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Biochemical evidence for ATPase activity in CFTR-enriched apical membrane vesicles from tracheal epithelium

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Abstract

In apical membrane vesicles from beef tracheal epithelia expressing up to 30% of the proteins as functional cystic fibrosis transmembrane conductance regulator (CFTR) – i.e. a voltage-independent and PKA-sensitive $^{36}Cl^-$ flux – an ATPase activity, different from P, F_0F_1 and V types, was reproducibly detected. Its specific activity averaged 20 μmol Pi h^{-1} mg^{-1} with an apparent affinity for ATP of 530 ± 30 μM. Its possible involvement in CFTR functions was supported by (1) the linear relationship between the ATPase activity and the magnitude of $^{36}Cl^-$ fluxes (turnover rate: 3 ATP hydrolyzed per CFTR per second), (2) the same rank of potency of ATP, ITP, GTP, UTP and CTP to be hydrolyzed and to open CFTR chloride channels, (3) the similar and parallel inhibition of the ATPase and CFTR Cl^- fluxes by NS004 (IC_{50}: 60 μM) and (4) the potency of anti-R domain antibodies to increase by 18% the ATPase activity.

Keywords: Cystic fibrosis; Cl^- channel; Cystic fibrosis transmembrane conductance regulator; NS004; ATP hydrolysis; Inhibition

1. Introduction

Cystic fibrosis (CF) is an inherited disease involving abnormalities of fluid and electrolyte transport in secretory epithelia [1]. This disorder resides in a mutated CF gene [2] which encodes the cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-regulated Cl^- channel of low and linear conductance (7 pS) located in apical cell membranes [3] where it regulates transepithelial Cl^- fluxes and other membrane channels like outwardly rectified chloride channels (ORCC) [4], amiloride-sensitive Na^+ channels [5,6] or K^+ channels [7,8].

The ATP binding cassette (ABC) protein [9] CFTR consists of two membrane-spanning domains involved in an ion-conductive pore, two cytoplasmic nucleotide binding domains (NBFs) and a regulatory R domain containing consensus phosphorylation sites for cAMP-dependent protein kinase (PKA) and Ca^{2+}-dependent protein kinase [10,11]. Most of

Abbreviations: ABC, ATP binding cassette; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); CFTR, cystic fibrosis transmembrane conductance regulator; DCCD, *N*,*N*′-dicyclohexyl-carbodiimide; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; ECL, enhanced chemiluminescence; IC_{50}, drug concentration leading to 50% inhibition; LDH, lactate dehydrogenase; NS004, 5-trifluoromethyl-1-(5-chloro-2-hydroxyphenyl)1,3-dihydro-2*H*-benzimidazol-2-one; ORCC, outwardly rectified chloride channels; PK, pyruvate kinase; PKA, cAMP-dependent protein kinase; PKI, protein kinase inhibitor; Pi, inorganic phosphate; pNPP, *para*-nitrophenyl phosphate; SDS, sodium dodecyl sulfate

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the mutations of CFTR are located in R and NBF domains.

For gating, CFTR requires phosphorylation on the regulatory domain by protein kinases and ATP binding and/or hydrolysis on NBFs [12,13]. Since CFTR channels passively conduct Cl^- ions down their electrochemical gradient, no energy would be required. This leads to controversial concepts about ATP hydrolysis for CFTR function [12–14].

Electrophysiological studies on CFTR regulation by ATP provided a model for CFTR gating in which ATP binding and hydrolysis on the NBFs open and close the chloride channel [13,15–17]. A low ATPase activity on the maltose binding protein–NBF_1 fusion protein has been previously described [18].

Li and coworkers [19] have reported that purified, reconstituted human CFTR was able to hydrolyze ATP. This low activity (ca. 3 μmol h^{-1} mg^{-1}) was Mg^{2+}-dependent and reacted differently towards ATP when CFTR was phosphorylated via PKA. The authors reported that CFTR channel and ATPase activity were strongly inhibited by 1 mM and 5 mM sodium azide, respectively. However, the time scales differed widely, the ATPase activity was assayed for 4–5 h and 5 mM sodium azide was necessary to inhibit it whereas 1 mM NaN_3 inhibited the CFTR channel within 90 s (see Fig. 4b in [19]).

We have previously shown [20] that voltage-independent and PKA-sensitive $^{36}Cl^-$ fluxes due to functional CFTR could be detected in inside-out apical membrane vesicles in which it represented up to 30% of the total proteins [20]. In these membrane vesicles purified from bovine tracheal epithelium, an ATPase activity was reproducibly detected. This activity was insensitive to vanadate, bafilomycin and oligomycin (i.e. different from P-, F_0F_1- and V-type ATPases, respectively). This activity was found to vary with the amplitudes of the CFTR-mediated chloride uptakes and to exhibit the same sensitivities to nucleotide triphosphates as CFTR did. The substituted benzimidazolone 5-trifluoromethyl-1-(5-chloro-2-hydroxyphenyl)1,3-dihydro-2*H*-benzimidazol-2-one (NS004), previously described as a general activator of both chloride and calcium-activated potassium channels [21,22], was found in our hands to inhibit several P-type ATPases (rat brain Na/K-ATPase $IC_{50} = 60$ μM and sarcoplasmic Ca^{2+}-ATPase $IC_{50} = 9$ μM). As a consequence, if NS004 could act either directly or indirectly on both chloride channels and ATP catalytic sites, it would represent an appropriate tool to determine a possible correlation between the ATPase activity in the preparation and the CFTR-linked chloride flux: NS004 exerted similar effects on both ATP hydrolysis and CFTR chloride flux. Antibodies reacting with the R domain of human CFTR increased by 18% the ATPase activity. This biochemical evidence argues in favor of an ATPase activity related to CFTR chloride channel gating.

2. Materials and methods

2.1. Apical membrane isolation procedure

Apical membrane vesicles of inside-out orientation were isolated from beef trachea epithelial cells as described by Dilda and Lelièvre [20]. In brief, fresh beef tracheas collected from adult animals were split and incubated for 15 min at 4°C and bubbled with air in a calcium-free buffer containing (mM) 250 sucrose, 2 ethylene glycol bis(b-aminoethyl ether)-*N*,*N*′tetraacetic acid, 1 dithiothreitol (DTT) and 40 imidazole–HCl, pH 7.8. Epithelial cells scraped off the tracheas were collected in 20 ml of (mM) 130 KCl, 250 sucrose, 1 DTT, 40 imidazole–HCl buffer pH 7.4 and then centrifuged at $800 \times g$ for 10 min. All fractionation procedures were carried out at 4°C in the presence of (μM) 15 aprotinin, 0.15 leupeptin, 1 pepstatin and 100 phenylmethylsulfonyl fluoride. The homogenized fraction (4.6 ± 0.6 mg ml^{-1}) was obtained with a Polytron PT10 (two bursts of 2 s at half-maximal speed) in 3 ml of (mM) 40 KCl, 1 DTT, 0.25 $MgCl_2$ and 40 imidazole–HCl buffer pH 7.4 and was gently stirred for 20 min at 4°C. Isotonicity was restored by adding appropriate amounts of concentrated solutions of (mM) 130 KCl, 1 DTT, 0.25 $MgCl_2$, 250 sucrose and 40 imidazole–HCl buffer pH 7.4. The $800 \times g$, 10 min supernatant was successively centrifuged at $8000 \times g$ for 15 min and at $31\,000 \times g$ for 40 min. The last pellet was resuspended in 100 mM mannitol and 40 mM imidazole–HCl buffer pH 7.4. Apical membrane vesicles were separated from basolateral ones by the magnesium precipitation procedure [20] carried out in the presence of imidazole–HCl instead of HEPES/Tris buffers and

at a final concentration of 2 mM ethylenediamine tetraacetic acid (EDTA). They were resuspended in (mM) 106 KCl, 6 $MgCl_2$, 4 NaCl and 40 imidazole–HCl, buffer pH 7.4, stored at −80°C and used within 1 week. About 0.5% of the initial proteins were recovered in the apical membrane fraction. Protein contents were measured as described by Lowry et al. [23].

2.2. ^{36}Chloride uptake

Chloride uptake through conductive pathways was evaluated by $^{36}Cl^-$ influx driven by a transmembrane Cl^- gradient [20]. To measure CFTR $^{36}Cl^-$ uptake, membrane vesicles were first phosphorylated during a 30 min preincubation with 300 nM PKA, 1 mM ATP and 1 mM adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS) at 37°C within the KCl buffer: (mM) 106 KCl, 6 $MgCl_2$, 4 NaCl, 40 imidazole–HCl, pH 7.4. ATPγS, a non-hydrolyzable ATP analog, was found to prolonged the CFTR chloride channel opening [16]. Therefore, we considered the CFTR chloride influx as maximal under these conditions. 12 μl (30 ± 6 μg of protein) of these vesicles were mixed at 37°C with 108 μl of gluconate buffer containing (mM): 106 K^+-gluconate, 6 Mg-(gluconate)$_2$, 4 $Na^{36}Cl$ (0.5 μCi $mmol^{-1}$) and 40 imidazole/gluconate, pH 7.4. After 2 min at 37°C, 100 μl aliquots were filtered on GF/C filters, washed with 2 ml of an ice-cold buffer (in mM: 250 sucrose, 5 NaN_3, 10 HEPES/Tris, pH 7.4) and counted in a Beckman LS-6000-IC liquid scintillation counter.

Fluxes measured with both PKA-phosphorylated membranes and valinomycin (4 μM) represented voltage-independent $^{36}Cl^-$ uptakes, i.e. those due to CFTR [20,24]. Membrane-bound radioactivity, measured after addition of 0.5% (v/v) Tween 20 to permeabilize the vesicles, was subtracted from total fluxes.

Competition experiments, in terms of Cl^- fluxes, between NS004 and ATP were carried out with final ATP concentrations varying from 1 to 10 mM and an optimal ATP/Mg^{2+} ratio of 1 to 6.

2.3. ATP hydrolytic activity

This was systematically assayed under all the experimental conditions used to measure the different $^{36}Cl^-$ movements in the medium: with or without PKA-induced phosphorylation and in the presence or absence of valinomycin (see above). No ATPase activity could be detected in Triton X-100-treated vesicles. The ionic conditions were identical to those used for $^{36}Cl^-$ fluxes, i.e. in 1 ml of a buffer containing (mM): 106 K^+-gluconate, 6 Mg^{2+}-(gluconate)$_2$, 4 NaCl, 1 ATP, 40 imidazole/gluconate, pH 7, 4.5 ± 1 μg membrane proteins plus an ATP-regenerating medium: pyruvate kinase (PK) (7 U ml^{-1}), lactate dehydrogenase (LDH) (10 U ml^{-1}), 0.4 NADH and 2 phospho-(enol)pyruvate. Addition of protein kinase inhibitor (PKI, H8 = 1 μg ml^{-1}) completely inhibited the residual PKA (final concentration: 0.6 nM) activity in the ATPase assay medium. Note that PKI alone did not affect the rate of ATP hydrolysis. The ADP formation coupled to the PK plus LDH activities was continuously recorded at 37°C by following NADH oxidation at 340 nm for 10 min in a Cary DMS 70 spectrophotometer. All the results were obtained from experiments in which enzyme activity was linear vs. time, i.e. from 0.5 to 10 min.

Apparent affinity for ATP and putative competition between NS004 and ATP were assayed with final ATP concentrations varying from 0.25 to 10 mM and an ATP/Mg^{2+} ratio of 1 to 6.

Aliquots of membrane vesicles were preincubated for 15 min at 37°C with drug solutions at final concentrations varying from 1 to 500 μM in different solvents (final concentrations: 0.5% (v/v) dimethyl sulfoxide (DMSO) or ethanol). The specific activity in the presence of drugs was calculated as the percentage of the total hydrolytic activity in the presence of solvent.

Polyclonal purified antibodies, PATG 1615 from Transgene (67 μg ml^{-1} as measured by Bio-Rad assay, dilution 1/100), reacting with the human CFTR amino acids 589–830 (R domain) and antibodies directed against the Na^+/K^+-ATPase α subunit (dilution 1/100) were incubated for 2 h at 37°C with apical membrane aliquots in a pH 8 Tris buffer, before ATPase assay.

2.4. Substrate specificity and pH dependence

Since PK and LDH used in the ATPase assays were highly sensitive to pH variations and to vanadate and ADP levels in the media, the coupled en-

zyme system was not used in these particular experiments. The substrate specificity and pH dependence of the hydrolytic activity were tested by monitoring inorganic phosphate (Pi) release at 37°C in a reaction medium (final volume 2.5 ml) containing (mM) 106 K^+-gluconate, 0.25–6 nucleotide triphosphates (sodium salts), 1.5–36 Mg^{2+}-$(gluconate)_2$, 4 NaCl, 0.2 orthovanadate and 40 imidazole/gluconate adjusted to the desired pH. The reaction was initiated by adding approximately 25 μg proteins. Aliquots (0.5 ml) were taken every 30 s and immediately mixed with 500 μl of Misson's reagent (2% (w/v) ammonium molybdate, 0.2% (v/v) NH_3, 0.2% (w/v) ammonium metavanadate and 13.5% (v/v) nitric acid) [25]. After 10 min at room temperature, membranes were discarded by centrifugation (10 000 × *g* for 5 min) and the optical density was measured at 470 nm. The release of Pi was calculated by comparison with a standard curve obtained with 75–750 μM KH_2PO_4 in the assay medium. The enzyme activity tested was linear versus time, from 0.5 to 2.5 min. The substrate specificity was expressed as the V_m to K_m ratio.

2.5. Alkaline phosphatase assay

This activity was determined at 20°C by continuously recording for 10 min *para*-nitrophenyl (pNP) formation at 405 nm in a medium containing (mM): 5 $MgCl_2$, 1 *para*-nitrophenyl phosphate (pNPP), 100 Tris–HCl pH 9.0, 0.1% (w/v) Triton X-100 and 6 μg of membrane proteins in a final volume of 1 ml. To test the NS004 effects, membrane preparations were preincubated for 10 min at 20°C before the assay with NS004 doses varying from 1 to 500 μM. The same NS004 levels were also present in the assay media.

2.6. Polyacrylamide gel electrophoresis (PAGE) and immunological characterization

Gel electrophoresis were performed using the procedure of Laemmli [26]. The samples, treated with solubilization buffer (for details, see legends) were resolved on a 8% polyacrylamide gel. After sodium dodecyl sulfate (SDS)–PAGE, proteins were electrophoretically transferred to nitrocellulose sheets [27]. Saturation of the blots were performed by overnight incubation at 4°C in a medium containing 5% fat-free milk. Then nitrocellulose sheets were incubated for 1 h at 4°C with the primary antibodies PATG 1615 (1/200). Blots were incubated with biotinylated anti-IgG followed by horseradish peroxidase-conjugated streptavidin. Visualization was achieved by treatment with enhanced chemiluminescence (ECL) reagents (Amersham France SA).

2.7. Reagents

NS004 was solubilized in DMSO, *N*,*N*′-dicyclohexyl-carbodiimide (DCCD) and digoxigenin in ethanol; ouabain, KNO_3, KSCN, levamisole, oligomycin, bafilomycin and KCN in bidistilled water. All the reagents and drugs were obtained from Sigma. Two lots of NS004 were used (generous gifts of Dr. V.K. Gribkoff). Polyclonal antibodies PATG 1615 were obtained from Dr. Pavirani (Transgene). Polyclonal antibodies against the α subunit of Na^+/K^+-ATPase were a generous gift of Dr. J. Ball, Department of Pharmacology and Cell Biophysics, University of Cincinnati.

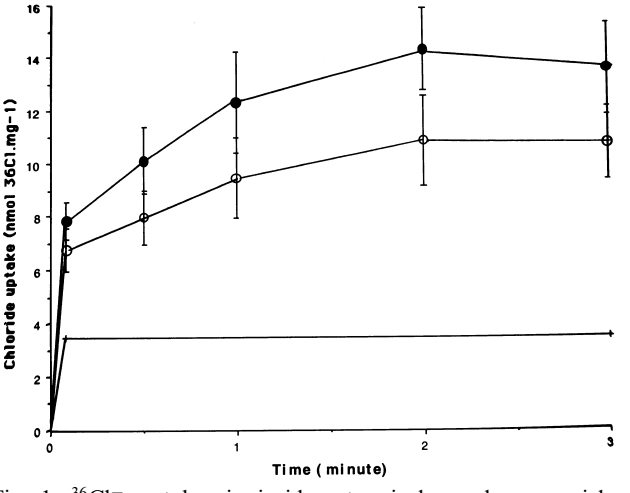

Fig. 1. $^{36}Cl^-$ uptakes in inside-out apical membrane vesicles. $^{36}Cl^-$ fluxes were carried out at 37°C, in the presence of 100 μM bumetanide with (○) or without (●) 4 μM valinomycin. Membrane-bound $^{36}Cl^-$ (+) 3.6 ± 0.2 nmol mg^{-1} was measured in the presence of the non-ionic detergent Tween-20 (0.5% w/v). Each point is the mean ± S.D. of triplicate assays carried out with five different preparations.

3. Results

3.1. Characterization of the $^{36}Cl^-$ uptakes due to CFTR

The $^{36}Cl^-$ uptakes remained stable in apical membrane preparations and could be accurately measured within 1 week after membrane isolation. The chloride flux due to CFTR being voltage-insensitive and PKA-stimulated [24], it was measured in the presence of both valinomycin, which clamps the transmembrane voltage [24] and cancels the ORCC, and bumetanide (100 μM), which inhibits the K/Na-dependent $^{36}Cl^-$ uptakes [28] from putative contaminations by basolateral membranes. These CFTR-specific chloride fluxes were in the range of 11.26 ± 1.25 nmol mg^{-1} per 2 min, consistent with values already reported in the same tissue [29]. The initial rates of $^{36}Cl^-$ uptakes, measured at 37°C within 5 s in the presence of bumetanide (100μM) with or without valinomycin, were 1.86 ± 0.19 and 1.51 ± 0.11 nmol ^{36}Cl mg^{-1}, respectively ($n = 11$). After phosphorylation of the membrane vesicles by PKA plus ATP and ATPγS, the initial rates of CFTR chloride

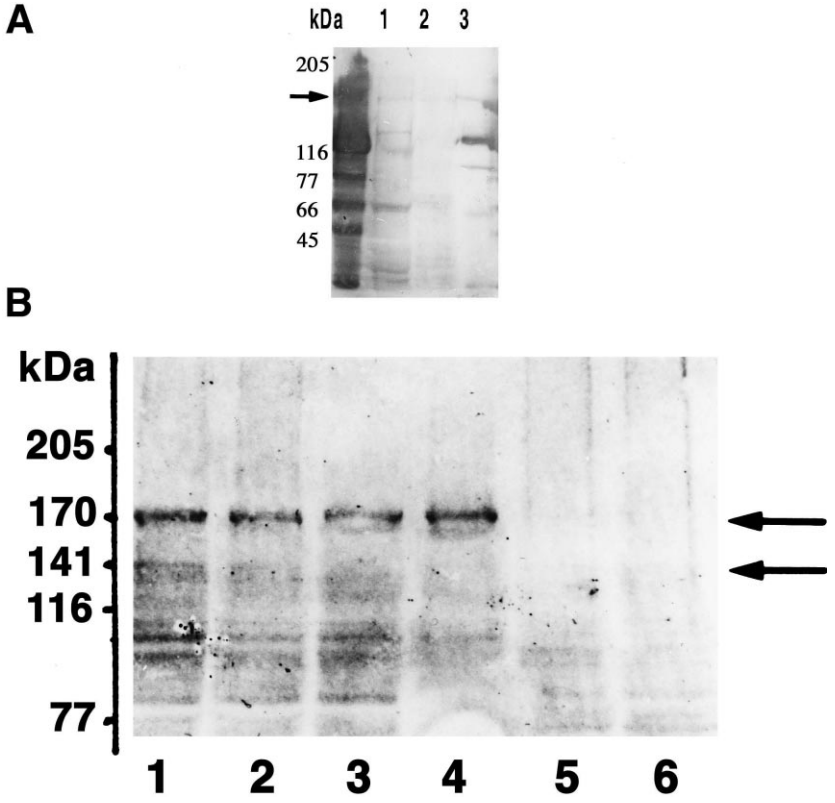

Fig. 2. (A) Immunodetection of bovine CFTR in apical membrane vesicles. Each sample was diluted in DTT (20 mM), SDS 10% (w/v), glycerol 10% (w/v) and 0.5 M Tris–HCl pH 6.8 before electrophoresis. Lane 1: standards: myosin (205 kDa), β-galactosidase (116.5 kDa), bovine albumin serum (77 kDa), albumin (66 kDa) and ovalbumin (46.5 kDa); lane 2: apical membrane proteins (50 μg); lane 3: basolateral membrane proteins (50 μg); lane 4: control β-galactosidase. This control was carried out because the mouse polyclonal antibodies (PATG 1615 from Transgene) were raised against the fusion protein β-galactosidase and the R domain of human CFTR. Bound antibodies (used at a 1/200 dilution) were detected by the biotin-conjugated second antibody in the presence of streptavidin and ECL reagents. The polyclonal antibodies against fusion proteins of β-galactosidase and the R domain of human CFTR revealed glycosylated CFTR (170 kDa) among other bands of immunoreactivity due to cross-reactions with either altered or non-mature CFTR and/or β-galactosidase. (B) SDS–PAGE of microsomes, apical and basolateral membranes. All samples were solubilized with an equal volume of 2% β-mercaptoethanol, 2% SDS, 10% glycerol, 1 mM EDTA and 0.5 M Tris–HCl, pH 6.8, before electrophoresis on a 6–12% linear gradient SDS polyacrylamide gel. Lanes 1 and 2: microsomes (75 μg and 100 μg); lanes 3 and 4: apical membranes (75 μg and 100 μg); lanes 5 and 6: basolateral membranes (75 and 100 μg).

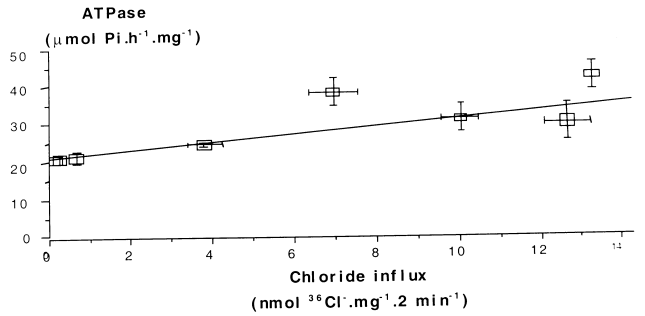

Fig. 3. Relationship between $^{36}Cl^-$ fluxes due to CFTR and ATPase activities. $^{36}Cl^-$ influx and ATPase activities were tested in the same PKA-phosphorylated vesicles and in the presence of valinomycin, i.e. functional CFTR in vitro. Assuming a linear relationship between ATPase activities and $^{36}Cl^-$ influx, the correlation range is 1. Seven membrane preparations are reported here, each experiment was carried out in septuplicate for $^{36}Cl^-$ flux and in triplicate for ATPase activity.

fluxes reached 4.86 ± 1.48 nmol ^{36}Cl mg^{-1} (Fig. 1). ATP or ATPγS alone failed to activate the chloride channel.

As shown in Fig. 2A, the fully glycosylated form of bovine CFTR (170 kDa) was present in the apical membranes in which the specific chloride uptakes were measured. However, note that CFTR was also detected in the basolateral membranes (lane 3) confirming a slight contamination of these latter by apical membranes [20]. A semiquantitative analysis by densitometry of the 170 kDa bands as revealed by SDS–PAGE (Fig. 2B) showed that between 116 and 210 kDa, the 170 kDa band represents about 30 and 4% of the total proteins in apical and basolateral membranes, respectively.

Inhibitors of voltage-sensitive chloride channels, anthracene 9-carboxylic acid, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid and 5-nitro-2-(3-phenylpropylamino) benzoic acid (up to 500 μM each), failed to inhibit the activatable Cl^- channel and did not affect the total chloride uptakes by more than 20%.

3.2. Correlation between $^{36}Cl^-$ transport capacities and ATPase activities

In all the apical membrane preparations tested, an ATPase activity could be detected (Fig. 3). The only experimental condition under which the ATPase activities paralleled $^{36}Cl^-$ flux amplitudes in the same preparations was when membranes were PKA-phosphorylated and studied in the presence of valinomycin, that is when CFTR was functional in vitro.

In fact, the ATPase activity measured here consisted of two components, one which paralleled the chloride flux amplitudes and one which was present even in the absence of chloride movements. Indeed, as shown in Fig. 3, control experiments with two sets of apical membranes kept for 3 weeks at −80°C showed that these vesicles exhibited (i) very low chloride fluxes – less than 1 nmol mg^{-1} per 2 min instead of 7–13 in the same freshly prepared membranes, and (ii) a constant ATPase activity in the range of 20 μmol Pi h^{-1} mg^{-1}, that is independent of the CFTR-specific chloride flux.

3.3. Characterization of the ATP hydrolytic activity by its affinity for ATP

As shown in Fig. 4, the Mg^{2+}-dependent ATP hydrolysis exhibited a Michaelis–Menten dependence

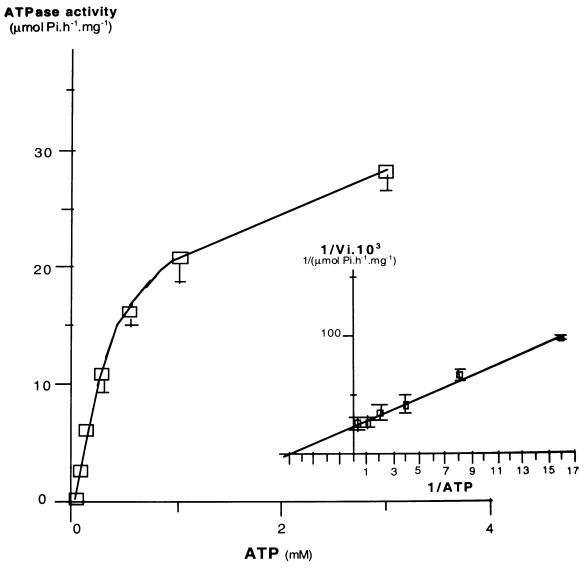

Fig. 4. ATP hydrolysis in apical membrane vesicles phosphorylated with 300 nM PKA (see Section 2). The ATP hydrolysis was measured in the presence of valinomycin in two PKA-phosphorylated membrane preparations exhibiting Cl^- fluxes of 6.8 and 13.4 nmol mg^{-1} per 2 min, respectively. Results depicted in this figure are means ± S.D. of three experiments with the former membrane preparation. A similar pattern was observed with the second vesicle population. (Inset) Lineweaver–Burk plot of ATP hydrolysis. The linear regression was drawn using the least squares method.

for ATP between 0 and 3 mM with a maximal rate of ATP hydrolysis at pH 7.4 of 22.1 ± 2.6 μmol Pi h^{-1} mg^{-1} proteins ($n = 2$) and a Michaelis constant (K_m) of 530 ± 30 μM ($n = 2$). The Hill coefficient calculated as $n_h = \log[ATP]/\log(V/(V_m - V))$ was 1. The maximal rate of ATP hydrolysis by the membrane vesicles was reached at pH 7.4 which is the pH at which $^{36}Cl^-$ fluxes were evaluated. The ATP analogs (1 mM each) adenosine 5′-(β,γ-methylene) triphosphate and adenosine 5′-(β,γ-imino) triphosphate were not hydrolyzed whereas adenosine 5′-(α,β-methylene) triphosphate containing a methyl group between the α and β phosphates was hydrolyzed ($V_{max} = 24.6$ μmol Pi h^{-1} mg^{-1}). ADP was not hydrolyzed by these apical vesicles.

3.4. Characterization of the ATPase activity by its sensitivity to different drugs

Several specific reagents were tested to determine whether this ATP hydrolytic activity could be related to well-known ATPases, kinases or phosphatases. The activity was insensitive to ouabain (2 mM) and digoxigenin (100 μM), two specific Na^+/K^+-ATPase inhibitors, to the PKI H8 (100 μM), to levamisole (1 mM) and orthovanadate (up to 200 μM) which are inhibitors of both phosphatases and P-type ATPases. The mitochondrial F_0F_1 ATPase inhibitors oligomycin (100 μM) and KCN (2 mM) had no effect on ATP hydrolysis. Sodium azide (10 mM) at 37°C inhibited one fourth of the PKA-sensitive chloride uptake within the first minute but did not affect the ATPase activity. Bafilomycin (20 μM), a vacuolar H^+-ATPase inhibitor, KNO_3 (500 μM) and KSCN (500 μM), V-type ATPase inhibitors, had no effect.

The H^+-ATP synthase inhibitor DCCD (100 μM) 30% inhibited the ATPase activity. Pi and PPi had similar effects when preincubated for 15 min at 37°C with the vesicles: 20 and 25% inhibition with 300 μM Pi and 1 mM PPi, respectively ($n = 3$). ADP led to a partial (30%) inhibition. Glibenclamide (500 μM) 30% inhibited the ATPase activity. This ATP hydrolysis was Mg^{2+}-dependent since a 30 min preincubation with 2.5 and 10 mM EDTA inhibited 60 and 100% of the total activity, respectively. However, Mg^{2+} chelation by EDTA did not significantly modify the PKA-sensitive chloride uptakes (data not shown). On the other hand, the CFTR chloride channel inhibitor *N*-phenylanthranilic acid (20% inhibition with 200 μM) did not affect the ATPase activity.

3.5. Substrate specificity of the ATPase

The substrate specificity, as evaluated by the K_m/V_m values, was studied with ATP, GTP, ITP, UTP and CTP. As shown in Table 1, this substrate specificity was: ATP > ITP = GTP > UTP ≫ CTP under our experimental conditions. According to the literature [12], the rank of potency of these nucleotide triphosphates (1 mM each) to open the phosphorylated CFTR chloride channel was ATP = GTP > ITP > CTP (100 = 100 > 93 > 85%) (Table 1).

3.6. Parallel inhibition of both ATPase activities and chloride uptake by NS004

Both ATPase activity and CFTR-associated Cl^- fluxes exhibited parallel responsiveness to the inhibitory potency of NS004, with the same IC_{50} value and the same complete inhibition (Fig. 5). The monophasic dose–response curve of ATPase activity spanned from 8–10 μM to 500 μM NS004. In contrast, CFTR-associated $^{36}Cl^-$ uptakes (13.4 ± 1.8 nmol $^{36}Cl^-$ mg^{-1} per 2 min) displayed a biphasic response to increasing levels of NS004 (from 0.1 to 500 μM): first, a 16 ± 10% stimulation ($n = 5$; $P < 0.02$) with 1 μM NS004 and at higher doses

Table 1
Effects of nucleotide triphosphates on the specificities of ATPase activity and CFTR Cl^- flux

Nucleotide	ATPase specificity (V_m/K_m)%	CFTR Cl^- flux (%)	
		This study[a]	Anderson et al. [12]
ATP	100[b]	100 ± 11	100
GTP	74	100 ± 11	60 ± 7
ITP	62	93 ± 9[ns]	48 ± 6
UTP	64	ND	42 ± 4
CTP	49	85 ± 6*	25 ± 5

*$P < 0.02$; ns: $P < 0.1$; unpaired *t*-test comparison to the experiments with ATP as a control value.
ND: not determined.
[a]Means ± S.D. of seven different duplicate experiments on two membrane preparations.
[b]100% represents a V_m/K_m value of 0.178 h^{-1} mg^{-1}.

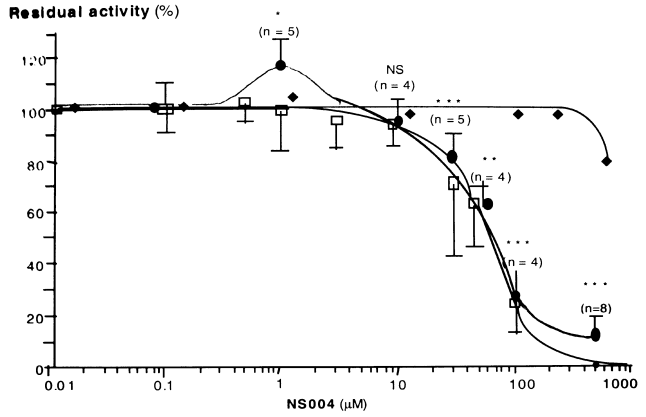

Fig. 5. Parallel inhibitions by NS004 of both voltage-insensitive phosphorylated CFTR-associated Cl^- fluxes and ATPase activity. (●) $^{36}Cl^-$ uptakes: 100% = 13.4 ± 1.8 nmol mg^{-1}; (□) ATPase activity: 100% = 21.5 ± 2.5 μmol Pi h^{-1} mg^{-1} over the basal level of 20 units; (♦) control: alkaline phosphatase activity assayed in two apical membrane preparations: 100% = 15.7 ± 1.2 μmol pNP h^{-1} mg^{-1}. Each point is the mean ± S.D. In parentheses: number of experiments. As compared to 100%: $*P < 0.02$; $**P < 0.01$; $***P < 0.001$ (unpaired *t*-test); NS: non-significant.

(10–500 μM), a drastic inhibition: up to 95 ± 5% with 500 μM (IC_{50} = 60 μM, n = 4). With 1 μM NS004, no ATPase stimulation could be revealed.

The alkaline phosphatase activity (15.7 ± 1.2 μmol pNP h^{-1} mg^{-1}, n = 3) present in the apical membrane preparations was not affected by NS004 (up to 400 μM final concentration).

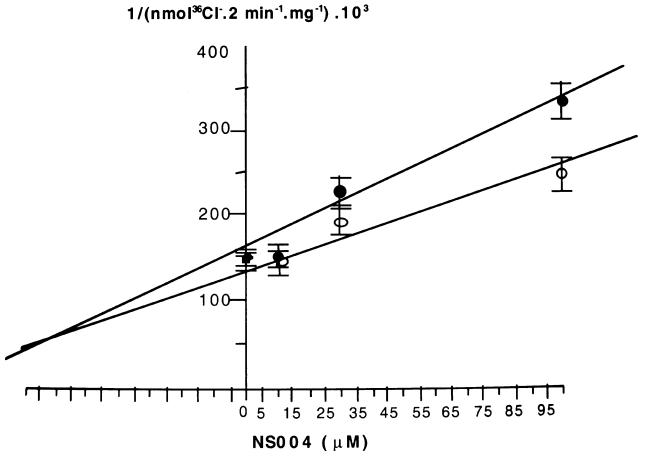

Fig. 6. Responsiveness of CFTR chloride fluxes to NS004 in the presence of (○) 1 mM and (●) 10 mM ATP. Dixon plot $1/v = f$[NS004]. Each point is the mean ± S.D. of seven experiments. The linear regression was calculated using the least squares method (1 mM ATP: r = 0.98, 10 mM ATP: r = 0.97).

3.7. Competition between NS004 and ATP on both ATPase activities and $^{36}Cl^-$ fluxes (Figs. 6 and 7)

High ATP concentrations (10 mM) partially relieved the inhibition of both chloride fluxes and ATP hydrolysis due to NS004 concentrations from 20 to 100 μM. Since on the Dixon plot $1/v = f$([NS004]) (Fig. 6), the intersections of the two straight lines for 1 and 10 mM ATP were distinct from the abscissa axis, it was concluded that NS004 competitively acted versus ATP on the CFTR chloride channel. The inhibition constant K_i was 60 μM. The inhibition of ATP hydrolysis by NS004 was partially relieved with 4 and 10 mM ATP. A similar Dixon plot reveals (Fig. 7) the competitive process between NS004 and ATP, the unique K_i value being 60 μM.

3.8. Action of antibodies directed towards the R domain of CFTR

The polyclonal antibodies PATG 1615, specifically reacting with amino acids 589–830 of the human CFTR R domain, increased by 18% the apical membrane ATPase activity (n = 3) whereas antibodies

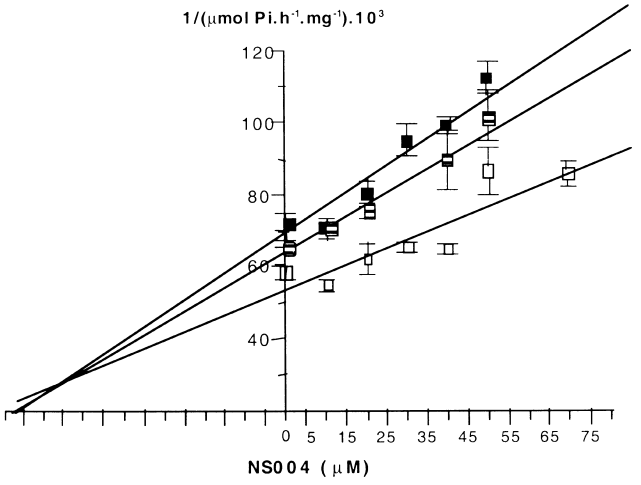

Fig. 7. Responsiveness of ATPase activity to NS004 in the presence of 1 (white squares), 4 (hatched squares) and 10 mM ATP (black squares). Dixon plot $1/v = f$[NS004]. Each point is the mean ± S.D. of three experiments on three membrane preparations. The linear regression lines were calculated using the least squares method (for both 1 and 4 mM ATP: r = 0.97, for 10 mM ATP: r = 0.90).

raised against the α subunit of the Na^+/K^+-ATPase had no effect.

4. Discussion

The present paper shows that ATPase activity would be associated with a functional CFTR chloride channel present in apical membranes of bovine airway epithelial cells.

The ATPase activity detected here would be related to the ABC protein CFTR for the following reasons.

1. A positive relationship was established between voltage-independent plus PKA-sensitive Cl^- fluxes (due to CFTR) and the specific activities of the ATPase in the same membrane preparations (Fig. 3). One may evaluate the minimal turnover rate of ATP split per CFTR molecule and per second in these preparations to be 3 s^{-1} if one considers (i) a V_{max} in the range of 20 μmol Pi h^{-1} mg^{-1} of membrane proteins (Fig. 4), (ii) CFTR proteins (170 000 Da, Fig. 2A) representing 30% of the total proteins ([20] and Fig. 2B) and assuming CFTR proteins are active. This value is higher than the value reported by Li et al. [19] (0.5–1 s^{-1}) but very low compared to the usual P-type ATPase turnover rates: at least 80–140 s^{-1} [30].
2. The specificity of the ATPase for four nucleotide triphosphates (Table 1) was similar to the rank of potency of these nucleotides to increase $^{36}Cl^-$ fluxes in relation to Cl^- secretion channel gating as previously described by Anderson et al. [12] for phosphorylated human CFTR chloride channel with which the bovine form exhibits 90% homology [31].
3. Both CFTR-specific $^{36}Cl^-$ fluxes and ATP hydrolytic activities were simultaneously inhibited by NS004 (Fig. 5) with a similar K_i value of 60 μM. A non-specific effect of NS004 via induced changes in the membrane environment seems unlikely since another phosphatase, alkaline phosphatase, which is also present in the same apical membrane fractions, was insensitive to NS004, even at high doses (Fig. 5).
4. Similar ATP doses competitively relieved both Cl^- fluxes (Fig. 6) and ATPase activities (Fig. 7) from inhibition by NS004, also suggesting that NS004 affects NBFs.
5. The ATPase activity was 18% increased by polyclonal PATG 1615. These results point out that at least the 18% activation of the ATPase activity would be related to the R domain of CFTR.

The nature of this ATPase remains questionable. Its lack of sensitivity to vanadate excludes any possible involvement of P-type ATPases of basolateral origin. This insensitivity also suggests that this activity differs from the multidrug resistance-associated ATPase activity which is 50% inhibited by 10 μM vanadate [32] and from the histidine periplasmic transport system, which is inhibited up to 70% by 500 μM vanadate [33]. The resistance to both KSCN and oligomycin excludes the existence of either vacuolar (inactivated by SCN^-) [34] or F_0F_1-type ATPases. The presence of unspecific phosphatases in the membrane preparations was also evaluated by the rates of pNPP hydrolysis under the same ionic conditions as for ATPase activity. The activities systematically detected in five membrane fractions were 14–21 μmol pNPP hydrolyzed h^{-1} mg^{-1} of protein but were fully inhibited by 200 μM vanadate. Thus, this ATP hydrolytic activity differs from a phosphatase and from P-, F_0F_1- or V-type ATPases.

Some of the functional characteristics of the ATPase activity found here have also been reported by Anderson et al. [12] and Li and coworkers [19] for the CFTR chloride channel. The unique K_m values for ATP hydrolysis calculated for phosphorylated CFTR are similar: 303 μM [19], 270 μM (EC_{50} in [12]) and 530 μM (present study). However, the Hill coefficient remained equal to 1 in bovine CFTR whereas it varied from 1 to 1.7 after phosphorylation of human CFTR [19]. This strongly suggests a cooperativity between two ATP hydrolytic sites in human phosphorylated CFTR whereas no cooperativity would be detected in phosphorylated bovine CFTR.

The inhibitory effect of EDTA on the rate of ATP hydrolysis measured here is consistent with that reported by Li et al. [19]. However, this Mg^{2+} chelator which blocked human CFTR chloride flux in liposomes [19] had no effect on the $^{36}Cl^-$ movements measured here. Our results fit the CFTR regulation model proposed by Gunderson and Kopito [17]:

Mg^{2+} would not be required for chloride channel opening but only for CFTR chloride channel closing.

In spite of common arguments from the properties of liposome-bound human CFTR [19] and bovine CFTR in intact apical membrane vesicles, complementary investigations would be necessary in order to fully understand the cellular function(s) of this ATPase and which phosphorylations may regulate it.

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