low affinity state. The affinities of isoprenaline for both the high and low affinity state of the β-adrenoceptor were not different in lung membranes of 6 BG and 9 mongrel dogs (Table 2). However, the BG lung membranes contained significantly greater numbers of β-adrenoceptors in the low affinity state than mongrel lung membranes (%R_L = 88±4.7% and 68±5.0% for BG and mongrel, respectively, $P<0.017$) (Figure 4a) and significantly



c

	BG	Mongrel
B_{max} (fmol mg^{-1} protein)	451 ± 66*	259 ± 38
−log K_d [^{125}I]-cyanopindolol	10.4 ± 0.39	10.6 ± 0.24

Figure 2 Total β-adrenoceptors and affinities for [^{125}I]-cyanopindolol in BG and mongrel lung membranes. (a) [^{125}I]-cyanopindolol (0–400 pM) resulted in a saturation in specific binding in lung membranes. (b) Representative Rosenthal transformations of saturations binding in BG (●) and mongrel (○) lung membranes. (c) β-Adrenoceptor numbers (B_{max}) were increased in BG lung membranes but affinities (K_d) for [^{125}I]-cyanopindolol were not different from mongrel lung membranes. $n=4$ for BG and $n=5$ for mongrel.*$P<0.05$.

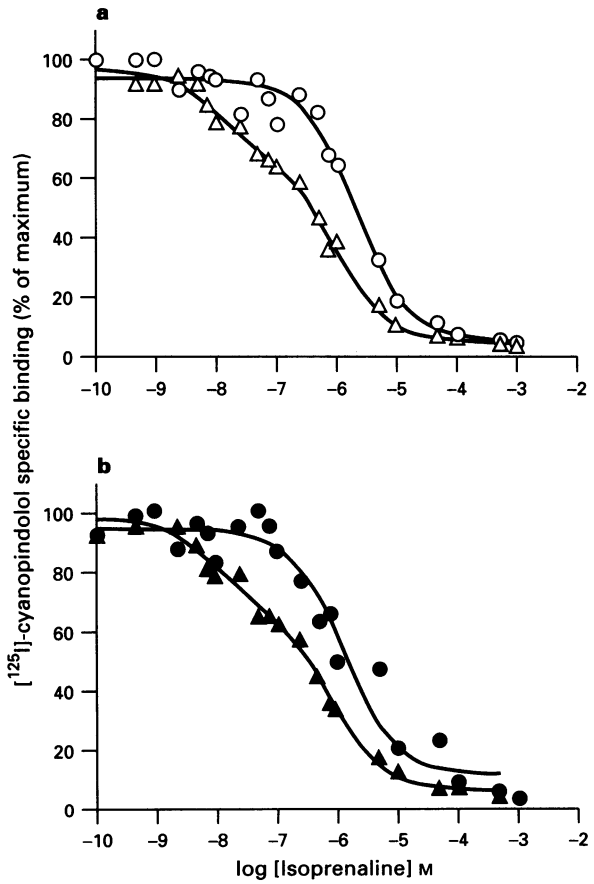


Figure 3 Representative isoprenaline competition displacement isotherms of [^{125}I]-cyanopindolol binding in lung membranes. Isoprenaline produced displacement of [^{125}I]-cyanopindolol binding in mongrel (△, a) and BG (▲, b) lung membranes that fit a two site model in the absence of Gpp(NH)p. Gpp(NH)p 100 μM resulted in a rightward shift and conversion to a single site model in both mongrel (○, a) and BG (●, b). This indicates that the two sites identified in the absence of Gpp(NH)p are high and low affinity sites for isoprenaline of a G protein coupled receptor. These displacement curves are representative of six experiments in (a) BG and (b) mongrel lung membranes.

Table 2 β-Adrenoceptor affinities for isoprenaline in lung membranes of BG and mongrel (M) dogs

	$K_H Iso$ $-pK_i$	$K_L ISO$ $-pK_i$
BG	7.6±0.16	6.1±0.15
M	7.5±0.21	6.0±0.07

Isoprenaline affinities for the β-adrenoceptor high (K_H) and low (K_L) affinity states were determined by competition binding of [^{125}I]-cyanopindolol with isoprenaline (1 mM–0.1 nM).

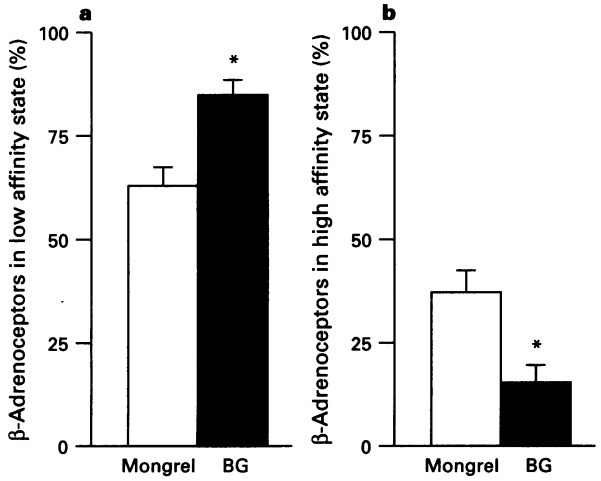


Figure 4 Percentage of β-adrenoceptors in the low and high affinity state for agonist in lung membranes. Isoprenaline competition binding in the presence of [^{125}I]-cyanopindolol was analyzed by non-linear regression curves being fitted to a two-site model to allow the determination of the percentage of receptors in the low (a) and high (b) affinity states for agonist. A greater percentage of β-adrenoceptors were in the low affinity state in BG ($n=6$) lung membranes than in mongrels ($n=9$). *$P<0.017$.

lower numbers of β receptors in the high affinity state (%R_H = 12±4.7 and 32±5.0% for BG and mongrels, respectively) (Figure 4b).

β-Adrenoceptor subtypes

Analysis of the competitive displacement of [^{125}I]-cyanopindolol by ICI 118.551 by nonlinear regression indicated that the data for all assays of both BG and mongrel membranes fit a two-site model better than a one-site model, allowing for the determination of the receptors that are of high affinity for ICI 118.551 (β_2-adrenoceptors) and receptors that are of low affinity for this antagonist (β_1-adrenoceptors). Competition of [^{125}I]-cyanopindolol binding by the β_2-selective antagonist, ICI 118.551, resulted in a biphasic dispacement of [^{125}I]-cyanopindolol by ICI 118.551 indicative of a mixed population of β_1- and β_2-adrenoceptors in lung membranes from both groups of dogs (Figure 5a and b). Lung membranes from 4 BG dogs had a greater percentage of receptors of the β_2 subtype than lung membranes from 5 mongrel dogs (82±1.0 and 66±1.3% β_2, respectively, $P=0.0087$, Figure 5c). Estimates of the number of β_1- and β_2-adrenoceptors based upon total receptors and the percentage of each subtype showed no difference in the number of β_1-adrenoceptors in BG and mongrel lung membranes (81±12 vs. 88±13 fmol mg^{-1} protein, respectively). However, BG lung membranes contained greater numbers of β_2-adrenoceptors than mongrel (370±54 vs. 171±25 fmol mg^{-1} protein, respectively, $P=0.0087$). The binding affinities of ICI 118.551 for the two receptor populations present were not different between BG and mongrel dogs.

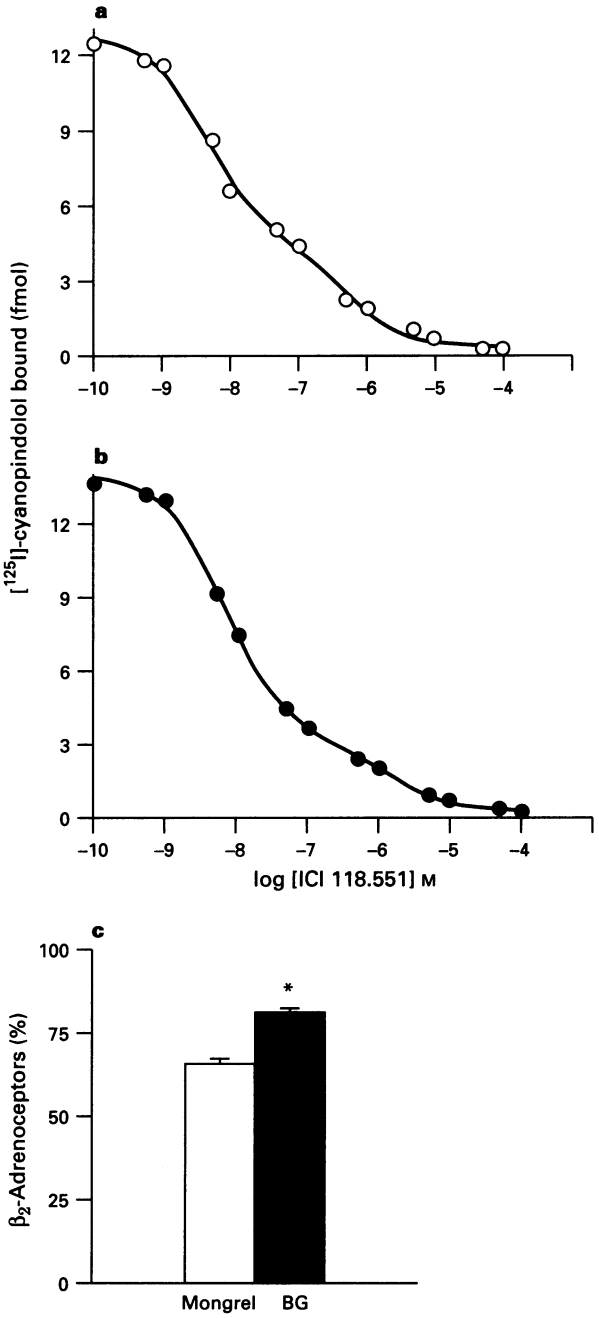


Figure 5 Percentage of β_1- and β_2-adrenoceptors in BG and mongrel lung membranes. Competition displacement curve between the β_2-adrenoceptor selective antagonist, ICI 118.551 and [^{125}I]-cyanopindolol. Shown is a representative assay from 1 of 4 (a) BG's and 1 of 5 (b) mongrels. Nonlinear regression analysis revealed that all assays fit a two-site model indicative of the high affinity binding of ICI 118.551 to β_2-adrenoceptors, and low affinity binding to β_1-adrenoceptors. (c) Nonlinear regression curves allowed the determination of percentage of receptors of each subtype. BG lung membranes contained a greater percentage of β_2-adrenoceptors than mongrel. *$P=0.0087$.

Sequestered β-adrenoceptors

Saturation binding revealed that the percentage of β-adrenoceptors (β_1 and β_2) present in the light membrane vesicle fractions were not different in 3 BG and 4 mongrel dogs and represented 17±1.0 and 15±1.3% of total cellular β-adrenoceptors, respectively, $P=0.39$ (Figure 6a). To verify that the method of discontinuous sucrose density centrifugation used in this study separated light membrane vesicles from plasma membranes, adenylyl cyclase activity was measured in each fraction. No NaF-stimulated (10 mM) adenylyl cyclase activity

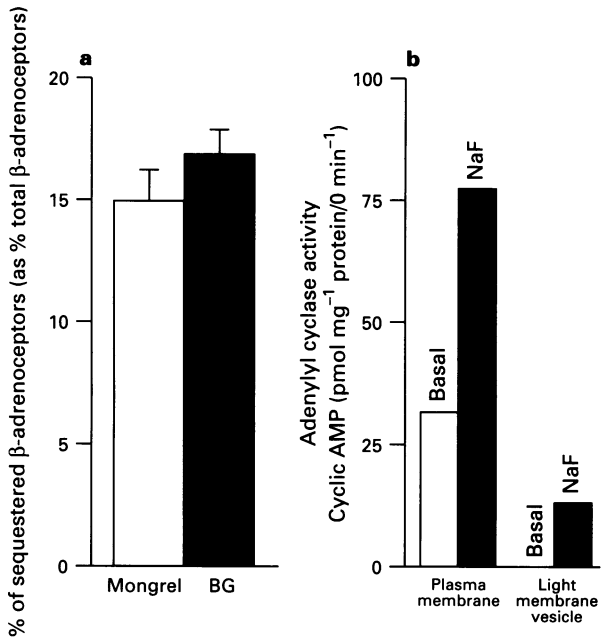


Figure 6 Percentage of β-adrenoceptors sequestered in light membrane vesicles. (a) Saturating doses of [^{125}I]-cyanopindolol (400 pM) ± 1 μM propranolol was used to define total specific binding in light membrane vesicles and plasma membrane fractions isolated on discontinuous sucrose gradients. The percent of sequestered β-adrenoceptors was not different in BG ($n=3$) and mongrel ($n=4$) lung membranes. (b) Adenylyl cyclase activity under basal and NaF-stimulated (10 mM) conditions revealed no activity in the light membrane fraction indicating the absence of contaminating plasma membranes in this fraction.

was found in the light membrane vesicle fraction indicating that it was free of plasma membranes. In contrast, the plasma membrane fraction had measurable adenylyl cyclase activity under both basal and NaF-stimulated conditions (Figure 6b).

Discussion

In this study, the coupling and binding affinities of the β-adrenoceptor were compared in lung membranes prepared from lung parenchyma of BG and mongrel dogs. Greater numbers of membrane-associated β_2-adrenoceptors were found in lung membranes from BG than mongrel dogs, but fewer of the receptors from the BG were in the high affinity state for agonist and less adenylyl cyclase activity resulted from receptor stimulation. A selective impairment of β-adrenoceptor agonist-stimulated adenylyl cyclase activity that was previously seen in trachealis muscle (Emala *et al.*, 1993) was found in lung parenchyma from BG dogs, as PGE_1 stimulation of adenylyl cyclase in lung membranes of BG and mongrels was not different.

The increased numbers of β-adrenoceptors with decreased function in BG lung parenchyma, agrees with previously published studies which utilized airway smooth muscle or lung parenchyma from human subjects with fatal asthma (Goldie *et al.*, 1986; Bai, 1990; 1991; Bai *et al.*, 1992). Bai *et al.*, found decreased β-adrenoceptor agonist-mediated function with increased numbers of total β-adrenoceptors in tracheal and bronchial muscle of patients with fatal asthma (Bai, 1990; 1991) which was associated with increased amounts of β_2-adrenoceptor mRNA in peripheral lung tissue (Bai *et al.*, 1993). Moreover, the current results agree with previously published studies using trachealis muscle from the BG dog model of airway hyperresponsiveness showing a selective impairment of β-adrenoceptor stimulated adenylyl cyclase activity with increased numbers of β-adrenoceptors, and suggest that with respect to the β-adrenoceptor-adenylyl cyclase pathway, the lung is a reasonable model of airway smooth muscle.

Three mechanisms could explain decreased function of β-adrenoceptors despite increased numbers in BG lung membranes. The greater number of receptors measured in BG membranes by saturation binding with [^{125}I]-cyanopindolol could include greater numbers of sequestered receptors which are unable to activate adenylyl cyclase upon binding of agonist due to the absence of G protein and adenylyl cyclase in vesicles (Stadel *et al.*, 1983). The increased total receptor numbers could include increased numbers of β_1-adrenoceptors which couple less efficiently to the stimulation of adenylyl cyclase in some cells (Levy *et al.*, 1993). Decreased coupling of the β-adrenoceptor to the G protein (G_s) (reflected by a decreased percentage of receptors in the high affinity state) would result in less stimulation of adenylyl cyclase despite increased β_2-adrenoceptors with normal agonist affinities.

The percentages of sequestered receptors found in the present study were similar to percentages reported in a human cultured epidermoid cell line (Lohse *et al.*, 1990). However, receptor sequestration could not account for the impaired β-adrenoceptor-stimulated adenylyl cyclase activity in the present study as no differences were found in numbers of sequestered β-adrenoceptors in light membrane vesicles prepared from BG and mongrel lung.

Lung parenchyma contains both β_1- and β_2-adrenoceptors, but no β_3-adrenoceptors (Krief *et al.*, 1993). In some cells β_1 adrenoceptors couple less efficiently to the stimulation of adenylyl cyclase than β_2-adrenoceptors (Green *et al.*, 1992; Levy *et al.*, 1993). A decreased percentage of the β_2 subtype and an increased percentage of the β_1 subtype could result in decreased stimulation of adenylyl cyclase despite a measured increase in total number of β-adrenoceptors. However, an increase, not a decrease, in the percentage of β_2-adrenoceptors was found in BG lung membranes compared to mongrel lung membranes, and thus decreased β_2-adrenoceptors cannot account for the decreased isoprenaline-stimulated adenylyl cyclase activity in BG lung parenchyma. A difference in number of β_1-adrenoceptors between BG and mongrel lung membranes would not explain our results since numbers of β_1-adrenoceptor numbers were not different in BG and mongrel lung membranes.

It is unlikely that decreased agonist affinity could account for reduced isoprenaline-stimulated adenylyl cyclase activity in BG lung membranes since the maximum adenylyl cyclase response, even in high concentrations of isoprenaline, was decreased in BG membranes. Competition binding studies revealed agonist affinities for the β-adrenoceptor that were not different in BG and mongrel lung membranes, and this agonist binding affinities could not account for decreased activation of adenylyl cyclase by β-adrenoceptor agonist in BG lung membranes. The findings in the present study are consistent with a study in human asthmatic smooth muscle by Bai *et al.* (1992) who found that the IC_{50} values for isoprenaline affinity were lower in the asthmatic muscle, suggesting that agonist affinity for the β-adrenoceptor was not decreased. Moreover, the agonist binding affinities for the low and high affinity states of the β-adrenoceptors in canine lung reported in the current study agree with those reported in rat (Vanscheeuwijck *et al.*, 1989) and mouse (Gavett & Wills-Karp, 1993) lung and turkey erythrocyte membranes (Stadel *et al.*, 1980).

In the current study, isoprenaline was used to displace [^{125}I]-cyanopindolol in plasma membrane fractions. Analysis of this competitive binding data revealed two affinity sites for isoprenaline. These two sites could represent a predominant subtype of β-adrenoceptor existing in two affinity states for agonist or two subtypes of β-adrenoceptors with differing affinities for isoprenaline. It is unlikely that these two affinity sites represent two receptor subpopulations (i.e. β_1- and β_2-adrenoceptors) as isoprenaline has a similar affinity for β_1- and β_2-adrenoceptors (Green *et al.*, 1992; Kusayama *et al.*, 1994). Competition binding studies performed in the presence of the

GTP analogue Gpp(NH)p caused a right-ward shift of the displacement curve and a better statistical fit of the data to a one site rather that a two site model. Thus, these data indicate that the two agonist affinity sites for isoprenaline represent a high and low affinity state of a predominating β-adrenoceptor population, rather than two different β-adrenoceptor subtypes with differing agonist affinities.

The proportion of β-adrenoceptors in the high affinity state are thought to represent the ability to the receptor to couple to G_s (DeLean *et al.*, 1980). The percentage of β-adrenoceptors in the high affinity state in the absence of guanine nucleotides depends on the cell type and the binding conditions employed, and ranges from 31–50% (Stadel *et al.*, 1980; Vanscheeuwijck *et al.*, 1989; Bouvier *et al.*, 1991; Hausdorff *et al.*, 1991; Samama *et al.*, 1993). In the present study lung membranes from mongrel dogs contained 32% of β-adrenoceptors in the high affinity state while lung membranes from BG dogs contained only 12% of receptors in the high affinity state. A 20% reduction of receptors in the high affinity state has been associated with decreased stimulation of adenylyl cyclase by isoprenaline (Bouvier *et al.*, 1991).

The mechanism for this uncoupling of the β-adrenoceptor from $G_s\alpha$ in BG lung is likely to reside either in the structure or the function of the β-adrenoceptor and not the $G_s\alpha$ protein, as function of the PGE_1 receptor which also couples through G_s was not different in BG and mongrel membranes. Our findings are consistent with either an alteration in the protein structure of the β-adrenoceptor that impairs conformational changes or an induced alteration in receptor coupling such as increased phosphorylation of the β-adrenoceptor.

β-Adrenoceptors are substrates from several kinases that result in uncoupling of the receptor from the G_s protein. Protein kinase A (Lohse *et al.*, 1990) and G-protein receptor kinases (GRK's, formerly referred to as βARK's) (Benovic *et al.*, 1988) and protein kinase C (Bouvier *et al.*, 1987) are known to phosphorylate the β-adrenoceptor and decrease the percentage of receptors in the high affinity state for agonist with resultant reduced stimulation of adenylyl cyclase by isoprenaline (Bouvier *et al.*, 1991). Thus, increased phosphorylation of the β_2-adrenoceptor could account for impaired isoprenaline-stimulated adenylyl cyclase activity despite increased numbers of β_2-adrenoceptors in BG lung membranes.

In summary, the present study has added further insight to the mechanism of impaired β-adrenoceptor stimulation of adenylyl cyclase in the BG dog model of airway hyperresponsiveness. Activation of increased numbers of β_2-adrenoceptors with normal agonist affinities and normal sequestration rates, generates less adenylyl cyxlase activity due to a reduced ability of the β_2-adrenoceptor to convert to a high affinity state and thus couple with the G_s protein.

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