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NCS 613, a potent and specific PDE4 inhibitor, displays anti-inflammatory effects on human lung tissues

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¹Le Bilarium, Department of Physiology and Biophysics, ²Service of Thoracic Surgery, and ³Department of Pathology, Faculty of Medicine and Health Sciences, Université de Sherbrooke, Sherbrooke, Québec, Canada; and ⁴UMR CNRS 7213, Laboratoire de Biophotonique et Pharmacologie, Faculté de Pharmacie, Université de Strasbourg, Illkirch Cedex, France

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Youbare I, Morin C, Senouvo FY, Sirois C, Albadine R, Lugnier C, Rousseau E. NCS 613, a potent and specific PDE4 inhibitor, displays anti-inflammatory effects on human lung tissues. *Am J Physiol Lung Cell Mol Physiol* 301: L441–L450, 2011. First published July 22, 2011; doi:10.1152/ajplung.00407.2010.—Chronic inflammation is a hallmark of pulmonary diseases, which leads to lung parenchyma destruction (emphysema) and obstructive bronchiolitis occurring in both chronic obstructive pulmonary disease and asthma. Inflammation is strongly correlated with low intracellular cAMP levels and increase in specific cAMP hydrolyzing activity. The aim of the present study was to investigate the role of the cyclic phosphodiesterase type 4 (PDE4) in human lung and to determine the effects of NCS 613, a new PDE4 inhibitor, on lung inflammation and bronchial hyperresponsiveness. High cAMP-PDE activities were found in the cytosol fractions from human lung parenchyma and distal bronchi. PDE4 (rolipram sensitive) represented 40% and 56% of total cAMP-PDE activities in the above-corresponding tissues. Moreover, PDE4A, PDE4B, PDE4C, and PDE4D isoforms were detected in all three subcellular fractions (cytosolic, microsomal, and nuclear) with differential distributions according to specific variants. Pharmacological treatments with NCS 613 significantly decreased PDE4 activity and reduced $\text{I}\kappa\text{B}\alpha$ degradation in cultured parenchyma, both of which are usually correlated with a lower inflammation status. Moreover, NCS 613 pretreatment potentiated isoproterenol-induced relaxations in human distal bronchi, while reducing TNF- α -induced hyperresponsiveness in cultured bronchi, as assessed in the presence of methacholine, U-46619, or histamine. This reducing effect of NCS 613 on human bronchi hyperresponsiveness triggered by TNF- α was related to a lower expression level of PDE4B and PDE4C, as well as a downregulation of the phosphorylated forms of p38-MAPK, CPI-17, and MYPT-1, which are known to control tone. In conclusion, specific PDE4 inhibitors, such as NCS 613, may represent an alternative and isoform-specific approach toward reducing human lung inflammation and airway overreactivity.

phosphodiesterase 4 isozymes; airways hyperresponsiveness

LUNG PARENCHYMA DESTRUCTION resulting from chronic inflammation and leading to emphysema and obstructive bronchiolitis are typical pathological changes related to both chronic obstructive pulmonary disease (COPD) (22, 29) and asthma (6, 36). These pathologies are functionally characterized by progressive airway inflammation, resulting in mucus secretion, cell proliferation, airway remodeling, and obstruction (7, 8). Downstream of surface membrane receptor activation, cyclic nucleotide phosphodiesterases (PDE) play a pivotal role in cAMP and cGMP signaling (9). This superfamily of enzymes

hydrolyses cyclic nucleotides as a feedback mechanism for returning local nucleotide concentration to basal levels. In mammals, 11 families of PDE are currently identified (PDE1–PDE11), most of which consist of several isozymes and numerous PDE isozyme-splice variants. Although all PDEs share a common structural feature of a catalytic core, they differ, however, in substrate affinity, kinetic properties, specific inhibitor sensitivity, and allosteric regulation (12, 17). By regulating local cyclic nucleotide levels, PDE contributes to their compartmentalization and governs many biology processes, including proliferation, inflammation, and regulation of smooth muscle tone (10, 21).

Phosphodiesterase type 4 (PDE4) has been suggested to be a critical factor in the pathogenesis of inflammation by metabolizing cAMP in human leukocytes and in both endothelial and epithelial lung tissues (3, 4). In animal, human, and cell models, PDE4 implication has been reported in lung diseases related to chronic inflammation such as smoking-induced lung injury, fibrosis, asthma, and COPD (8, 17, 22). It is well known that maternal exposure to secondhand cigarette smoke primes the lung for induction of phosphodiesterase-4D5 isozyme and exacerbated Th2 responses (34). In addition, cAMP and cGMP are also important second messengers associated with the control of smooth muscle relaxation (30). Mobilization of Ca^{2+} from intracellular stores or from the extracellular space triggers and maintains airway smooth muscle (ASM) tone, whereas relaxation is usually associated with activation of cyclic nucleotide signaling pathways, which regulate the interaction between myosin and actin as well as Ca^{2+} reuptake (31). The functional role of PDE in ASM tone has also been demonstrated in mice deficient in the PDE4D gene, which are believed to be refractory to muscarinic cholinergic stimulation (24). Consequently, PDE4 is thought to play a key role in lung inflammation and hyperresponsiveness. Despite increasing evidence, which depict PDE4 family enzymes as attractive targets for treatment of asthma and COPD (6, 22), little is known regarding their relative activities and expression in human lung tissue (16, 36, 41).

The main objectives of this study were as follows: 1) to characterize subcellular cAMP-PDE activities and PDE4 isoform expression in human lung parenchyma and 2) to assess whether NCS 613, a specific PDE4 inhibitor (1, 2), is able to modulate inflammation in human lung parenchyma and hyperresponsiveness triggered in distal bronchi. Herein, we report cAMP-PDE activities and for the first time the subcellular expression of PDE4 isozymes in human lung parenchyma, as well as anti-inflammatory effects of NCS 613 on this tissue.

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We also report the functional role of PDE4 in controlling smooth muscle tone derived from human distal bronchi.

MATERIALS AND METHODS

Materials. cAMP, EGTA, and erythro-9-(2-hydroxy-3-nonyl) adenine hydrochloride (EHNA) were purchased from Sigma (St. Louis, MO). [^3H]-cAMP and [^3H]-cGMP were from New England Nuclear (Boston, MA). Rolipram (a generous gift of Schering, Berlin, Germany), cilostamide (from Sigma), and NCS 613 (1, 2) were dissolved in 100% DMSO; the final concentration of DMSO did not exceed 1% for PDE activity assessment and 0.1% for tension measurements. All PDE antibodies were from FabGennix (Frisco, TX): anti-PDE4A (DP4-112AP), anti-PDE4B (PD4-201P), anti-PDE4C (PD4-301P), and anti-PDE4D (PD4-401P). Anti-GAPDH (#MAB374), anti-Histone H₃ (06-755), and anti-EGF receptor (06-847) were from Chemicon/Millipore. Anti-Ik β was from BD-Bioscience, and anti-phospho-CPI-17 and anti-total CPI-17 were from Upstate. Anti-p38-MAPK and anti- β -actin were from NEB-cell signaling. Horse radish peroxidase-conjugates were from Promega. Enhanced chemiluminescence (ECL) kit was from GE Healthcare.

Preparation of human parenchymal subcellular fractions. Cytosolic (c), microsomal (m), and nuclear (n) enriched fractions were prepared from human lung parenchyma as described previously (18, 19). The study was approved by our institution's Ethical Committee (Protocol number CRC 05-088). Human lung explants were obtained from 14 patients aged between 50 and 60 years undergoing surgery for lung carcinoma resection. Tissue samples were collected from fresh lobectomy and transported to the laboratory in physiological Krebs solution (26). In brief, 20 g of lung parenchyma were homogenized with a Polytron (1 $\times$ 30 s at 8,000 rpm followed by 2 $\times$ 30 s at 13,500 rpm) in 80 ml of the following buffer containing (in mM) 300 sucrose, 20 K-PIPES, 3 K-EGTA, 0.1 di-isopropylfluorophosphate (DIFP), 2 dithiothreitol, 1 ascorbic acid, plus 1 pepstatin, 1 leupeptin, and 2.5 UI/ml aprotinin (pH 7.4) and centrifuged at 6,500 g for 20 min in a TI 42.1 rotor (Beckman) at 4°C. The first pellet containing nuclei and mitochondria was kept in ice while the first supernatant was filtered through two layers of cheesecloth and centrifuged 1 h at 85,000 g in a TI 42.1 rotor at 4°C. The supernatant from the second centrifugation was thereafter pooled and aliquoted as cytosolic (c) fraction, whereas the corresponding pellet was resuspended in a preservation buffer containing (in mM) 300 sucrose, 400 KCl, 1 K-EGTA, 5 K-PIPES, 0.1 MgCl₂, 0.1 DIFP (pH 7.4) and preserved as the microsomal (m) fraction. The first pellet was thereafter resuspended in 2.4 M sucrose (pH 7.2) and filtered through four layers of cheesecloth and centrifuged 15 min at 1,000 g. This mixture was progressively dehydrated with 2.2 M sucrose and centrifuged 1 h at 100,000 g in a TI 35 rotor at 4°C. The supernatant was discarded and the pellet, comprising the nuclear (n) fraction, was resuspended in the preservation buffer described above and stored as 50 μ l aliquots. All samples were frozen in liquid nitrogen and stored at -81°C for subsequent use.

Preparation of human distal bronchi extracts. Either fresh or cultured distal bronchi from human lung were homogenized with a Polytron (1 $\times$ 30 s at 8,000 rpm followed by 2 $\times$ 30 s at 13,500 rpm) in the following buffer containing (in mM) 300 sucrose, 20 K-PIPES, 3 K-EGTA, 0.1 di-isopropylfluorophosphate, 2 dithiothreitol, 1 ascorbic acid, plus 1 pepstatin, 1 leupeptin, and 2.5 UI/ml aprotinin (pH 7.4) and centrifuged at 10,000 g for 10 min at 4°C. Supernatant aliquots were stored at -80°C until use. These cytosolic extracts were used for PDE assay and Western blot analysis (26).

Assessment of PDE activities in lung tissue. PDE activity was measured by a radioenzymatic assay as previously described (14) at a substrate concentration of 1 μ M cAMP or cGMP in presence of 10,000 cpm [^3H]-cAMP or [^3H]-cGMP as tracer and hydrolyzed between 10% to 20%. Contribution of the different isozymes to PDE activities were assessed with either 1 μ M cGMP in the presence of 10 μ M nimodipine for PDE1, 20 μ M EHNA for PDE2, and 0.1 μ M

DMPP0 for PDE5 or with 1 μ M cAMP in the presence of 1 μ M cilostamide for PDE3 and 10 μ M rolipram for PDE4. Alternatively, NCS 613 (0.1–10 μ M) was used as a PDE4 inhibitor, as previously reported (1, 2). All samples were also tested in the presence of 1 mM of EGTA and 1 μ M of substrate for cAMP-PDE or cGMP-PDE total activity. Protein concentration assessment enabled to calculate specific activity in picomoles per minute per milligram of protein. The remaining activity in the presence of a specific inhibitor was subtracted from total activity for each PDE family contribution.

Western blot analysis. Proteins from subcellular fractions derived from either lung parenchyma or human distal bronchi (30 μ g) were subjected to Western blot analysis. In brief, protein samples were solubilized and denatured for 5 min at 95°C in Laemmli buffer, electrophoresed on SDS 10% polyacrylamide gel, and electrotransferred onto polyvinylidene fluoride membranes. Immunodetection was carried out with anti-PDE4A, anti-PDE4B, anti-PDE4C, anti-PDE4D, anti-Ik β , anti-phospho-CPI-17, anti-p38 MAPK, anti- β -actin, and anti-GAPDH. Immobilized antigens were detected by chemiluminescence using horseradish peroxidase conjugates as secondary antibodies, an ECL kit, and autoradiography films. Autoradiography signals were captured on a GeneGenius Bio Imaging System (Syngene) using the GeneSnap software and analyzed using the GeneTools software.

Isolation and organ culture of human lung tissue. Human lung tissues were obtained from patients undergoing surgery for lung carcinoma (26). After lobectomy and transport in sterile physiological saline solution, lung samples, distant from the malignant lesion, were dissected by the pathologist. The absence of tumoral infiltration was retrospectively established in all bronchi by pathological analysis. Tissue samples were immediately placed in Krebs solution containing (in mM) 118 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 KH₂PO₄, 1.2 MgSO₄, 25 NaHCO₃, and 11.1 dextrose (pH 7.4) previously bubbled with 95% O₂-5% CO₂ at 22°C and then immediately transported to a level two-culture room. After removal of connective tissues and adhering parenchyma, paired rings of similar weight and length (inner diameter of 0.5–0.8 mm) were microdissected from the same bronchus segment. Bronchial rings were placed in individual wells of 24 well-culture plates, containing DMEM-F12 culture medium (1 ml/well) supplemented with 0.3% penicillin (100 IU/ml) and streptomycin (0.1 mg/ml) (11, 25). Culture plates were placed in a humidified incubator at 37°C under 5% CO₂. Distal bronchi explants were untreated (control) or treated (every 12 h for 48 h) with either 10 μ M NCS 613, 10 ng/ml TNF- α or 10 ng/ml TNF- α combined with 10 μ M NCS 613.

Isometric tension measurements. The mechanical tensions induced by specific agonists were measured as previously described (26, 27). Bronchial rings were mounted in isolated organ baths (Radnoti Mo-rovia CA), containing 6 ml of Krebs' solution at 37°C, bubbled with carbogen (95% O₂-5% CO₂). An initial load of 0.6 g was applied to the rings. Tissues were allowed to equilibrate for 1 h in Krebs' solution and washed out every 15 min. Passive and active tensions were assessed using transducer systems coupled to Polyview software (Grass-Astro-Med, West Warwick, RI) for facilitating data acquisition and analysis.

Immunohistochemistry experiments. Data have been obtained on lung parenchyma and human bronchi cryosections, exactly as previously described by our group (27), using rabbit anti-human PDE4B and PDE4C primary antibodies. Immunofluorescence staining was revealed by rabbit IgG antibody coupled to either Alexa fluor 647 (red) or Alexa fluor 488 (green), if not otherwise specified. DAPI 4',6'-diamino-2-phenylindole counterstaining was used to highlight cell nuclei.

Data analysis and statistics. Results are expressed as means $\pm$ SE with *n* indicating the number of experiments. Statistical analyses were performed using a one-way ANOVA followed by a Bonferroni post test. Differences were considered statistically significant (*) when *P* < 0.05. Data curve fittings were performed using

Sigma Plot 9.0 (SPSS-Science, Chicago, IL) and Prism graph Pad to determine IC₅₀ values.

RESULTS

Total cAMP-PDE and specific PDE activities in subcellular parenchymal fractions. Total cAMP-PDE as well as specific PDE2, PDE3, and PDE4 hydrolytic activities were detected in cytosolic (c), microsomal (m), and nuclear (n) subcellular fractions derived from human lung parenchyma (Fig. 1). Total cAMP-PDE activity was 100 pmol·min⁻¹·mg⁻¹ of protein in the cytosolic fraction, which was approximately two- and eightfold higher than the total activity measured in the microsomal and nuclear fractions, respectively (Fig. 1A). Of the PDEs assayed, PDE2 hydrolytic activity remained the most important cAMP-hydrolyzing enzyme detected in these three subcellular compartments, representing about 50% of total hydrolytic activities (Fig. 1B), followed by PDE4, which displayed a similar distribution (Fig. 1D). PDE3 activity was also detected in all three subcellular fractions but to a lesser proportion than both PDE2 and PDE4 (Fig. 1C). To assess the identity and quality of the subcellular fractions, three different biomarkers have been used, namely GAPDH, histone H₃ and EGFR antibodies. Specific protein immunostaining attests that chosen biomarkers were unique for cytosolic, nuclear, and microsomal fractions, respectively (Fig. 1E). Hence cross-contamination was fairly low. These observations validate the quantitative analysis of PDE isoform distribution, described above, and their identity and location are assessed below.

Subcellular localization of PDE4 isoforms in human parenchyma. The intracellular pattern of PDE4 expression was analyzed by Western blot and revealed several PDE4 isoforms in human lung parenchyma distributed among the three subcellular fractions (Fig. 2, A–D). PDE4A, PDE4B, PDE4C, and PDE4D were detected in all three subcellular fractions but with differential distributions. In the cytosolic fraction (left lane), PDE4 proteins were highly expressed and contained several PDE4 isoforms, comprised mainly of PDE4A and PDE4B, whereas the microsomal fraction appeared to be enriched in a 51 kDa PDE4A variant. The nuclear fraction also contained all four isoforms, with PDE4B variants of 60 and 71 kDa being the most prominent (Fig. 2, A–D, right lane). Finally, 63 kDa PDE4D was mainly present in the cytosol, whereas 112 kDa PDE4D and 84 kDa PDE4C were typical of the nuclear fraction (Fig. 2C).

Effects of NCS 613 pretreatment on PDE4 activity and IκBα expression. It has previously been reported that NCS 613 did not inhibit PDE1, PDE2, PDE3, and PDE5 isoforms (2). A 72-h treatment with NCS 613 induced a 64% decrease in PDE4 activity in human parenchyma (Fig. 3A). Complementary experiments revealed that NCS 613 did not modify the hydrolytic activity of PDE2 and PDE3 isoforms from human lung parenchyma (data not illustrated). Furthermore, among the four PDE4 isoforms analyzed by Western blot, 48-h NCS 613 treatments consistently decreased the 75 kDa PDE4C isoform (Fig. 3B). To assess the putative anti-inflammatory effect of NCS 613, we also verified the expression of IκBα, the repressor of transcription factor NF-κB. IκBα was largely expressed

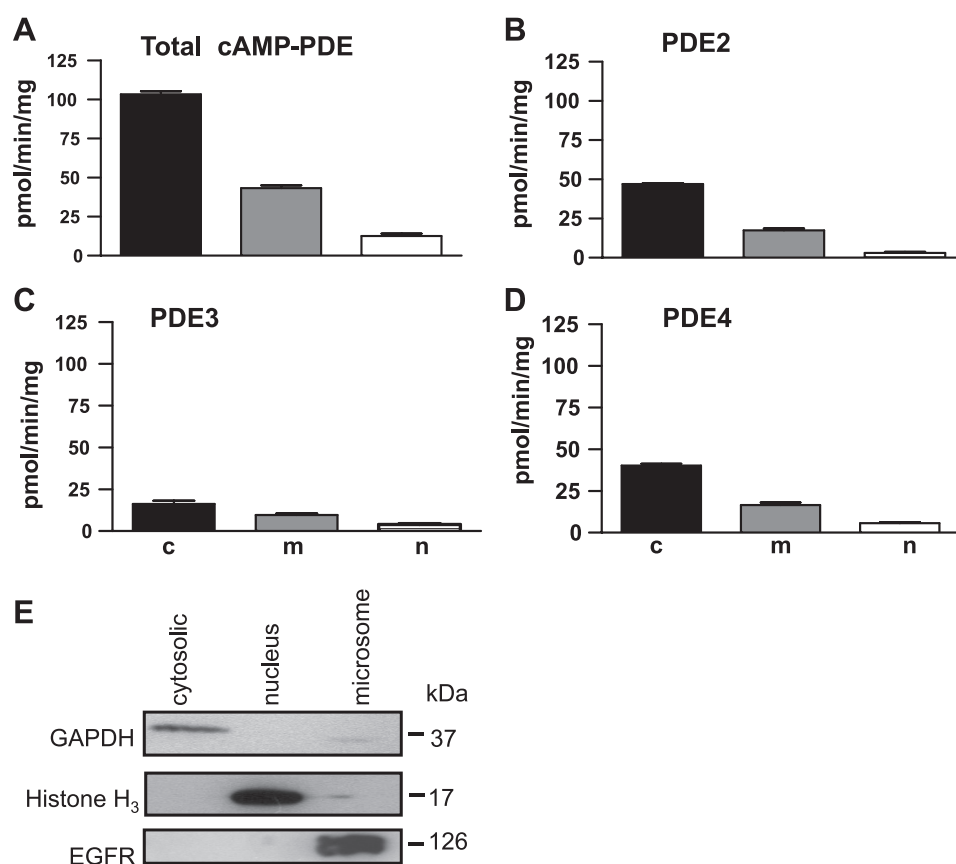


Fig. 1. Total cAMP-phosphodiesterase (PDE) and specific PDE2, PDE3, and PDE4 activities in subcellular parenchymal fractions. Total cAMP-PDE activity and contribution of 3 PDE isoforms were assessed at 1 μ M cAMP by radio-enzymatic assay. A: total cAMP-PDE activity was assessed in presence of 1 mM EGTA in human parenchyma subcellular fractions (c: cytosolic; m: microsomal; n: nuclear). B–D: relative distribution of PDE2 (B), PDE3 (C), and PDE4 (D) specific activity. Data are expressed as picomoles per minute per milligram protein and represent means $\pm$ SE of 3 independent experiments performed in triplicate. E: characterization of cytosolic, nuclear, and microsomal subcellular fractions was performed using specific antibodies against GAPDH, histone H₃, and EGF receptor, respectively. Data attest that the cross contamination was fairly low.

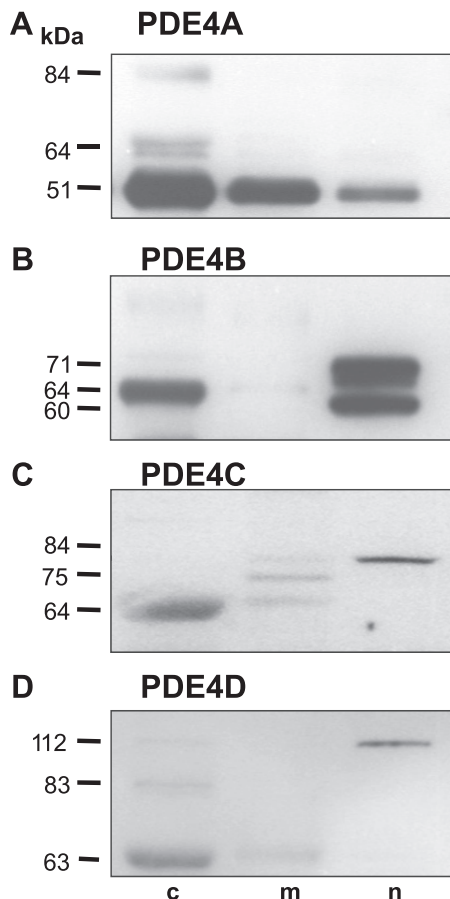


Fig. 2. Subcellular location of PDE4 isoforms in human parenchyma. Subcellular fractions from human parenchyma were prepared and subjected to Western blot analysis as described in MATERIALS AND METHODS. Cytosol (c), microsomal (m), and nuclear (n) fractions were probed with: anti-PDE4A (A), anti-PDE4B (B), anti-PDE4C (C), and anti-PDE4D (D) specific primary antibodies. Results are representative of 3 independent experiments. Note the different location patterns when comparing the distribution of PDE4 subtypes in cytosolic, microsomal, and nuclear subcellular fractions.

in native human parenchyma (Fig. 3C), whereas after a 72-h cultured period, I κ B α was no longer detectable in cultured parenchyma (Fig. 3C, second lane). By contrast, 72-h treatments of human lung parenchyma with NCS 613 (3 and 10 μ M) rescued I κ B α detection. Note that a reduced I κ B α detection is positively correlated with an activation of NF- κ B (26).

cAMP-PDE and PDE4 activities in human bronchi. With the use of cytosolic fractions, cAMP-PDE, PDE3, and PDE4 activities were detected in human distal bronchi (Fig. 4A). Total cAMP-PDE activity was consistently higher in human bronchi than that measured in the cytosolic fraction of human parenchyma described above (Fig. 1A). Cytosolic PDE4 controlled 56% of cAMP hydrolytic activity in human bronchi (Fig. 4A). Total cAMP-PDE in bronchi was approximately twofold higher than in pulmonary arteries (28), whereas bronchial PDE4 activity was 3.5-fold higher than its activity in pulmonary arteries (data not shown).

Immunolocalization PDE4 isoforms in human bronchi. Figure 4B reveals PDE4B detection in the human lung parenchyma. PDE4B immunofluorescence signals were very light in lung parenchyma tissues, as well as in bronchial cryosections. Figure 4, C-F, illustrates the immunolocalization of PDE4C,

including DAPI counterstaining in human bronchial cryosections. Parallel experiments, using a specific antibody against human PDE4B and a secondary antibody coupled to Alexa-fluor 647, revealed low staining levels of PDE4B in ASM cells, similar to the fluorescence recovered from lung parenchyma cryosections, as illustrated above (Fig. 4B). PDE4C isoform was consistently detected in epithelial and ASM cells of bronchi cryosections (green staining), since the secondary antibody was coupled to Alexa fluor 488 (Fig. 4C and F). PDE4C was also detected in pneumocytes 1 surrounding alveoli, in lung parenchyma, but to a lesser extent. Consequently, the presence of PDE4B and PDE4C isoforms in lung parenchyma and bronchi was ascertained, but it would be hazardous to compare immunofluorescence signals to staining levels detected following Western blot experiments where the immunoreactive bands were of high intensity (Fig. 2, B and C).

Effect of NCS 613 on human bronchial hyperresponsiveness. TNF- α -induced bronchi hyperresponsiveness was used as an in vitro model as reported previously (26, 27). TNF- α treatments (10 ng/ml for 48 h) increased the mechanical tension induced by either 1 μ M MCh, 30 nM U-46619, or 1 μ M histamine when compared with control cultured bronchi (26) (data not shown). Conversely, TNF- α -treated bronchi coupled to 10 μ M NCS 613 pretreatments resulted in a consistent reduction in reactivity and amplitude of the mechanical responses (Fig. 5,

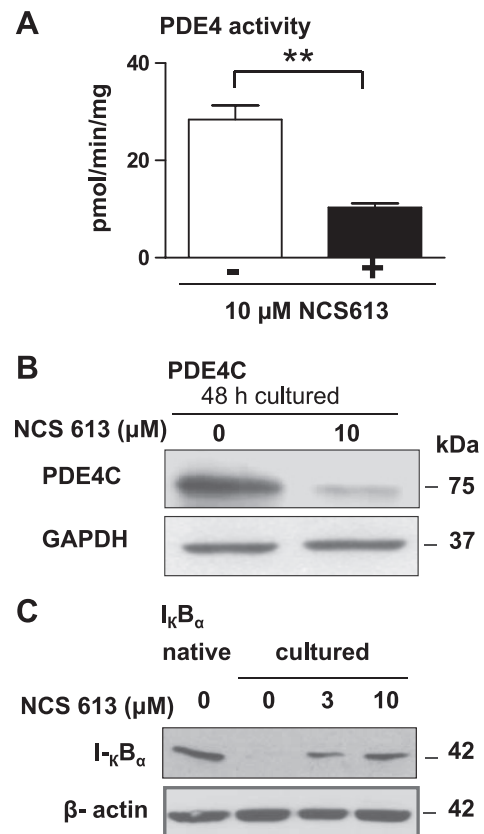


Fig. 3. Effects of NCS 613 treatment on PDE4 activity and I κ B α expression in parenchyma. A: specific PDE4 activity assessed in control and after NCS 613 treatment of parenchymal explants. $**P < 0.05$. B: PDE4C immunostaining level in control and after NCS 613 treatment of parenchymal explants over a 48-h period. C: detection of cytoplasmic I κ B α levels in native and cultured parenchymal explants in the absence and upon 10 μ M NCS613 treatment. Data are representative of 4 distinct experiments.

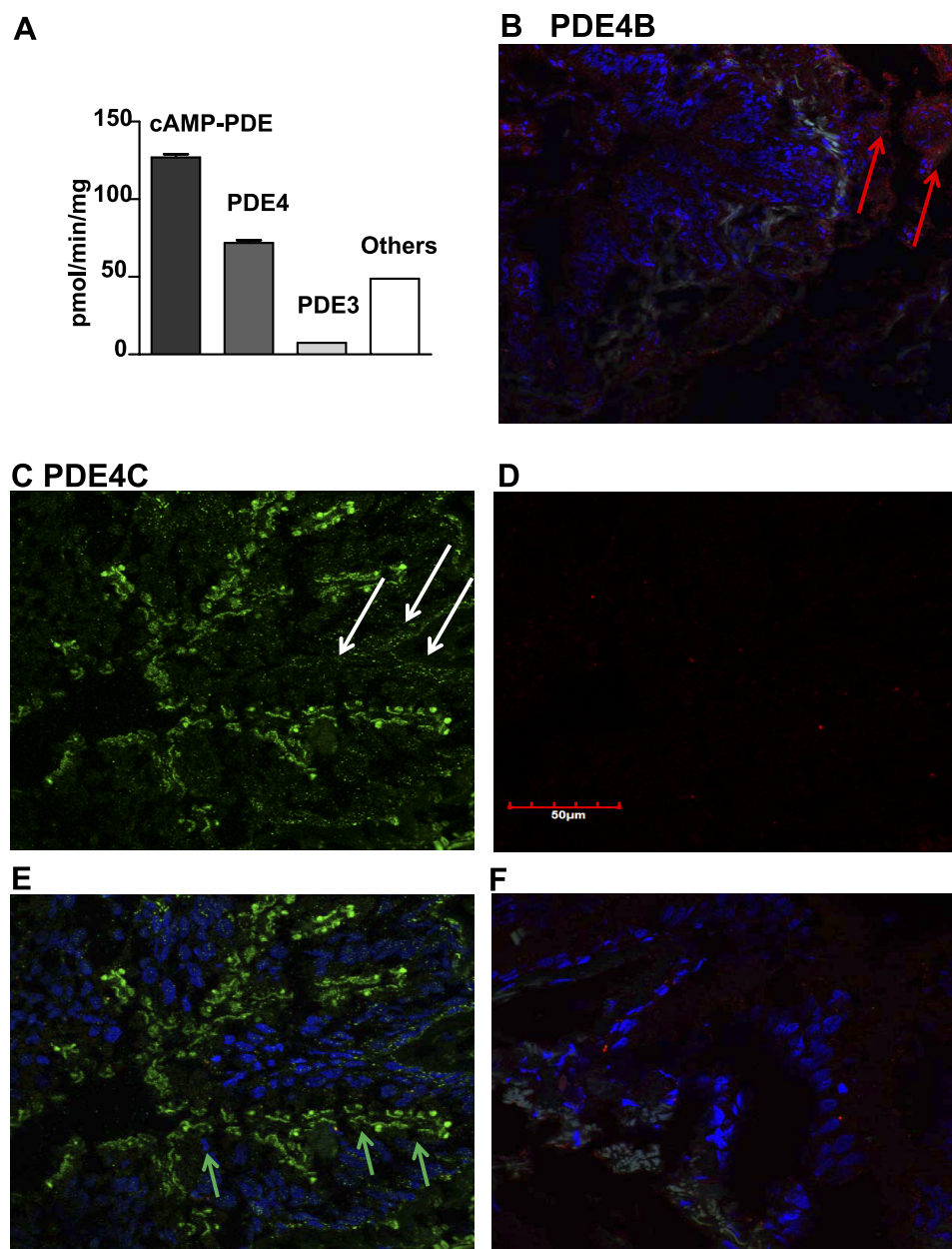


Fig. 4. Total cAMP-PDE and isoform specific hydrolytic activities in human bronchi as well as PDE4B and PDE4C immuno-localization in lung tissues. *A*: total cAMP-PDE activity and relative contribution of PDE4 and PDE3 were assessed by radio-enzymatic measurement in human airway smooth muscle. *B*: immunofluorescence staining revealed by rabbit anti-human PDE4B + anti-rabbit IgG coupled to Alexa fluor 647 (red arrows), including DAPI counter staining ($\lambda = 405$ nm). PDE4B specific staining corresponds to the red signal in human bronchi cryosection. *C*: immunofluorescence staining revealed following rabbit anti-human PDE4C and anti-rabbit IgG antibodies coupled to Alexa fluor 488 (white arrows: smooth muscle cell layer). *D*: putative auto-fluorescent signal at 647 nm on the same cryosection (negative control). *E*: superposition of *C* and *D*, including DAPI counterstaining ($\lambda = 405$ nm). Green arrows pointing toward decorated epithelial cell. PDE4C specific staining corresponds to the green signal. *F*: negative control in the presence of DAPI and secondary rabbit-IgG antibody coupled to Alexa fluor 488, but in the absence of rabbit anti-human PDE4C. Scale bar, 50 μ m.

A–C), thereby delaying the maximal amplitude. As can be seen in the representative histograms in Fig. 5 obtained from eight different patient lungs, TNF- α induced a hyperresponsiveness in human bronchi as shown by the significant increase in mechanical tension, whereas the combination of TNF- α and 10 μ M NCS 613 treatment significantly decreased the mean mechanical responses to all three agonists used (Fig. 5, *D–F*). Finally, NCS 613 had no effect on tone from control bronchial tissues (Fig. 5, *D–F*; white and light gray columns).

Effect of NCS 613 on relaxation properties of human bronchi. The effects of acute 10 μ M NCS 613 pretreatments on human bronchial relaxations induced by a β_2 adrenoceptor-agonist were assessed on freshly dissected human bronchi. In the absence of NCS 613 treatment, the mechanical tension induced by 1 μ M methacholine (MCh) was fully relaxed by 300 nM isoproterenol (Fig. 6*A*). However, acute pretreatment with 10 μ M NCS 613 reduced the basal tension of resting

bronchial rings (Fig. 6*B*). Of note, in the presence of 10 μ M NCS 613 treatment for 10 min, 100 nM isoproterenol fully relaxed the mechanical tension induced by 1 μ M MCh (Fig. 6*B*). After quantitative analysis, NCS 613 pretreatment reduced by twofold the apparent IC_{50} value to isoproterenol, from 40 nM in control bronchi to 20 nM in NCS 613-treated bronchi (Fig. 6*C*).

Effects of NCS 613 treatment on specific protein markers upon proinflammatory conditions. Inflammation marker, such as p65-NF- κ B, and phospho-proteins likely involved in human bronchial hyperresponsiveness were analyzed by Western blot under various experimental conditions. When compared with control conditions, TNF- α treatment alone increased translocation of p65-NF- κ B subunit into nuclear fraction, when compared with the basal levels in the cytosolic fractions (Fig. 7*A*). Higher p65-NF- κ B staining in nuclei is usually correlated with a higher I κ B α degradation (26). For instance, NCS 613 treat-

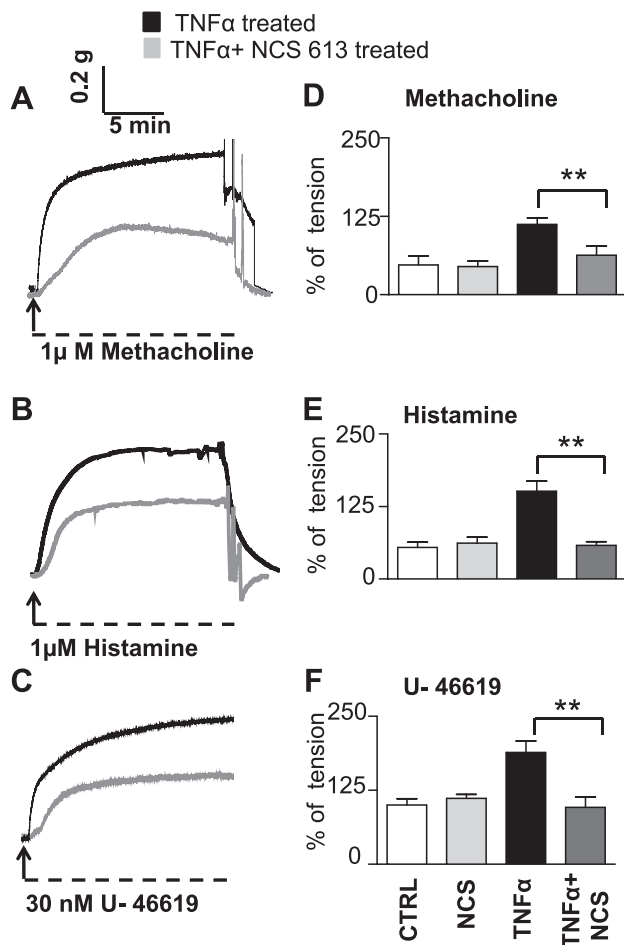


Fig. 5. Effects of NCS 613 (NCS) on human bronchi hyperresponsiveness. Human bronchi were treated for 48 h with 10 ng/ml TNF- α in the absence (black lines) or presence (gray lines) of 10 μ M NCS 613 (*left*). Representative superimposed recordings illustrating the tension induced by 1 μ M methacholine (A), 1 μ M histamine (B), and 30 nM U-46619 (C) on human distal bronchi. *Right*: bar histogram displaying the mean contractile responses induced by 1 μ M methacholine (D), 1 μ M histamine (E), and 30 nM U-46619 (F). Responses to pharmacological agonists were normalized to the responses triggered by 80 mM KCl. Human bronchi were cultured for a 48-h period under the following experimental conditions: untreated (control), + 10 μ M NCS 613, + 10 ng/ml TNF- α , or 10 ng/ml TNF- α + 10 μ M NCS 613. Each bar represents means $\pm$ SE, with $n = 8$ for each experimental condition. $^{**}P < 0.05$.

ment slightly increased I κ B α detection due to a lower degradation rate, as already illustrated in Fig. 3C. This observation was correlated with lower inflammation level in bronchi treated with the isoform-specific PDE4 inhibitor. Under proinflammatory condition, detection of PDE4B and PDE4C protein levels (Fig. 7B, top 2 rows), as well as phospho-p38-MAPK, were consistently increased (Fig. 7B, middle row); whereas 10 μ M NCS 613 treatments reduced the detection of these protein immunoreactive bands enhanced by TNF- α (Fig. 7B, lanes 3 and 4, respectively). On the other hand, the total p38 MAPK detection levels were fairly constant.

Figure 8, A–F, displays quantitative analysis of the Western blot experiments of the relative detection levels of p65 NF- κ B in the nuclear fractions, PDE4B, PDE4C, phospho/total-p38-MAPK, phospho/total-CPI-17, and GAPDH, respectively. Data demonstrate that a 48-h TNF- α treatment upregulates

protein detection levels, whereas addition of 10 μ M NCS 613 significantly decreases PDE4B and PDE4C detection, as well as the phosphorylated forms of p38-MAPK and CPI-17 under proinflammatory experimental condition (Fig. 7C and Fig. 8, D and E), which correlates well with changes in the pharmacological reactivity reported in Fig. 5.

DISCUSSION

Chronic inflammation is a deleterious process occurring in several pulmonary diseases (41). Specific cAMP hydrolyzing phosphodiesterases (PDE4) have been shown to be involved in the control of inflammatory responses (10, 33, 38). In the present study, we report for the first time the characterization of cAMP-PDE activities as well as the location of four PDE4 subtypes (4A, 4B, 4C, and 4D) in human lung parenchyma. We also demonstrate that a specific and novel PDE4 inhibitor, NCS 613, is able to decrease inflammation markers in lung parenchyma by restoring I κ B α detection, suggesting a potential buffering of NF- κ B signaling. This compound also reduced NF- κ B translocation into nuclei and pharmacological hyperre-

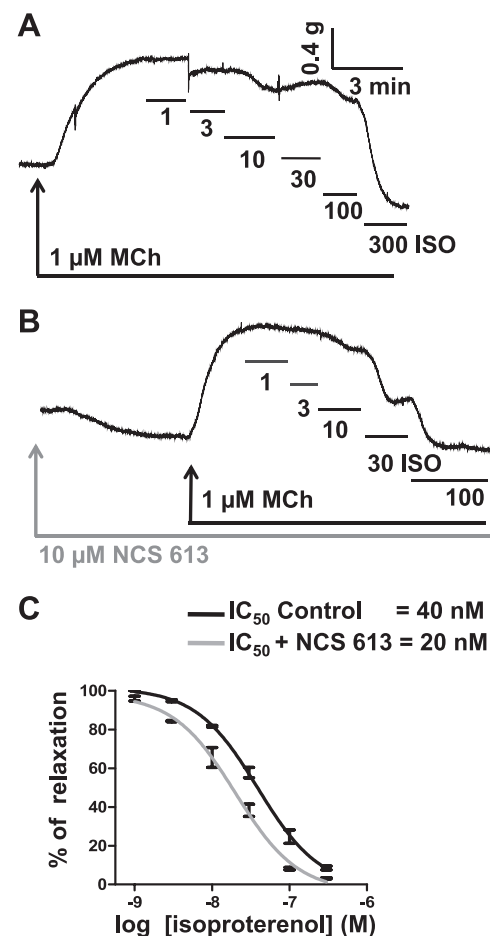


Fig. 6. Effects of NCS 613 on human bronchial relaxation induced by isoproterenol (ISO). A: representative recording illustrating the tension induced by 1 μ M MCh and subsequent relaxations in the presence of cumulative isoproterenol concentrations in fresh human bronchi. B: same experiment following acute pretreatment with 10 μ M NCS 613. C: mean relaxing responses to isoproterenol were assessed on human bronchi in the absence or presence of 10 μ M NCS 613 pretreatments after which corresponding IC₅₀ values to isoproterenol were calculated ($n = 12$ for each data point).

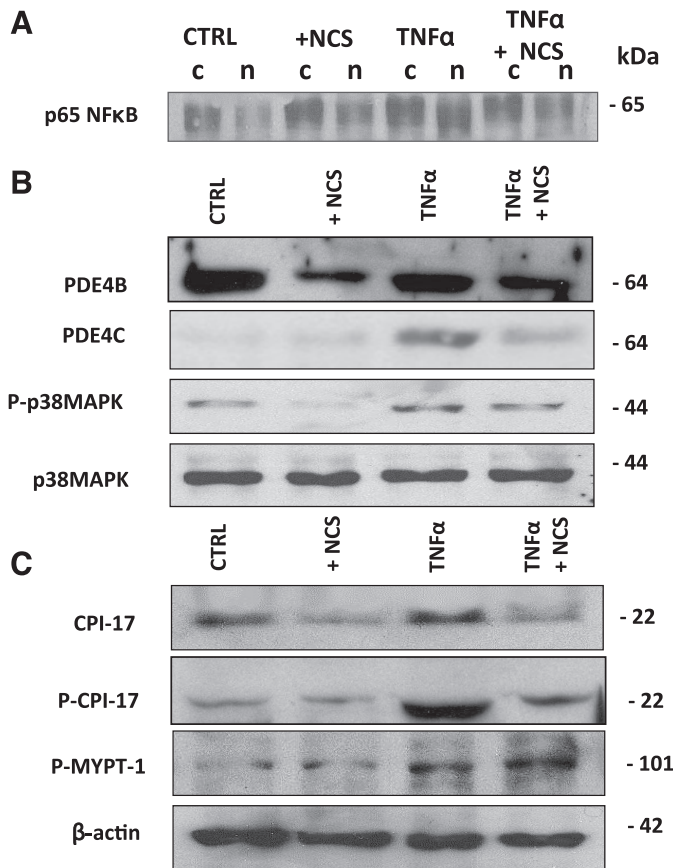


Fig. 7. Effects TNF- α and NCS 613 pretreatments on p65 NF- κ B and various intracellular proteins in human distal bronchi, including PDE4 isoforms. **A:** detection p65 NF- κ B in cytosolic (c) and nuclear (n) fractions in control conditions (CTRL), upon 10 μ M NCS 613, 10 ng/ml TNF- α , and 10 ng/ml TNF- α + 10 μ M NCS 613 pretreatments. **B:** comparative detection level of PDE4B, PDE4C, phospho-p38 MAPK, and total p38 MAPK, in the cytosolic fractions under the same experimental conditions, as described in **A**. All experiments were performed in bronchial explants cultured for a 48-h period. Data are representative of 5 independent experiments from 5 lung resections. **C:** total CPI-17, phospho-CPI-17, p-MYPT-1 (myosin light chain phosphatase catalytic subunit) and β -actin detection under the same experimental conditions as defined above in **A**. TNF- α induced a consistent increase of the assessed proteins in **A** and phosphorylated level of CPI-17, whereas NCS 613 treatment opposed the effects of TNF- α .

sponsiveness in TNF- α -treated bronchi, whereas potentiating isoproterenol induced relaxation in human distal bronchi.

cAMP-PDE activities and PDE4 subcellular locations in human parenchyma. Although results showed that cAMP-PDE activities were present in all three subcellular compartments from human lung parenchyma, mean cytosolic cAMP-PDE activity was nevertheless higher than that measured in both microsomal and nuclear fractions. These data are consistent with those of a previous report showing that both cGMP and cAMP hydrolytic activities in bovine main pulmonary artery were higher in the cytosolic fraction comparatively to the microsomal fraction (28). PDE4 isozymes, which predominantly catabolize cAMP in the cell, clearly displayed differential distribution profiles in the present study. In human parenchyma, PDE2 and PDE4 were found to be the major cAMP hydrolyzing enzymes with mean contributions representing 40% of total activity. Indeed, PDE4 activity, which was relatively low in the nuclear fraction, may be important for inac-

tivation of cAMP and its binding effectors, such as cAMP-dependent transcription factors. Previous studies have reported that PDE4 significantly contributes to cAMP hydrolysis in airways smooth muscle (30) and tracheal epithelial cells (31). Moreover, microsomal fractions from bovine trachea have been shown previously to contain three PDE isoforms: PDE1, PDE3, and mainly PDE4, the latter representing 48% of total cAMP hydrolytic activity (37).

In the present study, the distribution of PDE4 isoforms (PDE4A, PDE4B, PDE4C, and PDE4D) also revealed differential distributions according to specific variants. As expected, these isoenzyme proteins were abundantly found in the cytosolic fraction, which is likely correlated with the presence of high cAMP hydrolytic activity. PDE4A (64 and 84 kDa) was only found in the cytosolic fraction, whereas 64 kDa PDE4B and 63 kDa PDE4D were differentially expressed in this compartment (16). As for the microsomal fraction, 51 kDa PDE4A and mainly three PDE4C immunoreactive bands (84, 75, and 64 kDa) were the predominant forms, with 75 kDa PDE4C being exclusively expressed in this fraction. PDE4 activity was found to be lower in the nuclear fraction, although this compartment did reveal several PDE4 isoforms, with 60 and 71 kDa PDE4B and 84 kDa PDE4C being exclusively expressed in this fraction. Hence, according to their activity

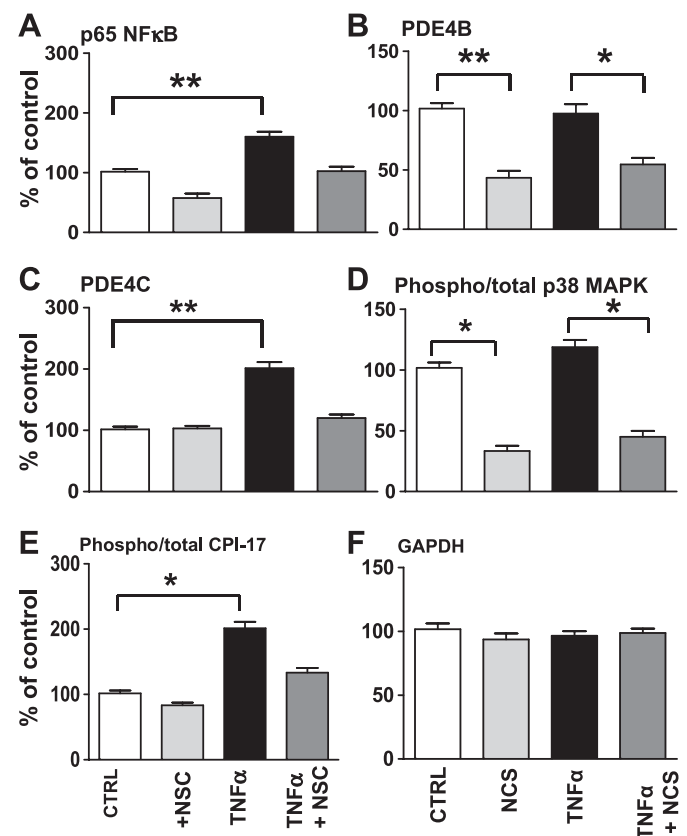


Fig. 8. Quantitative and statistical analysis of the mean TNF- α and NCS 613 effects on the protein profiles and phosphorylation levels shown in Fig. 7. Calculated density ratio in percentage of control value under the corresponding experimental conditions: in control (CTRL), upon 10 μ M NCS 613, 10 ng/ml TNF- α , and 10 ng/ml TNF- α + 10 μ M NCS 613 pretreatments. Bar histograms represent means of 3 experiments. **A:** p65 NF- κ B in the nuclear fractions. **B:** PDE4B. **C:** PDE4C. **D:** phospho/total-p38-MAPK. **E:** phospho/total-CPI-17. **F:** GAPDH in the cytosolic fractions ($n = 3$; * $P < 0.05$; ** $P < 0.01$).

and subcellular location, PDE4 protein variants may participate in cAMP compartmentalization and henceforth regulate the biological process, such as inflammation, gene expression, and ASM tone. Indeed, it has been reported that PDE4D-deficient mice are refractory to muscarinic cholinergic stimulation (24).

NCS 613 abolishes inflammation in human lung parenchyma. The present data showed that treatment with the specific PDE4 inhibitor NCS 613 significantly decreased PDE4 activity in cultured parenchyma. More specifically, this treatment significantly decreased 75 kDa PDE4C detection, which is in agreement with the high selectivity of NCS 613 for the PDE4C subtype. The use of NCS 613 induced a reduction of protein expression, which is likely due to a feedback mechanism at the nuclear level, since PDE4B and PDE4C isoforms are mainly localized in the nuclear fraction (Fig. 2, B and C). Hence, there is other evidence of negative regulation of PDE4 species by elevated levels of cAMP, which induce PKA-dependent upregulation of inducible cAMP early repressor (ICER) expression. For instance, the PDE4 inhibitor rolipram has been reported to stimulate ICER expression in mouse osteoblastic cells (5). In addition, in cardiac myocytes, cAMP elevation triggers the downregulation of transcripts and promoter activity for cyclic AMP PDE4A10 (23).

In addition, the NF- κ B transcription factor repressor I κ B α was consistently detected in native (fresh) parenchyma, whereas cultured lung tissue in the absence of NCS 613 resulted in I κ B α becoming undetectable, thus revealing a fast degradation rate, which is likely correlated with the translocation of NF- κ B subunits into the nuclei (26). Interestingly, NCS 613 treatment restored I κ B α detection in a concentration-dependent manner, suggesting that I κ B α binds to NF- κ B and prevents its nuclear translocation, which would result in a lower inflammation status. In contrast, cytokine stimuli and oxidative stress can activate the NF- κ B pathway. Under these inflammatory conditions, I κ B α is released from NF- κ B, ubiquitinated and degraded by proteasomes, thus decreasing its detection upon induced inflammation (13, 24). NF- κ B translocation thus allows gene transcription under its control and more specially those genes encoding for COX-1 and proinflammatory cytokines (TNF- α , IL-1, IL-6).

Lung inflammation is the first leading cause of mucus secretion, airway hyperresponsiveness, and fibrosis (27a, 35). Increasing evidence has also revealed the implication of PDE4 in lung disease in regard to inflammation and remodeling (39). During the fibrotic process, PDE4 inhibitors display epithelial mesenchymal transition, a critical event in this pathogenesis (15). Hence, the present data would suggest that NCS 613 may be a preventive agent for lung inflammation and injury.

cAMP-PDE in human distal bronchi. Patterns of cAMP-PDE activity were also assessed in human bronchi. PDE4 and PDE3 hydrolytic activities were confirmed in this tissue, with PDE4 being the predominant cAMP hydrolyzing enzyme in bronchi (56% of total cAMP-PDE). These results thus strongly suggest that PDE4 is a major cAMP hydrolyzing enzyme in lung smooth muscle and may represent a valuable pharmacological target through modulation of its functional properties (27a, 28).

NCS 613 potentiates isoproterenol relaxation in human distal bronchi. Relaxation induced by isoproterenol on human bronchi is mediated by β_2 receptors, adenylyl cyclase, and cAMP production. Herein, fresh human bronchi precontracted with 1

μ M MCh relaxed completely in response to cumulative concentrations of 1–300 nM isoproterenol. NCS 613 pretreatment, on the other hand, was shown to reduce basal tone. Moreover, when NCS 613-pretreated distal bronchi were contracted with 1 μ M MCh, only cumulative concentrations of 1–100 nM isoproterenol induced total relaxation. In addition, NCS 613 pretreatment decreased by twofold the IC₅₀ value to isoproterenol. This observation could be explained by the fact that NCS 613 pretreatment likely lowers cAMP degradation rate, thus amplifying the effect of isoproterenol. Thus PDE4 isozymes are clearly involved in controlling ASM tone as previously reported (37).

NCS 613 prevents TNF- α -induced human distal hyper-responsiveness. Another facet investigated in the present study was the putative role of PDE4 in bronchial hyperresponsiveness occurring in lung diseases such as asthma and COPD. The well characterized proinflammatory cytokine TNF- α was used to induce inflammation and hyperresponsiveness in an in vitro model of cultured human bronchi, which has been previously validated (26). In this particular model, TNF- α -treated bronchi exhibit rapid and large mechanical responses induced by pharmacological agents such as MCh, histamine, and U-46619. In the present study, micromolar concentrations of NCS 613 exhibited preventive effects on airway responsiveness since it reduced mechanical responses to agonists in TNF- α -treated bronchi, as witnessed by a shifting of the slope to the right, meaning a reduced sensitivity to agonists. Hence, NCS 613 significantly decreases hyper-responsiveness in TNF- α -treated bronchi by controlling cAMP hydrolytic rate. Such inhibitory action of NCS 613 may represent a valuable tool in addressing cytokine-induced bronchial remodeling and hyper-responsiveness, which are well documented in asthma (8, 32). Under inflammatory conditions, cytokines such as TNF- α and IL-1 β are known to induce upregulation of various signaling pathways and proliferation of ASM cells, hence resulting in an increased sensitivity to agonists (39, 40).

Finally, to better understand the role PDE4 inhibition on human bronchial hyper-responsiveness induced by TNF- α , the putative pathways involved in this process were also analyzed by assessing the expression of PDE4C, PDE4B, and phosphop38 MAPK. Indeed, TNF- α is known to activate inflammatory signaling through involvement of p38 MAPK phosphorylation and p65-NF- κ B translocation (26). Convincing evidence has now been reported along those lines using quantitative and statistical analyses, whereas NCS 613 treatments opposed TNF- α proinflammatory effects in human airways. TNF- α pretreatments also modified the phosphorylation level of contractile regulatory proteins. For instance, CPI-17 is a phosphorylation-dependent inhibitor of myosin light chain phosphatase (MLCP) and necessary for PKC-mediated Ca²⁺ sensitization (27). CPI-17 is believed to be implicated in bronchial hyper-responsiveness in that inflammation and/or stress leads to upregulation of its phosphorylated form. CPI-17 phosphorylation also results in an inhibition of MLCP activity, which in turn maintains steady state tension in ASM (27). Our data confirmed that TNF- α did in fact induce an increase in phosphorylated CPI-17 protein in cultured human bronchi (Fig. 7C). Addition of NCS 613 resulted in a decrease in CPI-17 phosphorylation, thus facilitating relaxation. Furthermore, TNF- α -induced bronchial upregulation of CPI-17 expression and hyper-responsiveness observed in our model was found to

occur under proinflammatory conditions since TNF- α also increased PDE4B and PDE4C expressions and phospho-p38 MAPK. In agreement with the functional data, NCS 613 treatments were found to reduce airway smooth reactivity and inflammation in TNF- α -treated bronchi (p65 NF- κ B translocation), which was correlated with a low inflammatory status.

Conclusion

The present study reports the presence of PDE4 activity and subcellular location of its isoforms in human lung parenchyma. Although PDE4 remained the major cAMP hydrolyzing enzyme in well-characterized cytosolic, microsomal, and nuclear fractions, PDE4 isoform variants were differentially expressed according to the investigated subcellular compartment. These PDE4 isozymes likely control important biological responses in the lung since their specific inhibition by NCS 613 resulted in anti-inflammatory effects in parenchyma (39, 40). Furthermore, NCS 613 demonstrated beneficial effects in airways reactivity to pharmacologically induced ASM tension, thus providing further insight into its current status (35). This new PDE4 inhibitor was also found to reduce human distal bronchial hyperresponsiveness by decreasing phospho-CPI-17, which in turn may be related to a downregulation of PDE4C, PDE4B, and other intracellular signaling pathways. Thus, specific PDE4C inhibitors, such as NCS 613, may represent an alternative approach to reducing bronchial hyperresponsiveness.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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