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Activation of the cystic fibrosis transmembrane regulator by cyclic AMP is not correlated with inhibition of endocytosis

George F. Santos, William W. Reenstra *

Research Institute, Children's Hospital Oakland, 747 52nd Street, Oakland, CA 94609, USA

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Abstract

Based on the observation (Bradbury et al. (1992) Am. J. Physiol. 262, C752–C759) that conditions known to activate the cystic fibrosis transmembrane regulator protein (CFTR) increase the rate of exocytosis and decrease the rate of endocytosis, it was proposed that activation of the CFTR may involve cAMP-dependent fusion of CFTR containing endosomes with the apical membrane. We have tested this hypothesis in two cell lines derived from epithelia that express defective chloride transport in cystic fibrosis (CF): the human colonic cell line, T84, and the tracheal cell line 9HTEo$^-$. The dose-dependence of forskolin- and CPT-cAMP-induced inhibition of endocytosis were compared with the dose-dependence of chloride channel activation. Endocytosis was determined from the uptake of FITC-dextran, and assayed in purified endosomes. Chloride channel activity was measured from the rate of I-efflux. If the fusion hypothesis is correct: (1) concentrations of agonist that inhibit endocytosis should activate chloride channel activity, and (2) the relationship between endocytosis and channel activation should be similar for forskolin and CPT-cAMP. Results in both cell lines were inconsistent with these postulates, suggesting that either chloride channel activation and the inhibition of endocytosis are separate effects of cAMP, or that the increase in apical CFTR resulting from agonist-dependent inhibition of endosomal fusion is minimal.

Keywords: Chloride channel; cAMP; FITC-dextran; T84 cell; 9HTEo$^-$ cell; Endosome; CFTR

1. Introduction

Cystic fibrosis (CF) is the most common lethal genetic disease in the Caucasian population [1]. It is the result of mutations in the cystic fibrosis transmembrane conductance regulator protein (CFTR) [2,3], a cAMP-activated chloride channel [4–6]. The CFTR protein is composed of five domains, an N-terminal membrane spanning domain; a cytoplasmic nucleotide binding domain; a cytoplasmic domain with multiple consensus sites for protein kinase A (PKA) dependent phosphorylation, the R-domain; a second membrane spanning domain; and at the C-terminal a second cytoplasmic nucleotide binding domain [2]. Electrophysiological studies have demonstrated that CFTR chloride channel activity is activated by a protein kinase A and ATP dependent mechanism that involves phosphorylation on R-domain [7,8], and the binding of ATP at one or both of the nucleotide binding domains [9,10]. The CFTR is expressed at the apical membrane of epithelial cells, where the loss of CFTR function leads to impaired chloride permeability [1]. This provides an obvious explanation for the primary physiological defect in CF, the loss of cAMP-dependent chloride secretion in several epithelia.

An additional mechanism for the regulation of CFTR channel activity, involving the cAMP-activated fusion of endosomes with the apical membrane has recently been proposed. It has been shown in intestinal, pancreatic, and tracheal cell lines that cAMP-dependent agonists can alter the rates of exocytosis and endocytosis [11–13] and in a pancreatic cell line that expression of the CF phenotype alters agonist-dependent exocytosis [12]. As in these tissues cAMP-dependent agonists are known to activate chloride channel

* Corresponding author. Present address: Department of Medical Cell Biology, Nemours Research Programs, P.O. Box 269, Wilmington, DE 19899, USA. Fax: +1 (302) 6516899.

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activity of the CFTR, it has been proposed that by altering the rates of exocytosis and endocytosis the number of CFTR channels in the apical membrane can be regulated, and that this process provides an additional level of regulation for apical CFTR activity. Additional support for the fusion hypothesis has been obtained from the following observations: (1) cAMP-dependent activation of the CFTR in T84 cells can be inhibited by agents that block endosomal fusion or disrupt microtubule polymerization [14,15]. (2) Clathrin coated vesicles from T84 cells contain the CFTR [16]. (3) Endocytosis of the CFTR is inhibited by forskolin, an activator of adenylate cyclase [17]. While evidence for intracellular pools of CFTR has been presented, there is also clear histological and biochemical evidence for an apical population of CFTR protein in nonstimulated cells [18,19]. Thus, it is likely that apical chloride channel activation can occur in the absence of endosomal fusion and the fusion hypothesis can only be tested by determining if maximal channel activity requires endosomal fusion. While quantitative comparisons of endosomal and apical CFTR have not been made, the total intracellular CFTR has been estimated to be at least equal to apical CFTR in nonstimulated T84 cells [19]. If true, this limits the maximal activation of apical CFTR activity that results from endosomal fusion to no more than 2-fold. These observations have led to the suggestion that endosomal fusion may play a relatively minor role in the regulation of apical CFTR activity [19].

Technical problems associated with quantitative measurements of apical and endosomal CFTR have made it difficult to obtain conclusive evidence for, or against, the fusion hypothesis. We have attempted to resolve this question by making quantitative comparisons of dose-response curves for cAMP-dependent inhibition of endocytosis and stimulation of chloride channel activity. We have used this approach because: (1) it is difficult to obtain quantitative measurements of the rate of exocytosis and (2) previous studies have suggested that agonist-dependent changes in the steady-state level of apical CFTR protein result from changes in the rates of both endocytosis and exocytosis [11,12]. In contrast to exocytosis, initial rates of endocytosis can be measured with relative ease from the uptake of a soluble marker into endosomal vesicles that can then be separated from other cellular components. It is our assumption that if decreased endocytosis results in an increase in apical CFTR, then dose-response curves for agonist-dependent channel activity will show concentration-dependent changes in channel activity at agonist concentrations that alter the steady-state rate of endocytosis. In addition, as the regulation of endosomal fusion and the subcellular distribution of CFTR protein between the apical membrane and intracellular endosomes may vary with cell type, we have carried out our studies in two cell lines derived from tissues that express the CF defect: T84 cells, an intestinal cell line, and 9HTEo$^-$ cells, a tracheal cell line. Studies with both of these cell lines have been cited as evidence for the fusion hypothesis [11,13].

In our studies we find no evidence for fusion dependent activation of the CFTR. For T84 cells, forskolin stimulated chloride channel activity and inhibited the rate of endocytosis while 8-(4-chlorophenylthiol)adenosine cyclic 3′,5′-monophosphate (CPT-cAMP) stimulated chloride channel activity to a similar extent without inhibiting the rate of endocytosis. In 9HTEo$^-$ cells the concentration of forskolin needed to stimulate chloride channel activity was 10-fold lower than the concentration needed to inhibit the rate of endocytosis. At these higher concentrations of forskolin where endocytosis was inhibited, no additional increase in chloride channel activity was observed. These results suggest that either chloride channel activation and the inhibition of endocytosis are separate effects, or that the increase in apical CFTR resulting from agonist-dependent inhibition of endosomal fusion is minimal.

2. Materials and methods

T84 cells at passages between 55 and 75 were maintained on 150 mm plates in a 50:50 mixture of Dulbecco's modified Eagle medium H16 and Ham's F12 supplemented with 5% new born calf serum, 2 mM glutamine, 10 mM glucose, 15 mM Hepes (pH 7.4), 100 μg/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin at 37°C in 5% CO_2 [20]. Cells were passaged at densities between 80 and 90% confluency and seeded onto 35 mm plates at 10^6 cells per cm^2. Media was changed 24 h after plating and thereafter every 48 h. Cells were used 5 to 8 days after seeding and in all cases were at densities greater than 90%. Similar procedures were used for the tracheal cell line 9HTEo$^-$ except that cells were cultured in Eagle's minimal essential medium supplemented with Earl's balanced salts and 10% fetal bovine serum.

Chloride channel activity was assayed by measuring the rate of I-efflux [21,22]. Cells were incubated in room air for 30 min at 37°C with Hepes-buffered Ringer's (140 mM Na, 5 mM K, 145 mM Cl, 2 mM PO_4, 1 mM Mg, 1 mM Ca, 1 mM SO_4, 10 mM glucose, and 10 mM Hepes/Tris, pH 7.4) containing 5 μCi/ml ^{125}I and then washed four times in Ringer's to remove extracellular ^{125}I. The loss of intracellular ^{125}I was determined by replacing and counting the bathing solution every 60 s for 12 min. Agonist or vehicle (0.2% DMSO) were added to all aliquots of bathing media after 4 min. Counts remaining in the cells after 12 min were extracted with 0.1 M HNO_3. Plots of $\log(^{125}I_t/^{125}I_T)$ against time were made where $^{125}I_t$ is intra-

cellular ^{125}I at time t and $^{125}I_T$ is intracellular ^{125}I at time 0. It should be noted that unlike previous studies [21] where a transient activation of the CFTR is seen, forskolin and CPT-cAMP activated channels remained active for the entire time course of our experiments [21]. This allowed steady-state rates to be calculated from linear least squares fits to the last five data points.

Endosomes were labeled with FITC-dextran by incubating cells for 10 min at 37°C with HCO_3-buffered Ringers (145 mM Na, 5 mM K, 125 mM Cl, 2 mM PO_4, 1 mM Mg, 1 mM Ca, 1 mM SO_4, 10 mM glucose, and 25 mM HCO_3 and 5% CO_2, pH 7.4); supplemented with 25 mg/ml 40 kDa FITC-dextran. Unless noted agonists were added 10 min prior to incubation with FITC-dextran and were present during the uptake period. Cells were then washed five times with 4°C TBS and scraped into 300 μl of homogenization buffer (300 mM mannitol, 10 mM Hepes/Tris, pH 7.5) containing 0.1 mM PMSF and 0.1 mg/ml aprotinin. Endosomes were prepared as described for rabbit renal cortex [23] with the exception that the final pellet was spun at $50\,000 \times g$ for 40 min. Intravesicular (endosomal) FITC-dextran was assayed with a Perkin-Elmer spectrofluorimeter. FITC fluorescence was excited at 488 nm and emitted light was filtered with a 525 nm cut-on filter. FITC-dextran labeled endosomes were added to homogenization buffer and extravesicular FITC-fluorescence quenched by adding a 5-fold molar excess of an anti-FITC antibody that quenches the fluorescence of bound FITC [24]. Intravesicular FITC-dextran was then determined from the decrease in fluorescence when intra-endosomal FITC-dextran was released after lysing endosomes with 0.1% Triton X-100. The fluorescence of the labeling media and total cell protein were measured, allowing endocytosis to be expressed in nl/mg cell protein.

Dose response curves for the rates of I-efflux and endocytosis of FITC-dextran were generated and relative values fit by non-linear least squares (Sigma Plot, Jandel) to,

$$R = (1 + R_{mx} \cdot ([Ag]/K_a))/(([Ag]/K_a) + 1) \qquad (1)$$

where R is the response relative to that at zero agonist, R_{mx} is the relative response at infinite agonist, [Ag] is the concentration of agonist, and K_a is the concentration of agonist that gives a half maximal change. Calculated values of K_a are presented as best fit values ± S.E.

2.1. Materials

FITC-dextran was obtained from Sigma and dialyzed against distilled water before use. The anti-FITC antibody was obtained from Molecular Probes. Carrier free ^{125}I (Na salt) was obtained from Amersham. T84 cells were obtained from ATCC and 9HTEo$^-$ cells were a gift from Dr. Dieter Greunert of the University of California at San Francisco.

3. Results

In this study the fusion hypothesis has been tested by comparing dose response curves for agonist-dependent chloride channel activation with agonist-dependent inhibition of the rate of endocytosis. To accomplish this it was first necessary to develop methods where steady-state measurements of chloride channel activity and the rate of endocytosis could be made under the same experimental conditions. This was done by measuring endocytosis with the uptake of FITC-dextran. As described in Materials and methods cells were exposed to FITC-dextran, homogenized and an endosomal membrane preparation prepared. Because our assay measures intravesicular FITC-dextran with minimal interference from membrane vesicles that do not contain FITC-dextran, the endosomal purification procedure was optimized for yield and not for purity. A typical assay of endosomal FITC-dextran is shown in Fig. 1. Purified endosomes are added to buffer (first arrow), and the fluorescence of extravesicular FITC-dextran is quenched by the addition of an anti-FITC antibody that quenches the fluorescence of bound FITC-dextran (second arrow). In control experiments (not shown) the fluorescence of bound FITC-dextran was determined to be less than 2% of that of free FITC-dextran. The subsequent addition of detergent

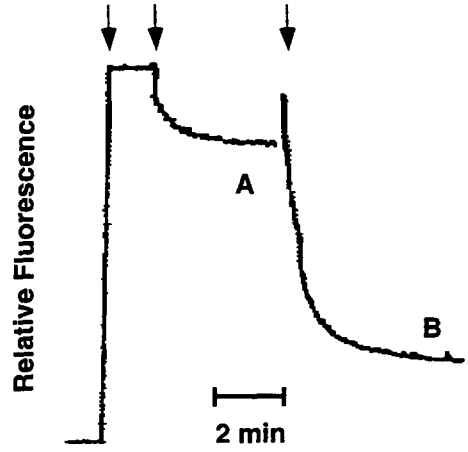

Fig. 1. Assay of endocytosed FITC-dextran. Purified endosomes from cells that were exposed to FITC-dextran for 10 min are added to assay buffer at the first arrow and fluorescence measured (excitation 488, emission > 510). At the second arrow a 5-fold molar excess of anti-FITC antibody is added. This quenches the fluorescence of extravesicular FITC-dextran. At the third arrow Triton X-100 is added to 0.1%, lysing endosomes and allowing the fluorescence of previously intravesicular FITC-dextran to be quenched. The difference between A and B is proportional to endosomal FITC-dextran.

(third arrow) lysed the vesicles and allowed antibody to bind and quench the fluorescence of previously intravesicular FITC-dextran. As described in Materials and methods, this decrease in fluorescence (A-B) is used to calculate total endosomal FITC-dextran and the rate of endocytosis.

In order to determine how rapidly the rate of endocytosis reaches a new steady-state after the addition of agonist, a time course for the onset of forskolin-dependent inhibition of endocytosis was measured as shown in Fig. 2. In all cases cells were exposed to FITC-dextran for a total of 2 min. FITC-dextran was added 0, 2, 4, or 10 min after the addition of 20 μM forskolin and uptake in nl/min per mg protein was normalized to a 2 min control uptake in the absence of forskolin. As shown in Fig. 2, 4 to 5 min after the addition of forskolin the uptake of FITC-dextran was maximally inhibited. The percentage inhibition in Fig. 2 when FITC-dextran was added 10 min after stimulation with forskolin, $35 \pm 4\%$, is not statistically different from the value obtained with a 10 min uptake of FITC-dextran (Fig. 4). These results suggest that when FITC-dextran is added 10 min after agonist, as in Figs. 4 and 5, the rate of uptake is time independent and therefore reflects the steady-state rate of FITC-dextran uptake. In a separate set of control experiments cells were exposed to FITC-dextran for times between 0 and 30 min (data not shown). Over the first 10 min endosomal uptake of FITC-dextran increased linearly but the measured rates of uptake were reduced at longer times. The reduction in the measured rate at longer times is likely to reflect exocytic recycling of FITC-dextran containing endosomal vesicles. Based on these observations FITC-dextran uptake in Figs. 4 and 5 was limited to 10 min and under these conditions the measured rate of uptake is likely to represent the rate of endocytosis and not be reduced by any exocytosis of FITC-dextran loaded endosomes.

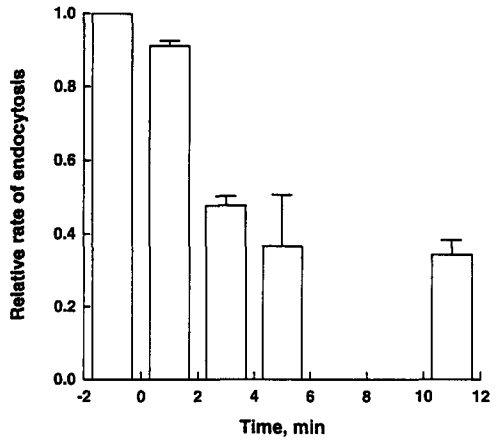

Fig. 2. Time-course for the establishment of a steady-state rate of endocytosis following stimulation with forskolin. T84 cells were exposed to FITC-dextran for 2 min prior washing with 4°C buffer and endosomal preparation. FITC-dextran was added 0, 2, 4 or 10 min after stimulation with 10 μM forskolin. Endocytosed FITC-dextran/mg of protein was compared to a 2 min uptake by nonstimulated cells. Values are means and S.E. values for three independent measurements.

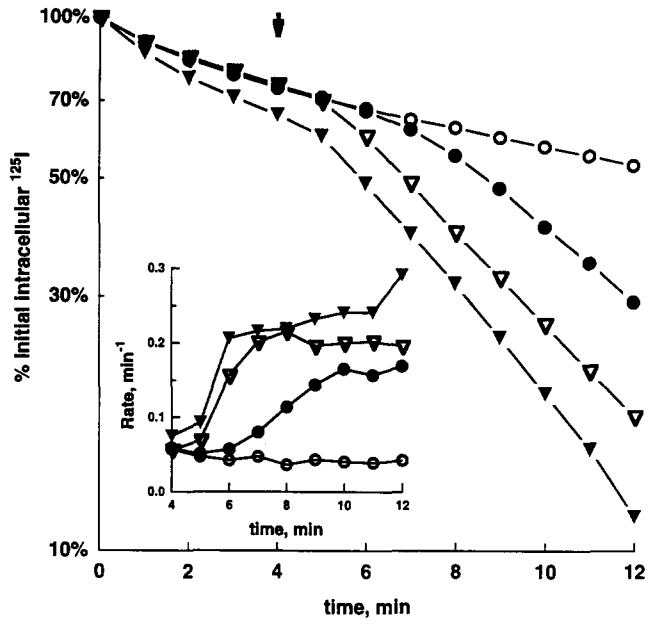

Fig. 3. Stimulation of I-efflux from T84 cells by forskolin. The percentage of ^{125}I remaining within T84 cells is plotted as a function of time. In all cases agonist (filled circles, 0.5 μM forskolin, open triangles, 5.0 μM forskolin, or filled triangles, 50 μM forskolin) or vehicle (open circles, 0.2% DMSO) is added at the arrow and present throughout the remainder of the experiment. (Inset) Calculated rates of I-efflux. Rates of $^{\text{I-efflux}}$ were calculated from the data in the main figure as rate $= (\ln(R_1) - \ln(R_2))/(t_1 - t_2)$, where R_1 and R_2 are the percentage of the initial cellular ^{125}I at successive time points t_1 and t_2. Data are from a representative experiment.

Chloride channel activity was studied by measuring the rate of I-efflux. Cells were incubated for 30 min with ^{125}I at 37°C in Hepes buffered Ringer's and room air, extracellular ^{125}I was removed by washing the cells with ^{125}I free buffer, and the rate of ^{125}I loss was determined by replacing the bathing solution every 60 s for 12 min. Agonist was added to the bathing solution after the fourth min. As shown in the inset, the forskolin-induced increase in I-efflux was sustained for the remaining 8 min of the experiment; similar effects were seen with CPT-cAMP (data not shown). In contrast to previous studies [21], transient stimulation of I-efflux was not observed. While we have no explanation for this difference, it allowed rates to be calculated from the slopes of linear least squares fits to the last five data points plots like those in Fig. 3. Since the rates of I-efflux did not vary with time, they were regarded as a steady-state measure of chloride channel activity. Thus, steady-state values for channel activity could be compared to steady-state values for the rate of endocytosis.

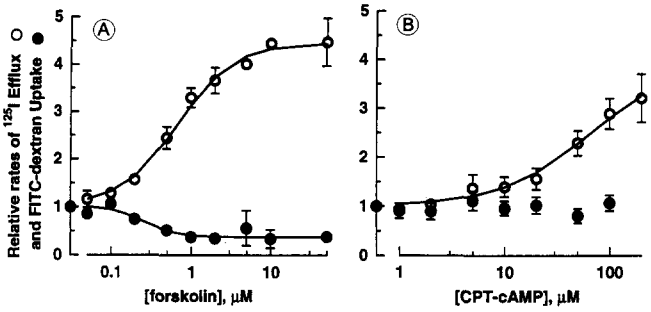

Fig. 4. Comparison of the relative changes in agonist-activated chloride channel activity and inhibition of endocytosis in T84 cells. Dose response curves for forskolin (A) and CPT-cAMP (B) induced changes in the steady-state rates of I-efflux (open circles) and endocytosis of FITC-dextran (filled circles). All points are mean values for four to six independent determinations, error bars are S.E. values. Lines are calculated from best fit parameters according to Eq. 1.

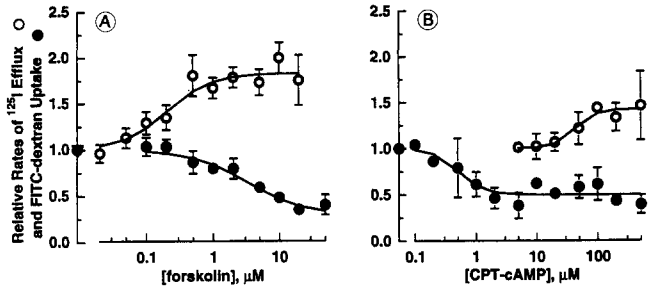

Fig. 5. Comparison of the relative changes in agonist-activated chloride channel activity and inhibition of endocytosis in 9HTEo$^-$ cells. Dose response curves for forskolin (A) and CPT-cAMP (B) induced changes in the steady-state rates of I-efflux (open circles) and endocytosis of FITC-dextran (filled circles). All points are mean values for at least three independent determinations, error bars are S.E. values. Lines are calculated from best fit parameters according to Eq. 1.

With the techniques described, dose response curves for agonist-dependent activation of I-efflux and inhibition of endocytosis were generated. Results with the intestinal cell line, T84, are shown in Fig. 4A and B. In Fig. 4A, T84 cells were stimulated with forskolin, while in Fig. 4B cells were stimulated with CPT-cAMP. Best fit parameters for the concentrations of agonist giving half-maximal effects and the maximal stimulation or inhibition were calculated and are given in Table 1. There was no significant difference between the maximal stimulation of I-efflux with forskolin and CPT-cAMP; however, unlike the results with forskolin there is no evidence for inhibition of endocytosis at concentrations of CPT-cAMP up to 100 μM. These results suggest that chloride channel activation can occur in the absence of an increase in apical CFTR resulting from the inhibition of endocytic removal. We have no explanation for the differences between forskolin and CPT-cAMP but if different forms of the regulatory subunit for PKA were involved in channel activation and inhibition of endocytosis, the results might reflect known differences in the binding of cAMP and CPT-cAMP to the two forms of the regulatory subunit of PKA [25].

As the extent that endosomal fusion plays in CFTR activation may vary with cell type a second cell line, 9HTEo$^-$, derived from human tracheal cells was also examined. Results are shown in Fig. 5A and B; forskolin-dependent effects are shown in Fig. 5A and CPT-cAMP-dependent effects are shown in Fig. 5B. Best fit parameters for the concentrations of agonist giving half-maximal effects and the maximal stimulation or inhibition were calculated and are given in Table 1. In 9HTEo$^-$ cells there are significant differences between the forskolin-dependent K_m values for chloride channel activation and the K_i values for the inhibition of endocytosis. With forskolin 80% of the maximal activation of I-efflux occurs at a forskolin concentration where less than 20% of the maximal inhibition of endocytosis has occurred; no additional chloride channel activity is seen at higher concentrations of forskolin that inhibit endocytosis by an additional 40%. With CPT-cAMP endocytosis is inhibited at a lower concentration of agonist than that required for activation of I-efflux.

4. Discussion

At present the extent that agonist-dependent fusion of endosomes containing the CFTR with the apical membrane plays in agonist-dependent activation of the

Table 1
Kinetic constants for agonist-induced changes in chloride channel activity and inhibition of endocytosis

Cell line	Agonist	^{125}I-efflux		Endocytosis of FITC-dextran	
		K_m (μM)	maximum rate (percent of control)	K_i (μM)	minimum rate (percent of control)
T84	forskolin	0.79 ± 0.16	430%	0.26 ± 0.05	33%
	CPT-cAMP	69 ± 13	400%	N.D.	N.D.
9HTEo$^-$	forskolin	0.20 ± 0.06	180%	3.3 ± 1.1	29%
	CPT-cAMP	70 ± 34	150%	0.5 ± 0.2	50%

N.D., no inhibition was observed at concentrations up to 100 μM CPT-cAMP. Data are from best fits to Eq. 1; means ± S.E. are given for K_m and K_i.

CFTR chloride channel is uncertain. Previous studies with T84 cells have led to conflicting conclusions. There is convincing evidence for the presence of an intracellular population of CFTR [15,16,19] and both biochemical and immunological studies have clearly demonstrated that the CFTR is present in the apical membrane of stimulated and nonstimulated T84 cells [18,19]. In addition, a recent report has claimed that in T84 cells forskolin inhibits endocytosis of the CFTR [17]. However, the noted correlation between cAMP-dependent activation of the CFTR and alteration of the rates of endocytosis and exocytosis does not establish that the two events are causally related. An alternative explanation for these results is that while endosomal fusion and activation of the CFTR are both cAMP-dependent processes, alterations in the rates of endocytosis and exocytosis have relatively minor effects on the steady-state level of apical CFTR because most of the CFTR is present in the apical membrane in the absence of agonist. This suggests that the only proper test of the fusion hypothesis is to determine if maximal channel activity requires endosomal fusion.

In these studies we have attempted to resolve these difficulties by comparing dose response curves for agonist-dependent activation of the CFTR and agonist-dependent inhibition of the steady-state rate of endocytosis. These studies were performed under conditions where changes in the steady-state rate of endocytosis will have measurable effects on the level of apical CFTR. The interconversion of apical and endosomal CFTR can be described by the kinetic scheme,

$$CFTR_e \underset{k_{exo}}{\overset{k_{endo}}{\rightleftharpoons}} CFTR_a$$

where $CFTR_e$ and $CFTR_a$ are endosomal and apical CFTR, respectively; and k_{exo} and k_{endo} are rate constants for exocytosis and endocytosis of the CFTR, respectively. Provided in the absence of agonist apical CFTR represents no more than 67% of total CFTR ($CFTR_a + CFTR_e$), a 3-fold decrease in the rate of endocytosis as in Fig. 4A with no change in the rate of exocytosis will increase apical CFTR by more than 30%; a 2-fold decrease in the rate of endocytosis will increase apical CFTR by 20%. The percentage change in apical CFTR induced by agonist-dependent inhibition of k_{endo} will be greater if the fraction of total CFTR in the apical membrane in the absence of agonist is less than 67%. Changes in apical CFTR are also dependent on k_{exo}. If k_{exo} is increased by agonist, agonist-dependent changes in apical CFTR will be greater than the values given above, while an agonist-dependent decrease in k_{exo} would decrease the predicted change in apical CFTR. We have arbitrarily limited our analysis to conditions where apical CFTR is less than 67% of total CFTR because under this condition the maximal activation by endocytosis would be no more than 50%. In addition we have not considered the possibility that the rate of endocytosis is inhibited by agonists because this is inconsistent with the central tennent of the fusion hypothesis. Thus, under these conditions, a 20 to 30% increase in apical CFTR is predicted by inhibitions of endocytosis of the magnitude observed in Figs. 4 and 5. As 20 to 30% changes in CFTR activity can be easily detected with the I-efflux assay, comparison of the steady-state rates of endocytosis and channel activity provides a rigorous test of the fusion hypothesis.

Three criteria for testing the fusion hypothesis have been used. (1) K_i values for agonist-dependent decreases in the steady-state rate of exocytosis should be similar to K_a values for agonist-dependent activation of apical chloride channel activity. (2) All agonists that activate chloride channel activity should have similar effects on the steady-state rate of endocytosis. (3) As the regulation of endocytosis and the role endocytosis plays in the regulation of apical CFTR activity may vary with cell type, we have compared dose responses for agonist-dependent endocytosis and CFTR activation in two cell lines, T84 and 9HTEo$^-$. Both have previously been cited as examples of cells where endocytosis plays a role in apical CFTR activation [11,13]. Evidence that is inconsistent with the fusion hypothesis was obtained in both cell lines.

In T84 cells forskolin inhibits endocytosis with a lower K_i than the K_a for chloride channel activation (Fig. 4A). These data could be consistent with the fusion hypothesis if at low concentrations of forskolin closed channels are retained in the apical membrane and at higher concentrations of forskolin these channels are activated. However, as shown in Fig. 4B, this does not appear to be the case. CPT-cAMP activates apical chloride channels to a maximal level of activity that is equivalent to the maximal activation with forskolin and this occurs without any detectable inhibition of endocytosis, suggesting that inhibition of endocytosis by forskolin is not required for apical CFTR activation. Previous descriptions of CPT-cAMP-dependent inhibition of endocytosis have used 1 mM CPT-cAMP [11], a concentration more than 10-times greater than the K_a for channel activation. In our study no inhibition of endocytosis was seen at CPT-cAMP concentrations that activate apical chloride channels. Thus, agonist-dependent inhibition of endocytosis does not appear to be required for activation of apical CFTR in T84 cells.

A different picture is observed in 9HTEo$^-$ cells. With forskolin, channel activation occurs at a significantly lower concentration than inhibition of endocytosis; 80% of the maximal channel activation occurs at a concentration of forskolin where less than 20% of the maximal inhibition of endocytosis has occurred. Additional activation of CFTR activity, attributable to in-

creased levels of apical CFTR are not seen at concentrations of forskolin that maximally inhibit endocytosis. Three explanations are possible: (1) endocytosis of the CFTR, as opposed to total endocytosis, is not inhibited at these concentrations of forskolin, (2) inhibition of exocytosis of the CFTR also occurs so that there is no net change in the level of apical CFTR, or (3) the fraction of total CFTR in the apical membrane in the absence of activation is so large that the relatively small changes caused by decreasing the rate of endocytosis has a minimal effect on the level of apical CFTR and apical chloride channel activity. In any case, endosomal fusion is not playing a significant role in the activation of apical CFTR activity, and the data in Fig. 5A are most consistent with CFTR activation being unrelated to endosomal fusion.

Our studies are most consistent with reductions in the steady-state rate of endocytosis playing at best a minimal role in the regulation of apical CFTR activity. However the data also show that cAMP is a potent regulator of the rate of endocytosis, as has been shown by others [11,13,14]. The most likely explanation is that CFTR activation and exocytosis are both regulated by cAMP-dependent mechanisms, but that they are separate processes. Thus, even though the CFTR is present in endosomes and therefore in principle its level in the apical membrane can be regulated by fusion, this appears to have a minimal effect on apical CFTR activity; most likely because in the absence of agonist, apical CFTR represents a significant fraction of total CFTR so that changes in the level of apical CFTR, due to changes in the rates of endocytosis and exocytosis, have little effect on the amount of total CFTR in the apical membrane.

Acknowledgments

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References

[1] Quinton, P.M. (1990) FASEB J. 4, 2709–2717.
[2] Riordan, R., Rommens, M., Kerem, B.-S., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J.-C., Drumm, L., Iannuzzi, C., Collins, F., Tsui, L.-C. (1989) Science 245, 1066–1072.
[3] Kerem, B.-S., Rommens, M., Buchanan, A., Markiewicz, D., Cox, T., Chakravarti, A., Buchwald, M., Tsui, L-C. (1989) Science 245, 1073–1085.
[4] Anderson, P., Gregory, J., Thompson, S., Souza, D., Paul, S., Mulligan, R., Smith, A., Welsh, M. (1991) Science 253, 202–205.
[5] Kartner, N., Hanrahan, W., Jensen, J., Naismith, S., Sun, C., Ackerley, F., Reyes, E., Tsui, L.-C., Rommens, J., Bear, C., Riordan, J. (1991) Cell 64, 681–691.
[6] Bear, C., Li, C., Kartner, N., Bridges, R., Jensen, T., Ramjeesingh, M., Riordan, J. (1992) Cell 68, 809–818.
[7] Anderson, M., Berger, H., Rich, D., Gregory, R., Smith, A., Welsh, M. (1991) Cell 67, 775–784.
[8] Tabcharani, T., Chang, X.-B., Riordan, J., Hanrahan, J. (1991) Nature 352, 628–631.
[9] Cheng, H., Rich, D., Marshal, J., Gregory, R., Welsh, M., Smith, A. (1991) Cell 66, 1027–1036.
[10] Quinton, P., Reddy, M. (1992) Nature 360, 79–81.
[11] Bradbury, A., Jilling, T., Kirk, K., Bridges, R. (1992) Am. J. Physiol. 262, C752–C759.
[12] Bradbury, N., Jilling, T., Berta, G., Sorscher, E., Bridges, R., Kirk, K. (1992) Science 256, 530–532.
[13] Schwiebert, M., Kizer, N., Gruenert, D., Stanton, B. (1992) Proc. Natl. Acad. Sci. USA 89, 10623–10627.
[14] Stanton, B., Gesek, F., Ercolani, L., Wjasow, C., Gruenert, D., Karlson, K., Schwiebert, E. (1993) Ped. Pul. Suppl. 8, 230.
[15] Fuller, C., Bridges, R., Benos, D. (1993) Ped. Pul. Suppl. 8, 224.
[16] Bradbury, N., Cohn, J., Venglarik, C., Bridges, R. (1994) J. Biol. Chem. 269, 8296–8302.
[17] Prince, L., Marchase, R. (1993) Ped. Pul. Suppl. 8, 203.
[18] Denning, D., Ostedgaard, L., Cheng, S., Smith, A., Welsh, M. (1992) J. Clin. Invest. 89, 339–349.
[19] Prince, L., Tousson, A., Marchase, R. (1993) Am. J. Physiol. 264, C491–C498.
[20] Reenstra, W. (1993) Am. J. Physiol. 264, C161–C168.
[21] Venglarik, C., Bridges, R., Frizzell, R. (1990) Am. J. Physiol. 259, C358–C364.
[22] Haws, C., Krouse, M., Xia, Y., Gruenert, D., Wine, J. (1992) Am. J. Physiol. 263, L692–L707.
[23] Reenstra, W., Sabolic, I., Bae, H.-R., Verkman, A. (1992) Biochemistry 31, 175–181.
[24] Lencer, W., Weyer, P., Verkman, A., Ausiello, D., Brown, D. (1990) Am. J. Physiol. 258, C309–C317.
[25] Dorstmann, W., Taylor, S., Genieser, H.-G., Jastorff, B., Doskeland, S., Ogreid, D. (1990) J. Biol. Chem. 265, 10484–10491.