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Increased expression of G protein-coupled receptor kinases in cystic fibrosis lung

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Abstract

A reduction in airway β-adrenoceptor density has been reported in cystic fibrosis lung but the mechanism underlying this defect remains unclear. In this study, we have investigated whether the decrease in β_2-adrenoceptor associates with altered G protein-coupled receptor kinase (GRK) levels. We assessed GRK activity by rhodopsin phosphorylation, and β_2-adrenoceptor and GRK at the mRNA and protein levels by Northern and Western blotting in peripheral lung samples from normal donors and patients with cystic fibrosis. GRK activity was significantly increased in peripheral cystic fibrosis lung with parallel increases in GRK2/5 mRNAs and protein expression. Functionally, isoproterenol-stimulated adenylyl cyclase activity was also diminished by 65% in cystic fibrosis lung homogenates. These data suggest that the increase in GRK activity may be one of the mechanisms underlying alterations in the coupling between β_2-adrenoceptor and adenylyl cyclase via G-protein and may thus contribute to the downregulation of β_2-adrenoceptor in cystic fibrosis lung.

Keywords: G protein-coupled receptor kinase; Adenylyl cyclase; Cystic fibrosis; Lung; Gene expression

1. Introduction

Cystic fibrosis is most commonly characterized by chronic bacterial infections of the upper respiratory tract, leading to intense inflammation, lung damage, respiratory failure, and eventual death. Abnormal expression of β-adrenoceptor has been implicated in cystic fibrosis (Sharma and Jeffery, 1990). Diminished adenylyl cyclase activity in response to isoproterenol stimulation has been reported in lymphocytes from severely ill cystic fibrosis patients (Davis and Laundon, 1980; Feldman et al., 1987). Diminished secretory responses in vitro to β-adrenoceptor agonists from salivary glands and sweat glands from patients with cystic fibrosis have been reported (Sato and Sato, 1984; McPherson et al., 1986). Cultured airway epithelial cells from patients with cystic fibrosis show a reduced Cl^- secretion in response to β-adrenoceptor agonists (Widdicombe, 1986; Welsh and Liedtke, 1986; Frizzell et al., 1986) and, in mucosal strips mounted in Ussing chambers, the secretion of mucus in response to isoproterenol is also reduced (Rogers et al., 1993).

β_2-Adrenoceptors belong to the G protein-coupled receptor family whose responsiveness is actively 'turned off' by members of the G protein-coupled receptor kinase (GRK) family, which consists of seven known members, GRK1 to GRK7 (Penn et al., 2000). These have been further classified into three subfamilies according to their sequence homology and functional similarity: (1) the rhodopsin kinase (GRK1) which is predominantly localized to the retina and phosphorylates light-bleached (agonist-activated) rhodopsin in rod outer segments; (2) the GRK2 subfamily including GRK2 and GRK3, which are more widely distributed; and (3) the GRK4 subfamily including GRK4 to GRK7. GRK5 and GRK6 are more ubiquitously expressed. GRKs phosphorylate serine/threonine residues in the carboxyl-tail and/or intracellular loops of receptors in an agonist-dependent manner. The phosphorylated receptors have enhanced affinity for a class of proteins called arrestins, the binding of which sterically inhibits further receptor/G protein coupling (Hausdroff et al., 1990). In addition, the receptor–arrestin complex may be internalized via a dyna-

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min/clathrin-dependent mechanism to result in resensitization and/or downregulation of the receptors (Gagnon et al., 1998). Of interest, GRK2, GRK3, and GRK5 target β-adrenoceptors and have been reported to be expressed in a variety of tissues including lung (McGraw and Liggett, 1997).

There is growing evidence to support the hypothesis that GRKs are important modulators of β-adrenoceptor signalling in vivo. Indeed, myocardial overexpression of GRK2 or GRK5 in mice impairs β-adrenoceptor/G protein coupling (Koch et al., 1995; Rockman et al., 1996). Furthermore, the β-adrenoceptor desensitisation observed in cardiac myocytes from human failing hearts and in lymphocytes from patients with hypertension is related to increased GRK activity (Ungerer et al., 1994; Gros et al., 1997). These data are in contrast to the decreased expression and activity of GRKs in peripheral blood mononuclear cells of patients with rheumatoid arthritis (Lombardi et al., 1999). However, the relationship between abnormal expression of β-adrenoceptor and GRK activity and gene expression has yet to be determined in cystic fibrosis.

In this study, we have assessed GRK activity and expression at mRNA and protein levels in human peripheral lung from normal donors and cystic fibrosis patients. We have also measured the isoproterenol-stimulated adenylyl cyclase activity to assess the functional significance on β-adrenoceptor signalling system in these peripheral lungs. A significant increase in both GRK activity and GRK2/5 expression was found in cystic fibrosis patients, which occurs concomitantly with a reduction in β_2-adrenoceptor expression and isopreterenol-stimulated adenylyl cyclase activity.

2. Materials and methods

2.1. Human lungs

Lung parenchyma was obtained from eight normal (four male; age 25.8 ± 3.9 years) potential donors for heart–lung/heart transplantation. Tissues were deemed normal on the basis of previous medical histories, macroscopic histological inspection and if the donors were nonsmokers with no previous history of chronic inflammatory lung disease. Tissues were also obtained from nine heart–lung transplant recipient patients with end-stage respiratory failure due to cystic fibrosis (five male; age 23.9 ± 2.3 years). All tissues were obtained in accordance with the approval of the Clinical Ethics Committee of the Royal Brompton NHS Trust. Written consents were obtained from each subject. All tissues were frozen in liquid nitrogen and stored at − 80 °C until use.

2.2. Lung cytosol and membrane preparations

For the preparation of cytosolic and membrane fractions, frozen lung was ground in liquid nitrogen and homogenized on ice for 2 × 30 s in 1–1.5 ml ice-cold 10 mM Tris–HCl (pH 7.4) containing 7.5 mM $MgCl_2$, 5 mM EDTA, 0.1 mM phenylmethyl-sulfonyl fluoride, 25 μg/ml aprotinin, 5 μg/ml leupeptin, 10 μg/ml pepstatin, 100 μg/ml bacitracin, 10 μg/ml soyabean trypsin inhibitor and 2 mM benzamidine. Unbroken debris was pelleted by centrifugation at 1000 × *g* at 4 °C for 10 min and discarded. The supernatant was then centrifuged at 100,000 × *g* at 4 °C for 60 min to separate plasma membrane from the cytosol. The resulting membrane pellet was resuspended in the homogenization buffer and the supernatant was collected, aliquoted and frozen in liquid nitrogen. Protein concentration was determined with a BioRad protein assay reagent, using bovine serum albumin as standard.

2.3. Measurement of G protein-coupled receptor kinase (GRK) activity

GRK enzymatic activity was assessed using light-dependent phosphorylation of rhodopsin (Benovic et al., 1987). Rod outer segment membranes were prepared from dark-adapted bovine retinas via stepwise sucrose gradient centrifugation, and then treated with 5 M urea to inactivate endogenous kinase activity as substrate. GRK-dependent phosphorylation was determined by incubating 60 μg of lung cytosolic protein with 0.5 μM rod outer segments in a buffer containing 20 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 2 mM EDTA, ~ 1 μCi [γ-^{32}P]ATP and 2 nmol ATP in a final reaction volume of 20 μl. The reactions were carried out at 30 °C for 30 min in the presence or absence of light. The incubations were terminated by the addition of 10 μl of 3 × sodium dodecyl sulfate (SDS) sample buffer (8% SDS, 50 mM Tris–HCl, pH 6.8, 20% glycerol, 5% β-mercaptoethanol, and 0.005% bromophenol blue). Samples were then electrophoresed on 10% SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoresis, the gel was stained with Coomassie blue, dried, and phosphorylated rhodopsin was visualized by autoradiography. Bands corresponding to rhodopsin (~ 38 kDa) were cut from the gel and quantitated by liquid scintillation counting. The extent of GRK-mediated phosphorylation was determined as the difference between light- and dark-dependent phosphorylation, each determined in triplicate. One unit of activity is defined as that amount of GRK that catalyzed the incorporation of 1 pmol phosphate from ATP into rod outer segment in 1 min/mg protein. In the initial experiments, rhodopsin phosphorylation was assessed in the presence of heparin (1 mg/ml) in the reaction buffer.

2.4. Northern blot analysis

Total RNA was isolated by the guanidinium–phenol–chloroform extraction and isopropanol precipitation (Chomczynski and Sacchi, 1987). PolyATtract mRNA isolation kit system IV (Promega, Southampton, UK) was used to prepare poly(A)$^+$ RNA according to the manufacturer's instructions. Samples of poly(A)$^+$ RNA were size-fractio-

nated on a 1% agarose-formaldehyde gel, transferred to nylon membrane (Magna, MA) by capillary action, and immobilized with a UV Stratalinker 2400 (Stratagene; Cambridge, UK).

The human β_2-adrenoceptor cDNA (439 bp *Sma*I fragment; kindly provided by Dr. R.J. Lefkowitz, Duke University, Durham, NC), GRK2 cDNA (284 bp *Eco*RI/*Sal*I fragment), GRK3 cDNA (281 bp *Eco*RI/*Xho*I fragment), GRK5 cDNA (188 bp *Eco*RI/*Xho*I fragment), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (1272 bp *Pst*I fragment) were radiolabeled by random primer labeling kit in the presence of [α-^{32}P]dCTP. The blot was prehybridized for 4–5 h in 50% formamide, 5 × standard sodium citrate (SSC), 5 × Denhardt's solution, 0.1% SDS, 10 mM NaH_2PO_4 and 100 mg/ml sonicated denatured salmon sperm DNA, and then hybridized with ^{32}P-labeled cDNA probes for 12–16 h at 42 °C. After hybridization, the blot was washed at high stringency in 0.1 × SSC/0.1% SDS at 55 °C for 30 min. The blot was exposed to Kodak OMAT XS film at − 70°C with an intensifying screen for up to 7 days. The blot was hybridized subsequently to each cDNA probe after stripping. Quantitative analysis of the autoradiograms was performed by densitometric scanning (UVP's Gel Documentation and Analysis System—GDS8000, Cambridge, UK) and normalized by GAPDH levels.

2.5. Western blot analysis

An aliquot of the cytosolic or membrane fraction was mixed with Laemmli sample buffer (Laemmli, 1970), boiled for 5 min and loaded onto 10% SDS/Tris polyacrylamide gel. Proteins were size fractionated at 80 mA and then transferred to presoaked Hybond ECL nitrocellulose membranes (Amersham, Amersham, UK) by electroblotting at 400 mA for 1 h in 50 mM Tris containing 200 mM glycine, 20% (v/v) methanol and 0.03% SDS. Efficiency of transfer was verified by Ponceau red staining. Membranes were soaked in a blocking buffer solution of 5% nonfat dry milk in TBS-T (10 mM Tris–HCl pH 7.5, 154 mM NaCl, 0.05% Tween-20) overnight at 4 °C, and then incubated with rabbit polyclonal antibody specific to either β_2-adrenoceptor, GRK2, GRK3, GRK5 (1:200 in all cases) or mouse monoclonal antibody specific to actin (1:500) diluted in blocking buffer for 1 h at room temperature. After extensive washing in TBS-T, membranes were incubated for 1 h at room temperature with either a donkey, anti-rabbit horseradish peroxidase-conjugated antibody at 1:4000 (for β_2-adrenoceptor and all GRKs antibodies) or a sheep anti-mouse horseradish peroxidase-conjugated antibody at 1:4000 (for actin antibody). Immunoreactivity was detected with an enhanced chemiluminescence detection system (ECL, Amersham) and bands were visualized after exposing blots to X-ray film. Same membranes were used for subsequent reporbing with specific antibodies after stripping. All protein bands were quantified by laser-scanning densitometry and normalized as a ratio to the "housekeeping" protein actin.

2.6. Adenylyl cyclase assay

Frozen lung tissue was homogenized in ice-cold 50 mM Tris–HCl buffer, pH 7.4, containing 0.25 mM EDTA. The homogenate was filtered through a single layer of nylon gauze and centrifuged at 1000 × *g* to pellet the unbroken cell debris. Only freshly prepared homogenates maintained at 4 °C were used for adenylyl cyclase assay. Adenylyl cyclase activity was determined using a modification of the method of Salomon et al. (1974). Briefly, the standard assay system contained 50 mM Tris–HCl buffer pH 7.4, 5 mM $MgCl_2$, 20 mM creatine phosphate, 10 IU creatine kinase, 1 mM cAMP, 0.25 mM Ro20-1724 (a phosphodiester inhibitor), 1 mM [α-^{32}P]ATP (i.e. 2μCi/tube), 4 μM GTP and 200–400 μg homogenate. The reaction was started by the addition of 30 μl of homogenate and incubated at 37 °C. After 15 min, the reaction was terminated by the addition of 800 μl of 6.25% (w/v) trichloroacetic acid. [8-^{3}H]cAMP (approximately 10,000 cpm in 100 μl of water) was added to each tube, and the mixtures centrifuged at 1000 × *g* for 20 min at 4 °C. [α-^{32}P]ATP and [^{32}P]cAMP were then separated using a two-step chromatographic procedure. The losses of [^{32}P]cAMP on the columns were corrected for by measurement of the recovery of [8-^{3}H]cAMP.

2.7. Materials

The radioisotopes [γ-^{32}P]ATP, [α-^{32}P]dCTP and [^{35}S]UTP (specific activities 20–40 Ci/mmol, >3000 Ci/mmol and >1000 Ci/mmol, respectively) were supplied by Amersham International. All primary and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and DAKO, respectively. All other reagents were from either BDH (Leicester, UK) or Sigma (Poole, UK).

2.8. Statistical analysis

Data are expressed as means ± S.E.M. Values were compared using the Student's *t* test for unpaired data. A value of $P < 0.05$ on a two-tailed test was used as a minimum level of significance.

3. Results

3.1. GRK activity in human lung

Incubation of rod outer segments as substrate with lung cytosolic fractions resulted in the phosphorylation of a ~ 38 kDa band, consistent with the phosphorylation of rhodopsin (18). Rhodopsin phosphorylation was dependent on light exposure since only very faint phosphorylation could be detected in the dark (data not shown). Furthermore, the light-dependent phosphorylation of rhodopsin was completely inhibited by the addition of 1 mg/ml heparin (Fig. 1A), which is known to inhibit GRK phosphorylation activity

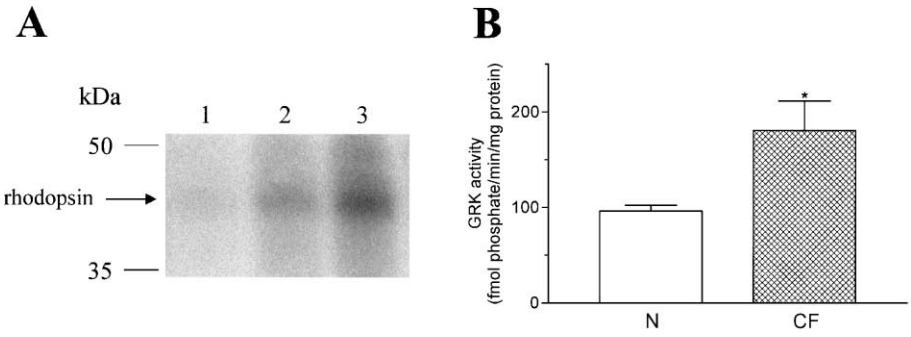

Fig. 1. Assessment of GRK activity in lung cytosolic fractions from normal donors (N) and cystic fibrosis patients (CF). (A) Autoradiograph depicting light-dependent phosphorylation of rhodopsin (~ 38 kDa). Cytosolic proteins from normal donor (lanes 1 and 2) and cystic fibrosis patient (lane 3) were incubated with rod outer segments as substrate in the light and in the presence (lane 1) or absence (lanes 2 and 3) of 1 mg/ml heparin. (B) GRK activity measurements of normal donors ($n=6$) and cystic fibrosis patients ($n=6$). The data are means ± S.E.M. * $P<0.05$ vs. normal donors.

(Chuang et al., 1992). In lung cytosolic fractions from cystic fibrosis patients, GRK activity was significantly increased (181.3 ± 30.7 fmol phosphate/min/mg protein, $P<0.05$) as compared with activity in lung samples from normal donors (96.5 ± 6.1 fmol phosphate/min/mg protein)(Fig. 1B).

3.2. Northern blot analysis

β_2-Adrenoceptor mRNA levels (~ 2.2 kb transcript) in lung from cystic fibrosis patients were significantly decreased from that of healthy donors (28.1 ± 5.9% of controls) in agreement with the receptor binding data (1) (Fig. 2). We also determined whether specific GRK mRNA expression levels were associated with the increase observed in GRK activity in lung from cystic fibrosis patients. GRK2 and GRK5 mRNA levels (~ 3.8 and ~ 2.8 kb transcripts, respectively) in lung from cystic fibrosis patients were significantly different from that of healthy donors (247 ± 14% and 222 ± 15% of controls, respectively)(Figs. 3 and 4) with no detectable levels of GRK3 mRNA in either group.

3.3. Western blot analysis

We determined whether β_2-adrenoceptor protein expression levels were associated with the decrease observed in mRNA expression in human lung from cystic fibrosis

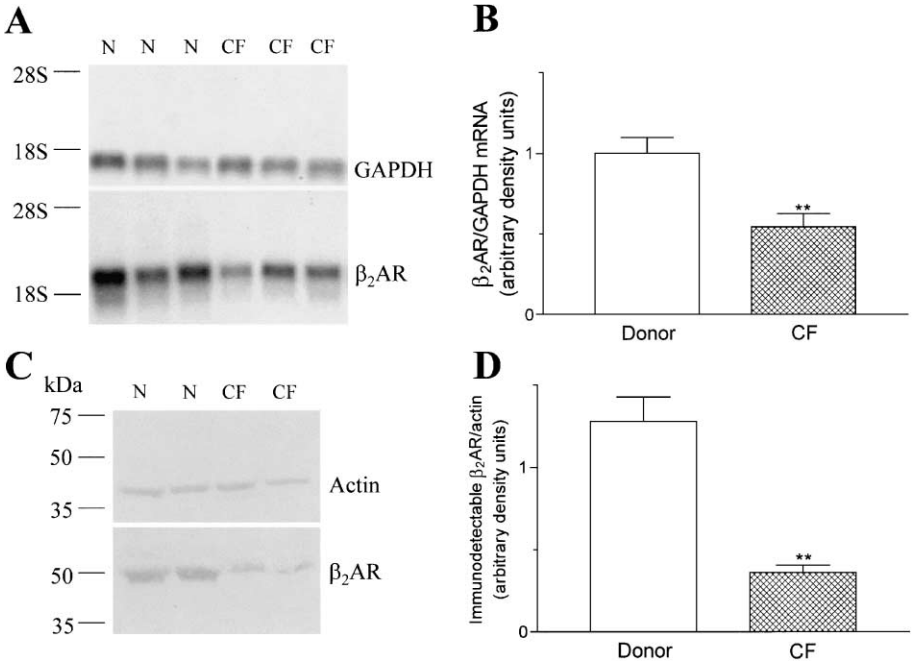

Fig. 2. (A) Representative autoradiographs of Northern blot for β_2-adrenoceptor (β_2AR) and glyceraldehyde-phosphate dehydrogenase (GAPDH) mRNA expression in lung from normal donors (N; $n=8$) and cystic fibrosis patients (CF; $n=8$). (B) Densitometric measurements of β_2AR mRNA expression normalized for GAPDH mRNA expression. (C) Representative autoradiographs of Western blot depicting immunodetectable β_2AR and actin in lung membrane protein from normal donors (N; $n=6$) and cystic fibrosis patients (CF; $n=6$). (D) Densitometric measurements of β_2AR immunoreactivity normalized to actin. The data are means ± S.E.M. ** $P<0.01$ vs. normal donors.

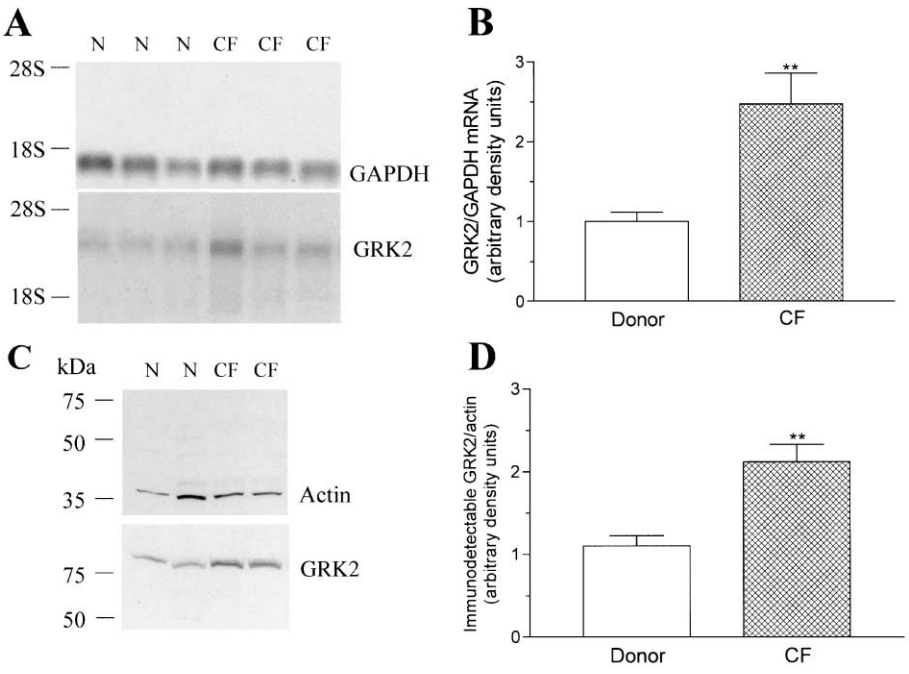

Fig. 3. (A) Representative autoradiographs of Northern blot for GRK2 and glyceraldehyde-phosphate dehydrogenase (GAPDH) mRNA expression in lung from normal donors (N; $n=8$) and cystic fibrosis patients (CF; $n=8$). (B) Densitometric measurements of GRK2 mRNA expression normalized for GAPDH mRNA expression. (C) Representative autoradiographs of Western blot depicting immunodetectable GRK2 and actin in lung cytosolic protein from normal donors (N; $n=6$) and cystic fibrosis patients (CF; $n=6$). (D) Densitometric measurements of GRK2 immunoreactivity normalized to actin. The data are means ± S.E.M. $^{**}P<0.01$ vs. normal donors.

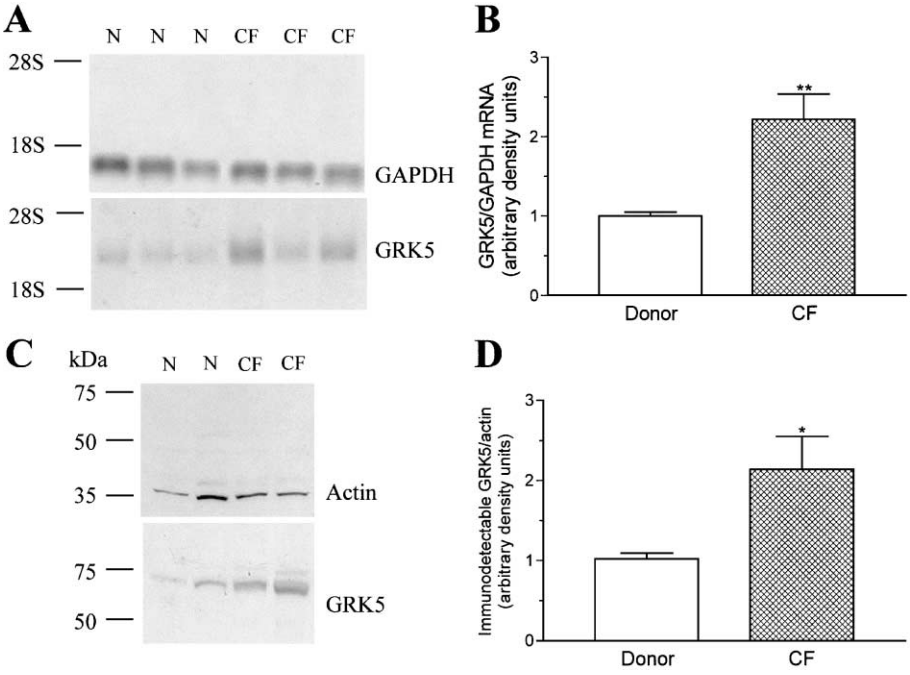

Fig. 4. (A) Representative autoradiographs of Northern blot for GRK5 and glyceraldehyde-phosphate dehydrogenase (GAPDH) mRNA expression in lung from normal donors (N; $n=8$) and cystic fibrosis patients (CF; $n=8$). (B) Densitometric measurements of GRK2 mRNA expression normalized for GAPDH mRNA expression. (C) Representative autoradiographs of Western blot depicting immunodetectable GRK5 and actin in lung cytosolic protein from normal donors (N; $n=6$) and cystic fibrosis patients (CF; $n=6$). (D) Densitometric measurements of GRK5 immunoreactivity normalized to actin. The data are means ± S.E.M. $^{*}P<0.05$, $^{**}P<0.01$ vs. normal donors.

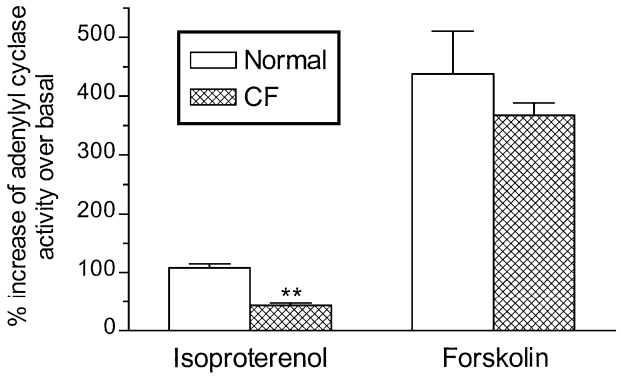

Fig. 5. Assessment of adenylyl cyclase activity stimulated by isoproterenol (10 μM) and forskolin (10 μM) in lung homogenates from normal donors ($n=4$) and cystic fibrosis patients (CF; $n=4$). The data are means ± S.E.M. ** $P<0.01$ vs. normal donors.

patients. Consistent with the previous radioligand binding data, β_2-adrenoceptor protein was reduced in lung membranes from cystic fibrosis patients by ~ 70% (Fig. 2). We next determined whether the increase observed in GRK-mediated phosphorylation of rod outer segment in lung from cystic fibrosis patients represented an increase in immunodetectable specific GRK protein. The anti-GRK2 and anti-GRK5 antibodies recognized proteins of apparent molecular masses of ~ 80 and ~ 68 kDa, respectively, with no detectable protein with anti-GRK3 antibody, consistent with the Northern blot analysis. Immunodetectable GRK2 and GRK5 were increased by 92 ± 10% and 109 ± 16%, respectively, in lung cytosolic fractions from cystic fibrosis patients as compared with healthy donors (Figs. 3 and 4).

3.4. Adenylyl cyclase assay

Isoproterenol-stimulated adenylyl cyclase activity was significantly less in lung homogenates from cystic fibrosis patients than from normal donors while there was no significant difference on forskolin-stimulated adenylyl cyclase activity between the two groups (Fig. 5).

4. Discussion

This study provides new findings. First, we demonstrated that human peripheral lung tissue contains significant amounts of GRK activity with the expression of GRK2 and GRK5. Second, we demonstrated a significant increase of GRK activity in cystic fibrosis lung compared with normal donors. This increase may be attributed to the induction of both GRK2/5 mRNA and protein expression. Moreover, cystic fibrosis patients showed both a significant decrease in β_2-adrenoceptor mRNA and protein expression, consistent with the findings in the literature (Sharma and Jeffery, 1990). However, it is difficult to say whether differences in GRK gene expression occur in a particular given cell type because peripheral lung tissue consists of many different cell types. Third, we established a correlation between increased GRK activity and impaired β_2-adrenoceptor/G protein coupling, by demonstrating a significant reduction in isoproterenol-stimulated but no change in forskolin-stimulated adenylyl cyclase activity in cystic fibrosis lung, suggesting that the defect was not at the level of adenylyl cyclase.

Cystic fibrosis is a condition characterized by disturbances in ion/fluid transport across secretory epithelia, including airway epithelial cells (Boucher et al., 1983; McPherson and Goodchild, 1988), which lead to alterations in the volume and composition of luminal secretions and resultant plugging of airways by viscid and tenacious mucus. The abnormally high negative electrical potential difference across airway epithelia in cystic fibrosis (Knowles et al., 1981; Hay and Geddes, 1985) is thought to be due to increased Na^+ resorption (lumen to serosa) and defective β_2-adrenoceptor regulation of apical cell membrane Cl^- secretion (Widdicombe, 1986; Boucher et al., 1986). Rogers et al. (1993) reported reduced responsiveness of cystic fibrosis airway tissue to β_2-adrenoceptor agonist-stimulated mucous secretions in vitro. Apart from a role in mucous secretion, the reduction in β_2-adrenoceptors in surface airway epithelium may be of significance in relation to the control of ciliary beat frequency (Clarke and Lopez-Vidriero, 1982). Several studies have indicated that pulmonary inflammation may occur early in the course of cystic fibrosis (Konstan et al., 1994; Balough et al., 1995; Khan et al., 1995).

We have characterized both GRK mRNAs and proteins in human peripheral lung tissues. GRK2 and GRK5 mRNAs identified by Northern blotting were of the reported size (Benovic et al., 1989; Kunapuli and Benovic, 1993). Characterization of GRK by Western blot analysis confirmed the presence of GRK2 and GRK5 with the absence of GRK3 in human peripheral lung. All protein identifications were validated by coimmunoblotting of the respective recombinant proteins. Previously, Benovic et al. (1991) indicated that GRK3 mRNA is present at much lower levels than GRK2 (< 10%) in peripheral tissues. Very low expression of GRK3 has also been reported in the heart, where GRK2 and GRK5 appear to be predominantly expressed (Inglese et al., 1993; Premont et al., 1994).

An increase in GRK function and expression leading to increased receptor phosphorylation would provide a mechanism for receptor uncoupling and downregulation in cystic fibrosis. Increased expression of recombinant GRK2 has been demonstrated to parallel an impairment of β_2-adrenoceptor stimulation of adenylyl cyclase (Pippig et al., 1993), which is consistent with our findings. The observed increase in GRK activity appears to be associated with an increase in GRK2 and GRK5 expression. The Northern blot analysis of GRK2 and GRK5 mRNAs suggests that the increase in GRK activity and GRK2 and GRK5 protein may be due to changes at the transcriptional level and/or on the level of mRNA stability. In contrast, the recent observation has found that GRK activity and expression are decreased in

patients with rheumatoid arthritis, which is a chronic inflammatory disease of the synovial joints (Lombardi et al., 1999). This may reflect differences in the cell types involved in these two different diseases. It would seem likely that our findings in cystic fibrosis might be secondary to bacterial infections and consequent airway inflammation.

The mechanisms underlying the increased GRK activity and impaired β_2-adrenoceptor function in cystic fibrosis may be important in our understanding of the role played by GRKs. It has been suggested that GRK-mediated regulation of G protein-coupled receptors can contribute to the setting of high level of catecholamines. In heart failure, a sustained activation of the sympathetic nervous system is associated with an increase of both GRK activity and GRK2 mRNA (Ungerer et al., 1993, 1994). The observation that an increase of GRK activity occurs in the liver of neonate rats after the transient physiological increase of catecholamines (Garcia-Higuera and Mayor, 1994) gives further support to the fact that GRK activity may be related to the degree of sympathetic stimulation in vivo.

Overall, our findings indicate a generalized defect in pulmonary GRK expression and function in cystic fibrosis, which could be an important factor in the impairment of β_2-adrenoceptor-mediated responsiveness. This is the first time that an increase in GRK activity and expression of specific GRKs has been associated to a decreased function and expression of β_2-adrenoceptors in a human airway disease.

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