Expression and function of cystic fibrosis transmembrane conductance regulator in rat intrapulmonary arteries

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ABSTRACT: The cystic fibrosis transmembrane conductance regulator (CFTR) gene encodes a cyclic adenosine monophosphate (cAMP)-dependent chloride channel located mainly at the apical membrane of epithelial cells. In myocytes of pulmonary arteries, numerous chloride channels have been identified and described, but not the CFTR. Thus the presence and function of the CFTR was investigated in rat intrapulmonary arteries.

CFTR expression, localisation and function were analysed in cultured smooth muscle cells using Reverse transcriptase (RT)-PCR and immunoprecipitation followed by protein kinase A phosphorylation, immunolocalisation and an iodide efflux assay, respectively. The role of the CFTR in pulmonary vasoreactivity was determined in arterial rings using an organ bath system.

RT-PCR and immunoprecipitation analyses, as well as the immunolocalisation study, revealed the expression of CFTR gene transcripts and protein. The iodide efflux assay showed the existence of functional cAMP-, calcium- and volume-dependent chloride channels. Furthermore, the following effects were found: 1) inhibition of forskolin/genistein-activated iodide efflux by glibenclamide, diphenylamine-2-carboxylic acid and CFTR-specific inhibitor (CFTR_{inh})-172; 2) activation of iodide efflux by the benzoquinolizinium derivative CFTR activators MPB-07 and MPB-91; and 3) inhibition of MPB-dependent efflux by CFTR_{inh}-172. Finally, CFTR activators induced concentration-dependent vasorelaxation in rings precontracted with phenylephrine, in the presence or absence of endothelium.

The present results are the first to reveal functional cyclic adenosine monophosphate-regulated cystic fibrosis transmembrane conductance regulator contributing to endothelium-independent vasorelaxation in rat intrapulmonary arterial myocytes.

KEYWORDS: Chloride channels, cystic fibrosis transmembrane conductance regulator, intrapulmonary arteries, iodide efflux, smooth muscle cells, vasoreactivity

The cystic fibrosis transmembrane conductance regulator (CFTR) is a cyclic adenosine monophosphate (cAMP)-dependent chloride channel expressed at the apical membrane of epithelial cells lining the tracheobronchial tree and the lumen of the digestive tract [1]. The CFTR was generally regarded as specifically expressed in epithelial cells until evidence for CFTR expression in nonepithelial tissues emerged. CFTR is expressed in cardiac muscle cells [2], brain [3] and endothelia [4], and has recently been found in tracheal smooth muscle cells (SMCs) [5] and aortic SMCs of rats and mice [6, 7]. Despite this CFTR expression profile, the clinical picture of patients suffering from the CFTR-related disease cystic fibrosis appears to be unrelated to cardiac, vascular and brain dysfunction. Since truly selective activators and

inhibitors are also lacking, the role of the CFTR in these tissues remains unresolved. However, the pharmacology of the CFTR has recently progressed, making available several CFTR activators, such as genistein and benzoquinolizinium derivatives [6–8], as well as blockers, such as the thiazolidinone compound CFTR-specific inhibitor 3-((3-trifluoromethyl)phenyl)-5-((4-carboxyphenyl)methylene)-2-thioxo-4-thiazolidinone (CFTR_{inh}-172) [9].

Several Cl[−] conductances have been described in smooth muscle, including the pulmonary artery. Among the Cl[−] channels expressed in SMCs, extensive studies have explored the implication of calcium-activated Cl[−] currents (I_{Cl,Ca}), evoked by a rise in intracellular calcium concentration ([Ca²⁺]_i) [10]. Although the molecular nature of

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the ICl_{Ca} is still unknown, its role in resting membrane potential and vascular tone has been described for pulmonary artery [11–13].

Regarding the volume-sensitive Cl^- channel (ICl_{swell}), members of the Cl^- channel (CIC) gene family appear to be good molecular candidates for ICl_{swell} , and CIC-3 was recently proposed to underlie ICl_{swell} in rat and canine pulmonary arteries [14]. Interestingly, CIC-3 is upregulated in pulmonary hypertensive rats [14]. It is also noteworthy that the contribution of Cl^- channels is enhanced in basal tone and norepinephrine-induced contraction in pulmonary hypertensive rats [15, 16].

A cAMP-dependent Cl^- channel has also been described in rat and bovine pulmonary arteries [17, 18]. This channel is implicated in cAMP-induced pulmonary vasodilation, pulmonary arterial SMC (PASMC) migration and morphological changes. However, the molecular identity of such a channel is still unknown in pulmonary artery and could be related to the CFTR.

Despite the fact that the CFTR is present in aortic SMCs [6, 7], and that Cl^- channels are important factors in the physiology as well as in the pathophysiology of the pulmonary artery, no studies have investigated the expression and functional role of the CFTR in pulmonary artery to date. Consequently, the present study focused on intrapulmonary artery (IPA) and investigated the expression of the CFTR in this vessel, and then explored cAMP-dependent iodide efflux, its pharmacology and the effect of CFTR activators on pulmonary vasorelaxation.

METHODS

Tissue preparation

Male Wistar rats (aged 8–10 weeks) (Janvier, Le Genest-Saint-Isle, France) were stunned and then killed by cervical dislocation according to the local animal care and use committee (agreement number AP2/11/2005 from the regional ethics committee of Aquitaine/Poitou-Charentes, France). The heart and lungs were removed and placed in Krebs–Henseleit (KH) solution, which comprised 118.4 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4 , 25 mM $NaHCO_3$ and 11.1 mM D-glucose (pH 7.4), saturated with 15% oxygen/5% carbon dioxide/80% nitrogen. IPA of the first order from the left lung was dissected free from surrounding connective tissues in KH solution.

Cell culture

The entire heart–lung preparation was rapidly removed and rinsed in culture medium (Dulbecco's modified Eagle medium/HEPES (pH 7.3) supplemented with 1% penicillin–streptomycin, 1% sodium pyruvate and 1% nonessential amino acids). IPA was dissected in culture medium under sterile conditions and cut into several pieces (1–2 mm²). SMCs were obtained from these explants and cultured as previously described [5]. All cells from these explants were immunostained using the monoclonal antibody anti-smooth muscle α -actin (Sigma-Aldrich, Saint-Quentin Fallavier, France), whereas they were negatively labelled with the endothelial nitric oxide synthase antibody (BD Transduction Laboratories, Le Pont-de-Claix, France; data not shown), demonstrating the presence of a population of SMCs.

Analysis of CFTR mRNA expression by reverse transcriptase-PCR

Total RNA was extracted using RNABle® (Eurobio, Courtaboeuf, France), according to the manufacturer's protocol. Complementary DNA was used as template in PCRs with PCR primers specific for the rat CFTR gene [19]: cfrex3 (5'-GCGATGCTTTGTCTGGAGATTC-3'), and cfrex6a (5'-CCAC-TTGTAAGGAGCAATCCATA-3'), spanning the region between nucleotides 222 and 625. The housekeeping β -actin gene (GenBank accession No. NM031144) was used as control, with the primers r-acti4 (5'-CTACCTCATGAAGATCCTGA-3') and r-acti5 (5'-TTTCATGGATGCCACAGGAT-3'), spanning the region between nucleotides 561 and 829. The primers were checked for matches in separate exons, by aligning their sequences with rat genomic sequences.

Immunoprecipitation and phosphorylation of CFTR

Chinese hamster ovary (CHO) cells stably expressing wild-type CFTR (used as a positive control for CFTR expression) and IPA SMCs were washed three times in PBS (pH 7.4), scraped in radioimmunoprecipitation assay buffer (50 mM Tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 7.5), 1 mM ethylenediamine tetra-acetic acid (EDTA), 100 mM NaCl and 1% Triton X-100) supplemented with protease inhibitors (20 μ M leupeptin, 0.8 μ M aprotinin, 10 μ M pepstatin and 2.1 mM 4-(2-aminoethyl)-benzenesulphonyl fluoride hydrochloride) and homogenised by several passes through a 23-gauge syringe needle. Cell lysates were then treated as previously described [7]. Briefly, lysates were incubated with CFTR mouse monoclonal antibody clone 24-1 (R&D Systems, Minneapolis, MN, USA) or nonimmune mouse immunoglobulin (Ig) G (Sigma-Aldrich). The CFTR was phosphorylated *in vitro* using two units of the catalytic subunit of protein kinase (PK)A (Sigma-Aldrich) and 370 kBq [γ -³²P] adenosine triphosphate (ATP; Amersham Pharmacia Biotech, Orsay, France; 111 TBq·mmol⁻¹). Phosphorylated proteins were visualised by autoradiography.

Immunofluorescence study

Immunofluorescent labelling of IPA SMCs using an anti-CFTR C-terminus monoclonal antibody (mouse anti-human clone 24-1; R&D Systems) with TO-PRO-3 iodide (Molecular Probes, Invitrogen Corporation, Cergy-Pontoise, France) for nuclear staining was performed as previously described [5].

Isometric tension measurements

The effect of CFTR activators on mechanical activity were measured using IPA rings (1.5–2.5 mm long) as previously reported [20]. In brief, KH solution, saturated with 15% oxygen/5% carbon dioxide/80% nitrogen, was used. Mechanical properties were assessed using an organ bath and transducer systems (EMKA Technologie, Paris, France), coupled to IOX software (EMKA Technologie) in order to facilitate data acquisition and analysis. As determined in preliminary experiments, tissues were set at optimal length by equilibration against a passive load of 0.8 g. IPA rings were then washed and precontracted with 0.3 μ M phenylephrine (PHE) in order to test the relaxant properties of CFTR activators (10-chloro-6-hydroxybenzo[c]quinolizinium chloride (MPB-07), 5-butyl-10-chloro-6-hydroxybenzo[c]quinolizinium chloride (MPB-91) and 10-fluoro-6-hydroxybenzo[c]quinolizinium chloride (MPB-80);

3–300 μM , prepared as described previously [21]) by constructing a cumulative concentration–response curve. Some experiments were performed in endothelium-denuded rings. Endothelium was removed by perfusing the lumen of the vessels with a solution containing 0.3% 3-((3-cholamidopropyl) diethylammonio)-1-propane sulphonate (CHAPS), followed by washout with the drug-free solution, as previously described [22]. The effect of CHAPS was confirmed by the absence of relaxation with 10 μM carbamylcholine of 0.3 μM PHE-induced precontraction. All experiments were performed at 37°C.

All chemical agents were dissolved in dimethylsulphoxide (DMSO; final concentration $\leq 0.1\%$), except 5,11,17,23-tetrasulphonato-25,26,27,28-tetramethoxy-calix[4]arene (calixarene; generously provided by A.K. Singh and R.J. Bridges (University of Pittsburgh, Pittsburgh, PA, USA)), carbamylcholine, CHAPS, MPB-07, MPB-80 and PHE, which were dissolved in water. The maximal concentration of DMSO used in the experiments was $\leq 0.1\%$ for all of the chemicals and 0.3% for MPB-91 and had no effect on the mechanical activity of the rings.

Iodide efflux

CFTR Cl^- channel activity was assayed by measuring the rate of ^{125}I efflux from cultured cells as previously described [6, 7]. Cells were washed with efflux buffer, which comprised 136.9 mM NaCl, 5.4 mM KCl, 0.3 mM KH_2PO_4 , 0.3 mM NaH_2PO_4 , 1.3 mM CaCl_2 , 0.5 mM MgCl_2 , 0.4 mM MgSO_4 , 5.6 mM glucose and 10 mM HEPES (pH 7.4).

In order to stimulate volume-sensitive Cl^- transport, the osmolarity of the efflux buffer was reduced from 300 to 150 $\text{mOsm}\cdot\text{L}^{-1}$. Cells incubated in efflux buffer containing Na^{125}I (New England Nuclear, Boston, MA, USA; 37 $\text{kBq}\cdot\text{mL}^{-1}$) for 1 h at 37°C were then washed with efflux medium in the presence or absence of 0.3 μM PHE to remove extracellular ^{125}I . Loss of intracellular ^{125}I was determined by removing the medium with the efflux buffer every minute for up to 8 min. The first three aliquots were used to establish a stable baseline in efflux buffer alone. Medium containing the appropriate drug was used for the remaining aliquots. The fraction of the initial intracellular ^{125}I lost at each time-point was determined, and time-dependent rates of ^{125}I efflux were calculated thus:

$$\ln (^{125}\text{I}_{t_1}/^{125}\text{I}_{t_2})/(t_1-t_2) \quad (1)$$

where $^{125}\text{I}_t$ is the intracellular ^{125}I concentration at time t , and t_1 and t_2 are successive time-points. Curves were constructed by plotting the rate of ^{125}I efflux *versus* time. All comparisons were based on the maximal time-dependent rate (k ; in min^{-1}), excluding the points used to establish the baseline ($k_{\text{peak}}-k_{\text{basal}}$).

Statistical analysis

Data are presented as the mean $\pm$ SEM of n observations, or the number of rings for the tension recordings. Sets of data were compared using ANOVA or an unpaired t -test. Differences were considered significant when $p < 0.05$.

RESULTS

Expression of CFTR in IPA

CFTR expression in IPA was revealed using three experimental approaches. First, the presence of CFTR mRNA was detected by reverse transcriptase-PCR (fig. 1a) in intestine, an organ known to express CFTR and used as a positive control for expression of the CFTR (lane 1), and PASMCs (lane 3), but not in rat skeletal muscle (lane 2). Secondly, immunoprecipitation using the anti-CFTR antibody followed by *in vitro* PKA phosphorylation analysis demonstrated that, as in the CHO cell line used as a positive control, mature CFTR was phosphorylated *in vitro* by PKA in cultured PASMCs (fig. 1b). The major CFTR form (band C) was a 175-kDa protein, as determined using molecular mass standards. Controls with nonimmune mouse IgG are also shown (lanes 2 and 4). Thirdly, an immunocytochemical approach was used to determine the presence and location of the CFTR in PASMCs. Cells were stained with the anti-CFTR C-terminus monoclonal antibody and anti-smooth muscle actin antibody, whereas no staining could be detected in control experiments (fig. 2). Taken together, these results demonstrate that the

FIGURE 1. Expression of the cystic fibrosis transmembrane conductance regulator (CFTR) in cultured smooth muscle cells (SMCs) from rat intrapulmonary arteries (IPAs). a) Reverse transcriptase-PCR analysis of CFTR mRNA (403 bp) expression in rat intestine (lane 1), skeletal muscle (lane 2) and isolated pulmonary arterial SMCs (PASMCs; lane 3). Lane 4: no cDNA (water control). The expected size of the β -actin housekeeping gene was 269 bp. CFTR mRNA was expressed in rat intestine and isolated PASMCs but not in skeletal muscle. b) The CFTR (band C) was identified by immunoprecipitation followed by *in vitro* cyclic adenosine monophosphate-dependent protein kinase A phosphorylation in Chinese hamster ovary cells stably expressing wild-type CFTR (lanes 1 (positive control) and 2) and IPA SMCs (lanes 3 and 4). Lanes 2 and 4: CFTR antibody omitted (negative controls). This experiment was performed in duplicate. M: molecular mass standards.

FIGURE 2. Immunolocalisation of the cystic fibrosis transmembrane conductance regulator (CFTR) in cultured smooth muscle cells from rat intrapulmonary arteries. Confocal microscopic images showing: a) smooth muscle α -actin staining (red; Cy3 was used as the secondary antibody); b, c) CFTR staining using anti-CFTR C-terminus monoclonal antibody (green); and c, f) nuclei labelled in blue with TO-PRO-3 iodide. Negative controls (d, e) were obtained by using the secondary antibody without the primary antibody. The merged images (c and f) show both nuclei and CFTR fluorescence. Scale bars=10 μ m.

CFTR is endogenously expressed in IPA SMCs and can be detected as PKA-phosphorylated mature protein.

Analysis of chloride transport in cultured myocytes from IPA

In order to verify that the iodide efflux method can be applied to IPA cultured cells, the cells were first exposed to hypo-osmotic bath solution (150 mOsm·L⁻¹) to stimulate the endogenous $I_{Cl,swell}$ (fig. 3a). The cAMP agonists vasoactive intestinal peptide (500 nM) and forskolin (LC laboratory, PKC Pharmaceuticals, Inc., Woburn, MA, USA; 10 μ M) plus the isoflavone genistein (30 μ M) and agents that raise $[Ca^{2+}]_i$, such as the Ca^{2+} ionophore A23187 (1 μ M) and ATP (100 μ M), significantly stimulated iodide efflux ($p < 0.001$; $n = 4$), demonstrating the functional presence of multiple Cl^- transporters dependent on cAMP, calcium and/or cell volume in rat IPA (fig. 3).

Inhibitors of cAMP-dependent chloride transport in cultured intrapulmonary arterial myocytes

Since the CFTR is activated by cAMP, it was hypothesised that the cAMP-dependent forskolin/genistein-activated Cl^- transport would be supported by the CFTR. Glibenclamide and diphenylamine-2-carboxylic acid (DPC) are two nonspecific

inhibitors of Cl^- channels, including the CFTR [23], the stilbene derivative 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS) is a nonspecific blocker of Cl^- channels but does not inhibit the CFTR from the extracellular side of the plasma membrane [23], and calixarene is an inhibitor of outwardly rectifying Cl^- channels but not of the CFTR [23, 24]. Forskolin/genistein-dependent iodide efflux was fully inhibited by 100 μ M glibenclamide and 500 μ M DPC, but by neither 100 nM calixarene nor 500 μ M DIDS ($n = 4$ for each; fig. 4). CFTRinh-172 (Calbiochem, VWR International, Fontenay-sous-Bois, France; 10 μ M), a specific CFTR blocker [9], also strongly inhibited the iodide efflux response to forskolin/genistein (fig. 4), suggesting that the CFTR is likely to be responsible for the forskolin/genistein-activated Cl^- transport in cultured IPA cells.

Role of CFTR in intrapulmonary arterial vasorelaxation

Since cAMP-related agonists induce pulmonary vasorelaxation [25, 26], the effect of CFTR activators were investigated in rat IPA rings precontracted with 0.3 μ M PHE. MPB-07 and -91 are good specific CFTR activators, whereas MPB-80 is a very poor CFTR activator but a useful control [6, 7, 21]. Unlike MPB-80, MPB-07 and MPB-91 induced strong vasorelaxation in IPA rings whether endothelium-denuded or not (figs 5 and 6b and figs 5 and 6a, respectively).

FIGURE 3. Analysis of chloride transport in cultured rat intrapulmonary arterial smooth muscle cells. Stimulation of iodide efflux with time for: a) a hypo-osmotic solution (HOS) (150 mOsm·L⁻¹; ■); and b) calcium-related compounds (▲: 1 μM A23187; △: 100 μM adenosine triphosphate (ATP)) and cyclic adenosine monophosphate agonists (▼: 10 μM forskolin (FSK) plus 30 μM genistein (GEN); ◇: 500 nM vasoactive intestinal peptide (VIP)). Horizontal bars represent period during which drugs were present in bath (○: iso-osmotic vehicle alone). c) Maximal time-dependent rate (k ; from a and b). Data are presented as mean $\pm$ SEM ($n=4$ for each) with the basal rate subtracted from the peak rate ($k_{\text{peak}} - k_{\text{basal}}$). NS: nonsignificant. **: $p < 0.01$; ***: $p < 0.001$ versus basal.

FIGURE 4. Pharmacological inhibition of iodide efflux stimulated by forskolin plus genistein. Cultured smooth muscle cells were stimulated by cAMP agonists (10 μM forskolin plus 30 μM genistein) in the presence or absence of 100 nM calixarene (Calix), 500 μM 4,4'-diisothiocyanostilbene-2,2'-disulphonate (DIDS), 500 μM diphenylamine-2-carboxylic acid (DPC), 10 μM CFTR inhibitor (CFTRinh-172 or 100 μM glibenclamide (Glib). Data are presented as mean $\pm$ SEM ($n=4$ for each set of experiments) with the basal time dependent rate subtracted from the peak rate ($k_{\text{peak}} - k_{\text{basal}}$). Basal: vehicle alone; NS: nonsignificant. *: $p < 0.05$; **: $p < 0.01$.

In the presence of 500 μM DIDS, a Cl_iCa blocker [11] that had no effect on the cAMP-dependent Cl^- transport (fig. 4), 200 μM MPB-07 still induced strong relaxation ($59.9 \pm 4.5\%$ of original precontraction with PHE ($n=15$); data not shown), indicating that the effect of MPB is mainly independent of Cl_iCa activity.

Finally, in cultured rat IPA myocytes pretreated with 0.3 μM PHE, 250 μM MPB-07 and MPB-91 stimulated iodide efflux ($n=4$; fig. 7), whereas MPB-80 induced low CFTR activation (fig. 7b). As with forskolin/genistein, the iodide efflux stimulated by MPB-91 was insensitive to 100 nM calixarene and 500 μM DIDS, but was fully inhibited in the presence of 100 μM glibenclamide, 500 μM DPC (data not shown) and 10 μM CFTRinh-172 (fig. 7). CFTRinh-172 also inhibited the response to MPB-07 and MPB-80 (fig. 7b).

Taken together, the present results show that activation of the CFTR induces endothelium-independent vasorelaxation in rat IPAs, suggesting a potential and unexpected role of the CFTR in pulmonary vascular tone.

DISCUSSION

The current report presents evidence that a functional CFTR is endogenously expressed in IPA smooth muscle. First, it was shown that agonists of the cAMP pathway, such as forskolin, stimulated CFTR Cl^- channel activity. Secondly, the pharmacology of the CFTR is very similar to that of the epithelial CFTR, in terms of both activation (using MPB derivatives and genistein) and inhibition (using glibenclamide, DPC and CFTRinh-172). Moreover, the structural and pharmacological specificity of MPBs (*i.e.* the different activity of MPB-80, MPB-07 and MPB-91) are similar in IPA cells from rat and mouse aortas [6, 7] and in epithelia [21]. Thirdly, activation of the

FIGURE 5. Role of the endothelium in the mechanical effect of cystic fibrosis transmembrane conductance regulator activators in rat intrapulmonary arteries (IPAs) in the presence (a) and absence (b) of the endothelium. Typical traces showing isometric tension measurements as a function of time in IPA rings precontracted with 0.3 μM phenylephrine (PHE). Bath application of 10 μM carbamylcholine (Carb) demonstrated the presence or otherwise of the endothelium. After washout (W), the tension returned to basal levels and 0.3 μM PHE was again added. The effect of stepwise increases in the benzoquinolizinium derivatives MPB-07 (a) and -91 (b) concentration were determined in these precontracted vessels (figures above vertical arrows indicate micromolar concentrations). Horizontal bars indicate presence of benzoquinolizinium derivative.

CFTR in IPA leads to endothelium-independent vasorelaxation. The present study is the first to show the presence and function of CFTR channels in primary cultured PASMCS and their implications for pulmonary vasorelaxation.

The presence of ICl_{Ca} and ICl_{swell} in rat PASMCS was also confirmed, and the use of cultured IPA SMCs to study Cl^- transport consequently validated.

The presence of functional cAMP-dependent Cl^- channels has previously been suggested in bovine pulmonary arterial smooth muscle by the use of rather nonspecific Cl^- channel blockers, such as phenylanthranilic acid, 9-anthracene carboxylic acid, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) and DIDS, on cAMP-dependent responses induced by 5-hydroxytryptamine [17, 27]. The molecular origin of this

FIGURE 6. Concentration-dependent vasorelaxation of cystic fibrosis transmembrane conductance regulator activators (●: the benzoquinolizinium derivative MPB-07; ■: MPB-91; ▲: MPB-80) in rat intrapulmonary arteries in: a) the presence; and b) the absence of endothelium. Data are presented as mean $\pm$ SEM ($n=9-15$ rings (a) and 8-14 rings (b)) percentage of original precontraction with 0.3 μM phenylephrine. **: $p < 0.01$; ***: $p < 0.001$.

channel has not been identified, although the cAMP-related 5-hydroxytryptamine responses were inhibited by phenylanthranilic acid and 9-anthracene carboxylic acid but not by DIDS and NPPB. The CFTR is insensitive to DIDS [23], and this result was confirmed in the present study, suggesting that the channel observed in rat IPA may be similar to that observed in bovine pulmonary arterial smooth muscle [17]. The pharmacological profile of CFTR activation in IPA using MPB activators (MPB-91 > MPB-07 > MPB-80) studied with the iodide efflux and isometric tension techniques (in the present report) is similar to that previously obtained in rat or mouse aortic myocytes [6, 7], epithelial cells [21] and tracheal myocytes [5]. Moreover, endothelium removal did not influence pulmonary arterial vasorelaxation in response to MPBs, a result in agreement with that previously reported for rat and mouse aorta [6, 7]. Finally, the activation of cAMP- and MPB-dependent iodide efflux was fully inhibited by the CFTR-specific inhibitor CFTRinh-172 in IPA, as in epithelial cells [9] and aortic SMCs [6]. All of these results contributed to the conclusion that the CFTR, as a cAMP-dependent Cl^- channel expressed in PASMCS, is involved in endothelium-independent pulmonary vasorelaxation.

FIGURE 7. Effect of benzoquinolizinium derivative activators of the cystic fibrosis transmembrane conductance regulator (CFTR) on iodide efflux. a) Iodide efflux evoked by 250 μ M MPB-91 in the presence (□) or absence (■) of 10 μ M CFTR inhibitor (CFTRinh)-172 as a function of time. Horizontal bar indicates bath application of the drug. b) Effect of 10 μ M CFTRinh-172 on the MPB-stimulated efflux in the absence (□) or presence (■) of CFTRinh-172. All experiments were performed in the presence of 0.3 μ M phenylephrine. Data are presented as mean $\pm$ SEM ($n=4$ for each) with the basal time dependent rate subtracted from the peak rate ($k_{\text{peak}}-k_{\text{basal}}$). *: $p<0.05$; ***: $p<0.001$ versus absence of CFTRinh-172 (unpaired t-test).

In epithelial cells, cAMP agonists stimulate transepithelial Cl^- transport *via* phosphorylation and opening of apical CFTR channels [1, 28]. In SMCs from the systemic circulation, *i.e.* aortas [6, 7], or pulmonary circulation (present study), activation of the CFTR was evidenced after precontraction of the muscle cells. These observations are consistent with previous studies showing that cAMP is implicated in the relaxation of vascular SMCs in response to vasodilators such as β -adrenergic agonists or vasoactive intestinal peptide [25, 26]. Since the current study demonstrated the presence of the CFTR in IPA, it may explain, at least in part, why an increase in cAMP concentration would induce pulmonary vasorelaxation *via* activation of the CFTR.

It is noteworthy that the present study was conducted in IPAs, which thus exhibit a functional CFTR. It is tempting to hypothesise that altered CFTR function in pulmonary arteries may be linked to the development of pulmonary hypertension

since: 1) IPAs, compared with extrapulmonary arteries, are particularly sensitive to hypoxia and thus strongly involved in the pathogenesis of pulmonary hypertension; 2) patients affected by the genetic disease cystic fibrosis also develop some respiratory diseases, eventually leading to pulmonary hypertension; and 3) some Cl^- channels are involved in pulmonary hypertension (see Introduction section). Although this is beyond the scope of the present study, further investigations are required to explore the expression and function of the CFTR in pulmonary arteries from pulmonary hypertensive animals.

In summary, the current report presents the first time direct evidence of functional cystic fibrosis transmembrane conductance regulator expression in rat intrapulmonary arterial primary cultured smooth muscle cells. In a more integrated model, such as intrapulmonary arterial rings, activation of the cystic fibrosis transmembrane conductance regulator induces endothelium-independent vasorelaxation, indicating a potential role of cystic fibrosis transmembrane conductance regulator in the regulation of pulmonary vascular tone. An interesting new therapeutic development could arise from the discovery that cystic fibrosis transmembrane conductance regulator activators are able to relax pulmonary arterial smooth muscle cells, thus being potential anti-hypertensive agents.

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