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CFTR Is Involved in Membrane Endocytosis but Not in Fluid-Phase and Receptor-Mediated Endocytosis in Human Respiratory Epithelial Cells

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Cystic fibrosis transmembrane conductance regulator (CFTR) protein has been reported to be a cAMP-regulator of plasma membrane recycling in epithelial cells overexpressing CFTR. To assess its role in the different endocytic processes in human respiratory epithelial cells, the rates of internalization of membrane, fluid-phase and receptor-mediator tracers were compared, under control conditions and after treatment with the cAMP agonist forskolin in normal and cystic fibrosis (CF) cells. In both control and treated-cells, CFTR was only present in the plasma membrane of normal but not in CF cells. Similarly, activation of Cl^- efflux only occurred in normal and not in CF-treated cells. The rate of membrane endocytosis was significantly decreased by 35% in normal treated-cells, whereas it was not significantly decreased (12%) in CF-treated cells. Upon forskolin treatment, the decrease of the rate of both fluid-phase and receptor-mediated endocytosis was not significantly different between normal and CF cells. These results demonstrate that CFTR is involved in membrane endocytosis but not in fluid-phase and receptor-mediated endocytosis in human respiratory epithelial cells.

Cystic fibrosis transmembrane conductance regulator (CFTR) protein is a cAMP-activated transmembrane channel regulating chloride transport through the apical membrane of various epithelial cells (1). In cystic fibrosis (CF), a lethal genetic disease, mutations in the gene encoding the CFTR result in an impairment of the gating properties and a mislocalization of the CFTR protein (2, 3). Presence of functional CFTR protein in the membrane of endocytic vesicles (4-6) has suggested that the appropriate number of CFTR channels in the apical membrane of epithelial cells may be regulated by an endocytosis/exocytosis process. Recent studies showed that plasma membrane recycling is associated with a cAMP-dependent inhibition of endocytosis in pancreatic and intestinal cell lines overexpressing the wild-type CFTR but not in cells expressing the mutated ΔF508/ΔF508 CFTR (7, 8). These results led to the conclusion that CFTR is involved in regulating plasma membrane recycling of epithelial cells. However, additional studies (9-11) have recently reported contradictory conclusions with regard to the direct involvement of CFTR in the endocytosis/exocytosis mechanisms. Therefore, it seems likely that the mechanisms of endosomal fusion and the subcellular distribution of CFTR protein between the endocytic vesicles and the apical membrane may differ according to the cell type studied.

We recently demonstrated that abnormalities of CFTR protein expression and subcellular distribution previously observed in CF airway surface epithelial cells can also be observed in normal airway epithelial cells (12). Accordingly, determining whether CFTR protein expression

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Abbreviations used: CFTR, cystic fibrosis transmembrane conductance regulator; CF, cystic fibrosis; SPQ, sulfopropyl-quinolinium; TMA-DPH, trimethylamino-diphenylhexatriene; FITC, fluoresceine-isothiocyanate; PBS, phosphate-buffered saline; MATG, monoclonal antibody; cAMP, cyclic-AMP.

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does regulate endocytic mechanisms in human airway epithelial cells is of importance since endocytosis represents potential pathways to internalize drugs, proteins or genes in order to correct airway diseases. Because endocytosis may occur by either non-specific processes which internalize extracellular solutes or by receptor-mediated processes, the present study was aimed to investigate whether or not the cAMP activated CFTR protein regulates the rate of membrane, fluid-phase and receptor-mediated endocytosis in both normal and CF human respiratory epithelial cells.

MATERIALS AND METHODS

Cell culture. Normal (JME/Nl7) and CF (JME/CF15, ΔF508/ΔF508 CFTR) human nasal polyp cell lines were kindly provided by Dr. Douglas M. Jefferson (13). Cells were cultured on 35 mm culture dishes or glass coverslips coated with type I collagen in DMEM/Ham's F-12 (1:1) medium supplemented with 10% fetal calf serum, antibiotics and growth factors in an 5% CO_2 atmosphere of humified air at 37°C (13). Cell cultures were used for the experiments between passage 20 and 30 at confluency.

CFTR immunocytochemistry. Confluent cells were incubated with 50 μM forskolin or appropriate amounts of dimethyl sulfoxyde (vehicle for forskolin) for 30 min at 37°C and fixed with 3.7% formaldehyde in phosphate buffered saline (PBS). Cells were firstly incubated with the primary anti-CFTR antibody MATG 1031 (Transgene, Strasbourg, France) raised against the CFTR external loop (1/20 in PBS/0.5% ovalbumin (Sigma, St Louis, MO USA)) for 1 hour. Then, they were successively incubated with a goat anti-mouse biotinyled secondary antibody (1/50 in PBS/0.5% ovalbumin) for 30 min and with streptavidin-FITC (1/50 in PBS/0.5% ovalbumin) for 30 min. Cells were then observed with a standard epifluorescence oil-immersion microscope (Zeiss Axiophot, Oberkochen, Germany).

Measurement of cell chloride efflux by SPQ analysis. Confluent cells were incubated with 3.5 mM of 6-methoxy-N-(3-sulfopropyl) quinolinium (SPQ, Sigma) in a deionized water chloride buffer solution (1:1) for 10 min at 37°C according to a transient permeabilization procedure (14). SPQ-loaded cells were then incubated in a nitrate buffer (14) in the absence or presence of 25 μM forskolin. Intracellular SPQ was excitated at 365 nm through a X32 planachromat objective and emission light at $>$ 395 nm was recorded for 2 s every min for 15 min with a low level video-camera (Lhesa SIT 4036, St Ouen l'Aumone, France). Variation of fluorescence intensity was analyzed by a multivariate statistical technique from the temporal image series using a Sparc-Classic workstation (X-video card, Parallax Graphics, Moutain View, CA USA).

Membrane endocytosis. Internalization of 1-[4(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH, Molecular Probes, Eugene, OR USA) was performed according to Illinger and Kuhry (15). Briefly, confluent cell cultures were incubated at 37°C for various times with 2×10^{-6} M TMA-DPH in the presence or absence of 50 μM forskolin. Peripheral labeling was removed with cold PBS. The cells were resuspended in PBS after gentle scraping with ''cell lifters'' (Costar, cambridge, MA USA). The fluorescence intensity was measured with the help of MPF-66 Perkin-Elmer spectrofluorimeter (excitation 360 nm, emission 435 nm, bandwidth 5 nm). The results were expressed as % of the fluorescence intensity of the peripheral labeling measured in parallel on unwashed cells after a 10 s incubation of TMA-DPH at room temperature.

Fluid-phase endocytosis. Confluent cells were incubated with 1 mg/ml of FITC-dextran (Sigma, 40 kDa, St Louis, MO USA) previously dialyzed against deionized water for various intervals at 37°C in the presence or absence of 50 μM forskolin. Free label was removed by four extensive washings with cold PBS. The internalized FITC-dextran was extracted with cold 100% ethanol (16) and the fluorescence intensity was measured at 520 nm (excitation 480 nm ; bandwidth 5 nm). The background contribution was measured on unlabeled cells. Protein content was estimated according to the Bradford protein assay (17).

Receptor-mediated endocytosis of transferrin. Human transferrin (Tf, Sigma, St Louis, MO USA) was radiolabeled according to the method of Bolton and Hunter (18) to a specific radioactivity (^{125}I) of 5×10^{10} cpm/mg of Tf. Confluent cells were incubated with Tf-free medium for 30 min and then with medium containing 5×10^6 cpm of ^{125}I-Tf, for the indicated periods, in the presence or absence of 50 μM forskolin. Cell surface Tf was removed (95%) by an acid/neutral rinse procedure (19). Intracellular Tf was determined by radioactivity measurements with a Tri-Carb 1900CA analyzer (Packard, Meriden, CT USA) after cell recovery by scraping.

Statistical analysis. All data is expressed as mean $\pm$ S.E.M. and statistical analysis was performed by Student's *t* test. The significance of a variation between groups of different incubation time was analyzed using analysis of variance. P values $<$ 0.01 were considered significant.

RESULTS

CFTR protein expression. Normal (JME/Nl7) and CF (JME/CF15) respiratory epithelial cells were analyzed by immunofluorescence using a monoclonal antibody (MATG 1031) specific to the extracellular loop of CFTR. Normal cells only showed a labeling at the plasma

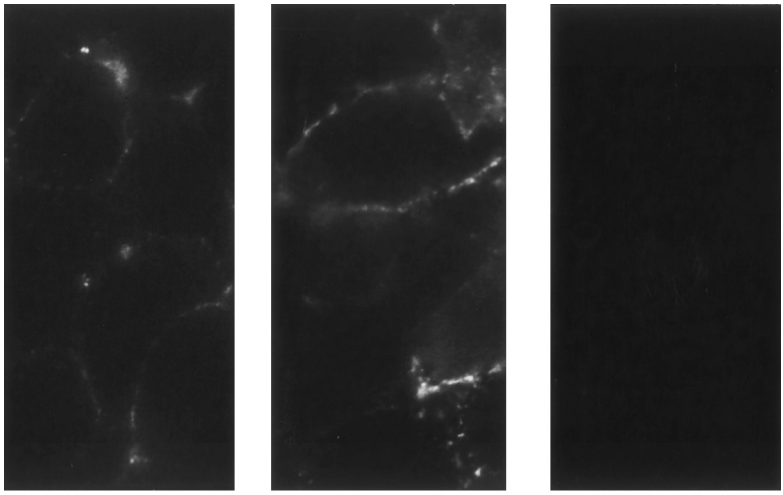

FIG. 1. Immunofluorescent localization of CFTR. In A, normal cells (JME/Nl7) incubated with monoclonal antibody to CFTR show a weak staining reaction in the plasma membrane. In B, normal cells stimulated with 50 μM forskolin exhibit an enhanced staining reaction in the plasma membrane. In C, CF cells (JME/CF15) do not express any membrane labeling.

membrane (Fig. 1A) which was increased upon forskolin treatment (50 μM, 30 min) (Fig. 1B). In contrast, CF cells lacked the plasma membrane labeling, even after forskolin treatment (Fig. 1C).

CFTR chloride channel activity. In order to ensure that normal cells used in these studies express functional CFTR chloride channel activity, we assessed the Cl^- efflux expressed as percentage of fluorescence variation of SPQ probe upon forskolin treatment. As reported in Fig. 2, Cl^- efflux was found to be significantly increased in normal cells (69 $\pm$ 14% [n=20] and 87 $\pm$ 22% [n=20] after forskolin treatment) compared to CF cells (82 $\pm$ 12% [n=18] and 78% $\pm$ 23% [n=18] after forskolin treatment).

Endocytosis pathways. To investigate whether CFTR chloride channel is involved in the regulation of distinct endocytosis processes, series of experiments were carried out to assess the rate of membrane, fluid-phase and receptor-mediated endocytosis in both control and treated normal and CF cells.

In control cells, the rate of internalization of membrane endocytosis tracer TMA-DPH was similar in normal (36 $\pm$ 3% [n=6]) and CF (35 $\pm$ 3% [n=6]) cells after a 30 min incubation period (Fig. 3A). Upon forskolin treatment (50μM, 30 min) a high significant decrease (26%) of membrane endocytosis was observed in normal cells. In contrast, only a slight decrease (8%) was detected in CF cells. Other series of experiments using a longer incubation time with TMA-DPH (a 60 min incubation period) similarly showed a high significant decrease of membrane endocytosis (35%) in control cells and a slight decrease (12%) in CF cells (Fig. 3B).

As shown in Fig. 4A, after a 30 min incubation period, fluid-phase endocytosis of FITC-dextran was similar in normal and CF cells. Upon forskolin treatment, the rate of fluid-phase endocytosis was significantly decreased in both normal (41%) and CF (45%) cells as compared with control cells. A similar significant decrease in fluid-phase endocytosis was observed (Fig. 4B) after a longer incubation time with FITC-dextran (a 60 min incubation period) in normal (42%) and CF (41.5%) cells, upon forskolin treatment.

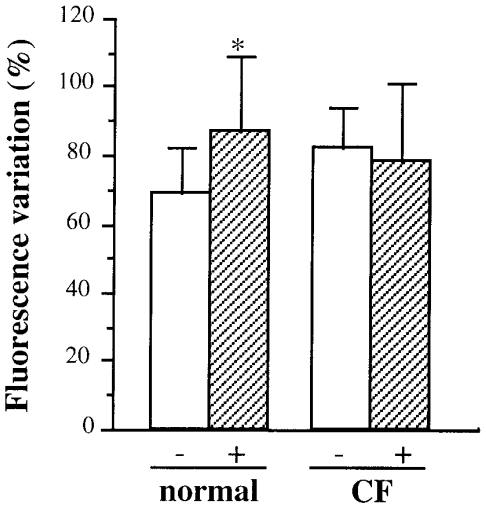

FIG. 2. Chloride efflux measurements evaluated by SPQ fluorescence variation in normal (JME/Nl7) and CF (JME/CF15) cells in the absence (−) and presence (+) of 25 μM forskolin. Values are means ± S.E.M. from the multivariate statistical analysis of 15 temporal images obtained 15 min after stimulation relative to time 0 (*$p<0.01$).

Fig. 5A and B show the rate of receptor-bound Tf endocytosis in normal and CF cells after forskolin treatment . As it has been observed in the case of fluid-phase endocytosis (Fig. 4), the decrease of the rate of receptor-mediated endocytosis in normal cells (20% and 31% after a 5 and 10 min period, respectively) was similar in CF cells (26% and 36,5% after a 5 and 10 min incubation period, respectively) upon forskolin treatment.

DISCUSSION

Conflicting results about the direct involvement of CFTR protein in the regulation of cAMP-dependent inhibition of endocytosis and stimulation of exocytosis have been reported in several

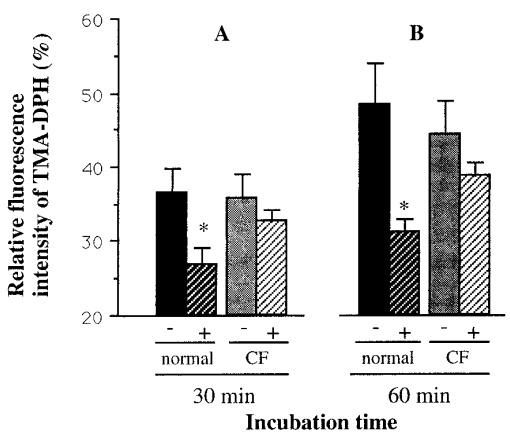

FIG. 3. Relative fluorescence of the membrane endocytic marker TMA-DPH in normal (JME/Nl7) and CF (JME/CF15) cells in the absence (−) and presence (+) of 50 μM forskolin after a 30 min (A) and 60 min (B) incubation time. Results are expressed as % of the intracellular fluorescence intensity of the peripheral labeling. Values are means ± S.E.M. of triplicate determination from each of two separate preparations (*$p<0.01$).

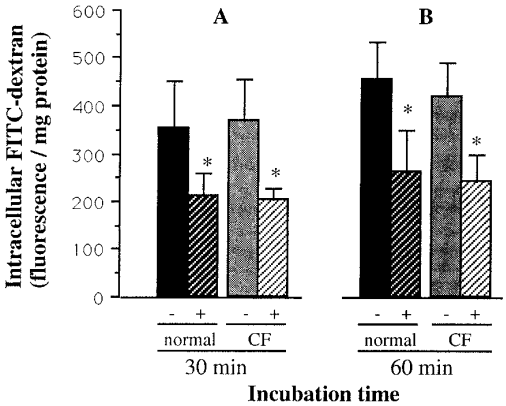

FIG. 4. Internalization of the fluid-phase endocytic marker FITC-dextran in normal (JME/Nl7) and CF (JME/CF15) cells in the absence (−) and presence (+) of 50 μM forskolin after a 30 min (A) and 60 min (B) incubation time. Values are means ± S.E.M. of triplicate determination from each of two separate preparations (*$p<0.01$).

CF-cell lines which have been transfected with the wild-type CFTR (8). The first studies of Bradbury et al. (8) have shown that CFTR was involved in cAMP-activated membrane trafficking in a pancreatic ductal cell line CFPAC. In this latter study, membrane recycling and fluid-phase endocytosis were shown to be regulated by cAMP in CFPAC-1 cells expressing wild-type CFTR, but not in mutant CFTR. In contrast, more recent studies of Santos and Reenstra (9) and those of Dho et al. (10) demonstrated that, in T84 and CHO cell lines, respectively, the expression and activation of CFTR by cAMP was without effect on plasma membrane recycling endocytosis. Therefore, it is possible that overexpression of CFTR in a heterologous expression system may lead to different results according the transformed cell line tested. In the present work, we used non-transfected normal and CF established human respiratory epithelial cells (13), which more closely express endogenous CFTR than transfected cells in order to investigate the effect of cAMP agonist forskolin on the various conventional aspects of endocytosis by using appropriate probes, i.e.: TMA-DPH for membrane endocytosis, FITC-dextran for fluid-phase endocytosis, and ^{125}I-Tf for receptor-mediated endocytosis.

Evidence for the presence of CFTR protein in the plasma membrane of normal but not CF

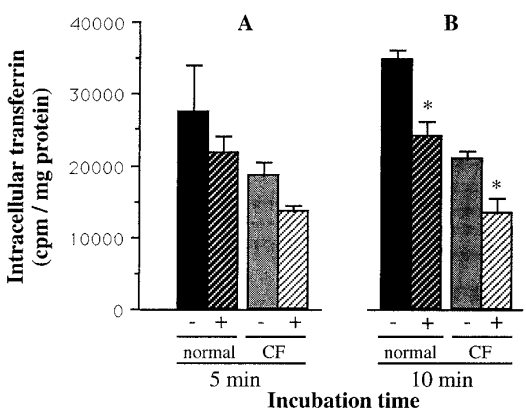

FIG. 5. Internalization of the receptor-mediated marker ^{125}I-Tf in normal (JME/Nl7) and CF (JME/CF15) cells in the absence (−) and presence (+) of 50 μM forskolin after a 5 min (A) and 10 min (B) incubation time. Results are means ± S.E.M. of triplicate determination of one preparation (*$p<0.01$).

human respiratory epithelial cells was found by immunofluorescence labeling using a specific anti CFTR (MATG 1031) antibody to the extracellular loop of CFTR. In normal cells, forskolin treatment increased the labeling intensity in plasma membrane (Fig. 1B), which, according to other authors (20), may result from either endocytosis inhibition and/or exocytosis stimulation mechanisms. In addition, we observed, in agreement with earlier results (13) that normal but not CF human respiratory epithelial cells, showed a cAMP-dependent increase in Cl^- permeability upon forskolin treatment (Fig. 2). These data were consistent with the assumption that an increase in Cl^- secretion in normal respiratory epithelial cells may result from a greater amount of CFTR resident at the cell membrane after inhibition of endocytosis and/or activation of exocytosis by the cAMP agonist forskolin (21). In the same way, retrieval inhibition and/or insertion activation of intracellular vesicles occuring within the plasma membrane should result in a decreased endocytic vesicle number during membrane recycling upon forskolin stimulation. Upon forskolin stimulation (Fig. 3), the significant reduced amount of the membrane endocytic marker TMA-DPH found in normal but not in CF respiratory epithelial cells supports this assumption and moreover reveals that CFTR is involved in the fusion processes of intracellular vesicles with the plasma membrane during regulating plasma membrane recycling. These results agree completely with our immunocytochemical findings (Fig. 1) showing an increased number of CFTR protein in the plasma membrane of normal cells after forskolin treatment.

Wild-type CFTR has also been reported to be involved in the forskolin-dependent regulation of the fluid-phase endocytosis (8). In contrast, our results with FITC-dextran as fluid-phase endocytosis tracer (Fig. 4) showed no significant difference on the rate of fluid-phase endocytosis in normal and CF human respiratory epithelial cells. Moreover, forskolin stimulation induced a similar significant decrease of the internalized fluid-phase marker in both cell lines. These data provide evidence that CFTR is not involved in the regulation of fluid-phase endocytosis in non transfected respiratory epithelial cells.

In receptor-mediated endocytosis (Fig. 5), the higher amount of transferrin internalized in normal compared to CF cells may result from a different number of transferrin receptors present on the cell surface of the two cell lines. The fact that forskolin induced a similar significant decrease in both normal and CF cells indicated furthermore that CFTR is not implied in the receptor-mediated endocytic process in respiratory epithelial cells . This finding is in agreement with a previous study (11) which showed that transferrin internalization was unaffected by forskolin neither in normal nor in CF pancreatic cells.

In conclusion, our data indicate that CFTR is involved in membrane endocytosis but not in fluid-phase and receptor-mediated endocytosis in non transfected human respiratory epithelial cells.

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