Regulation of Plasma Membrane Recycling by CFTR

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The gene that encodes the cystic fibrosis transmembrane conductance regulator (CFTR) is defective in patients with cystic fibrosis. Although the protein product of the CFTR gene has been proposed to function as a chloride ion channel, certain aspects of its function remain unclear. The role of CFTR in the adenosine 3′,5′-monophosphate (cAMP)–dependent regulation of plasma membrane recycling was examined. Adenosine 3′,5′-monophosphate is known to regulate endocytosis and exocytosis in chloride-secreting epithelial cells that express CFTR. However, mutant epithelial cells derived from a patient with cystic fibrosis exhibited no cAMP-dependent regulation of endocytosis and exocytosis until they were transfected with complementary DNA encoding wild-type CFTR. Thus, CFTR is critical for cAMP-dependent regulation of membrane recycling in epithelial tissues, and this function of CFTR could explain in part the pleiotropic nature of cystic fibrosis.

The cystic fibrosis (CF) transmembrane conductance regulator (CFTR) can function as a cAMP-activated Cl^- channel within epithelial tissues, where it regulates plasma membrane Cl^- conductance (*1–10*). We tested the hypothesis that CFTR is important in the cAMP-dependent regulation of endocytosis and exocytosis (that is, plasma membrane recycling) in epithelial cells. This hypothesis is based on the following observations: (i) cAMP regulates plasma membrane recycling, in addition to Cl^- conductance, in a colonic epithelial cell line (T_{84}) that expresses large amounts of CFTR (*11, 12*) and (ii) Cl^- channels have been implicated as facilitators of exocytosis in a variety of cell types (*13, 14*).

We used both optical and biochemical techniques to assay the endocytosis and subsequent recycling of fluid and membrane markers in three epithelial cell lines: (i) CF pancreatic cells (CFPAC) that were cultured from a pancreatic adenocarcinoma of ductal origin isolated from a patient homozygous for the most common CF mutation, ΔF508 (*15*), (ii) CFPAC cells that were stably transfected with a retroviral vector (PLJ) that contained the full-length cDNA encoding wild-type CFTR (CFPAC-PLJ-CFTR) (*3*), and (iii) CFPAC cells that were mock-transfected with vector alone (CFPAC-PLJ) (*3*). The CFPAC-PLJ-CFTR cells expressed detectable wild-type CFTR mRNA (Fig. 1). Chloride ion permeability, determined with an anion efflux assay (*3*) and a fluorescence assay of halide influx (*4, 16*), was stimulated by the adenylate cyclase activator forskolin in CFPAC-PLJ-CFTR cells, but not in parental CFPAC cells or the CFPAC-PLJ cells.

The expression of wild-type CFTR in CFPAC cells confers not only a forskolin-stimulated halide permeability but also a forskolin-dependent regulation of the endocytosis of fluid phase markers such as horseradish peroxidase (HRP) (Fig. 2). We monitored HRP endocytosis by measuring latent peroxidase activity that was trapped within a specific membrane vesicle fraction from a sucrose gradient—that is, endosomes (*12*). Forskolin treatment inhibited the degree of HRP endocytosis by CFPAC-PLJ-CFTR cells but had no effect on HRP endocytosis in either the mock-transfected or untransfected CFPAC cells (Fig. 2A). Because data were obtained from a number of different CFPAC-PLJ-CFTR and CFPAC-PLJ clones (*3*), it is unlikely that the forskolin-dependent inhibition of endocytosis, seen only in cells that expressed wild-type CFTR, is due to clonal variation. Forskolin-dependent inhibition of endocytosis was also seen in crude microsomal fractions from CFPAC-PLJ-CFTR cells, which confirmed a net reduction in endocytic rates rather than a redistribution of marker to different fractions on a sucrose gradient (*17*). Similar results were obtained with quantitative fluorescence microscopy to monitor the internalization of fluorescent dextran into endocytic vesicles (*12*). Again, forskolin inhibited endocytosis only in CFPAC cells that expressed wild-type CFTR (Fig. 2B).

In order to examine the involvement of CFTR in controlling the other limb of membrane recycling, exocytosis, we assayed the recycling of a previously internalized membrane marker (biotinylated wheat germ agglutinin, or bWGA) back to the plasma membrane. Cells that had endocytosed bWGA at 37°C were allowed to recycle the marker back to the cell surface in the presence or absence of activators of cAMP-

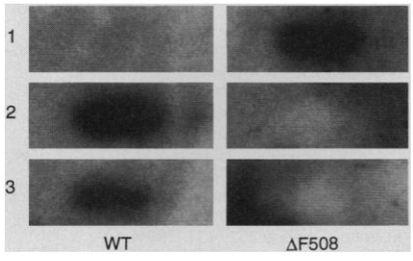

Fig. 1. Expression of wild-type CFTR mRNA in CFPAC-PLJ-CFTR cells. Allele-specific oligonucleotide hybridization was performed with probes specific for wild-type CFTR cDNA (WT) and ΔF508 CFTR cDNA (ΔF508) (*21*). Hybridization of WT and ΔF508 probes to the PCR product after amplification of cDNA that contained the ΔF508 mutation (lane 1); of wild-type CFTR cDNA from T_{84} cells (lane 2); and of CFTR cDNA from CFPAC-PLJ-CFTR cells (lane 3).

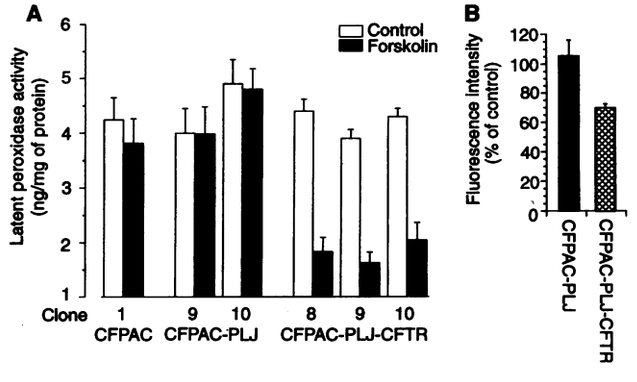

Fig. 2. Effect of expression of wild-type CFTR in CFPAC cells on forskolin-dependent endocytosis. (**A**) Endocytosis of HRP (*22*) by untransfected CFPAC cells (CFPAC clone 1) (n = 16 observations), mock-transfected CFPAC cells [CFPAC-PLJ; clones 9 (n = six observations) and 10 (n = six observations)], and cells transfected with virus that contained wild-type CFTR [CFPAC-PLJ-CFTR; clones 8 (n = four observations), 9 (n = six observations), and 10 (n = four observations)]. (**B**) Effect of forskolin on endocytosis of rhodamine-dextran (*22*) by mock-transfected CFPAC cells (CFPAC-PLJ) (n = three experiments, five measurements per experiment: clone 6 = two experiments; clone 10 = one experiment), and cells transfected with virus that contained wild-type CFTR (CFPAC-PLJ-CFTR) (n = three experiments, five measurements per experiment: clone 6 = two experiments; clone 20 = one experiment). Shown are the changes in fluorescent marker uptake induced by forskolin (50 μM) relative to untreated controls. Results are the mean ± SEM.

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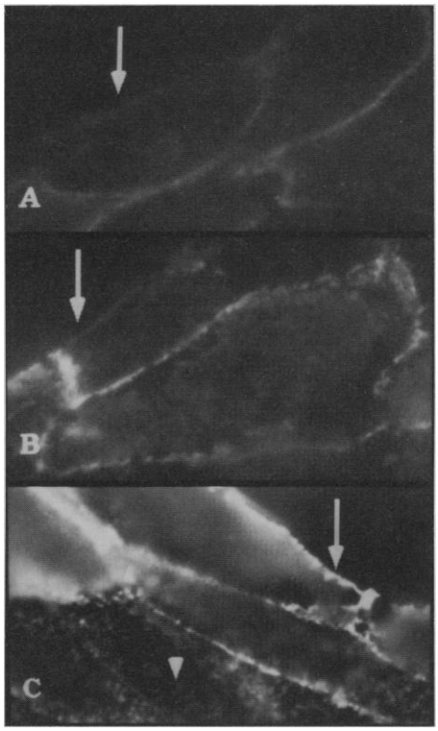

Fig. 3. Effect of cAMP on the recycling of internalized bWGA to the plasma membrane of CFPAC-PLJ-CFTR cells. Recycling of internalized marker to the cell surface (*23*) was performed under the following conditions: (**A**) 4°C, (**B**) 37°C, and (**C**) 37°C plus CPTcAMP (500 μM). Arrows, edges of cell islands; arrowhead, microvillus on cell surface. Magnification, ×1160.

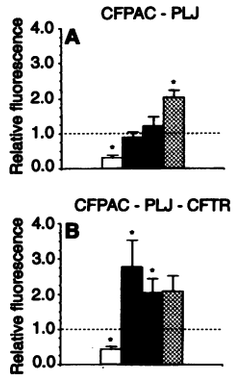

Fig. 4. Effect of forskolin, cAMP, and ionomycin on bWGA recycling to the plasma membrane. (**A**) CFPAC-PLJ cells (clones 6 and 10) and (**B**) CFPAC-PLJ-CFTR cells (clones 7 and 20). Cells were treated with Texas Red–avidin as described (*23*). For each experiment, four cover slips of cells were treated identically except that during the 30-min recycling period, cells were exposed to either 4°C, 37°C without any treatment, 37°C plus ionomycin (1 μM), or 37°C with either forskolin (50 μM) or CPTcAMP (500 μM). Fluorescence was normalized to the untreated control at 37°C. Values greater than 1.0 represent recycling rates greater than those in untreated controls (*21*). Data are mean ± SEM and were obtained as described (*24*). White bars, 4°C; dark bars, forskolin; gray bars, CPTcAMP; crosshatched bars, ionomycin. CFPAC-PLJ: 4°C (n = 10), forskolin (n = 7), CPTcAMP (n = 3), and ionomycin (n = 6); CFPAC-PLJ-CFTR: 4°C (n = 9), forskolin (n = 6), CPTcAMP (n = 3), and ionomycin (n = 6). Asterisk, $P < 0.05$ compared to untreated control by paired t test. No differences between CFPAC-PLJ clones or between CFPAC-PLJ-CFTR clones were detected; thus, the reported data are averaged over the two clones examined for each group of cells (*25*). Results obtained with a 10-min bWGA recycling period also showed stimulation by CPTcAMP (2.42 ± 0.78–fold over untreated control at 37°C; n = 4) and ionomycin (3.33 ± 0.36–fold; n = 4) of marker recycling in CFPAC-PLJ-CFTR cells (clones 10 and 14).

dependent protein kinase. The recycled bWGA was visualized by fluorescence microscopy after treatment of the cells with a fluorescent avidin conjugate, which bound the bWGA on the cell surface. During a 30-min period of bWGA recycling, CFPAC-PLJ-CFTR cells were incubated either in the cold (4°C) or at 37°C with or without a membrane-permeant cAMP analog, 8-(4-chlorophenylthio)cAMP (CPTcAMP) to directly increase cellular cAMP levels (Fig. 3). These images illustrate that the degree of bWGA recycling to the plasma membranes of CFPAC-PLJ-CFTR cells was stimulated by CPTcAMP and was inhibited at low temperature, as expected for membrane recycling.

We used digital image processing to quantify the degree of cell surface fluorescence attributable to bWGA recycling in both CFPAC-PLJ and CFPAC-PLJ-CFTR cells (Fig. 4). Neither forskolin nor CPTcAMP stimulated bWGA recycling in CFPAC-PLJ cells. In addition, the Ca^{2+} ionophore ionomycin, which stimulates the Cl^- permeabilities of both CFPAC-PLJ-CFTR and CFPAC-PLJ cells (*3*), also stimulated bWGA recycling in both cell types, which indicates that the membrane recycling machinery was intact. The degree of bWGA recycling to the surfaces of CFPAC-PLJ-CFTR cells was stimulated by either forskolin or CPTcAMP. Thus, activation of the cAMP-mediated second messenger cascade stimulated the recruitment of marker to the cell surface only in those cells that expressed wild-type CFTR. The Ca^{2+} ionophore appears to stimulate membrane recycling [and presumably Cl^- permeability as well (*15, 18*)] by means of a process that is independent of CFTR.

Our results demonstrate that expression of normal CFTR in pancreatic cells from a patient with CF confers upon these cells a cAMP-dependent regulation of plasma membrane recycling. Endocytosis and exocytosis were coordinately regulated by cAMP in those cells that expressed wild-type CFTR; specifically, elevations in cAMP levels led to decreases in endocytosis and increases in the exocytic insertion of previously internalized membrane into the plasma membrane. This coordinate regulation of membrane turnover could result in a net recruitment of membrane and membrane proteins to the cell surface in response to cAMP. We have observed a similar cAMP-dependence of membrane recycling in native (that is, untransfected) colonic epithelial cells that express wild-type CFTR (*11, 12*), which implies that this mode of regulating membrane turnover may be a general consequence of CFTR expression in epithelial cells.

Our results suggest that there are consequences of CFTR function that extend beyond the regulation of plasma membrane Cl^- conductance. Although the mechanism by which CFTR controls both membrane recycling and Cl^- conductance is unclear, it is possible that CFTR functions as a Cl^- channel, not only in the plasma membrane, but also within intracellular membrane compartments where it could influence the trafficking of membrane and associated membrane proteins. The possibility that mature CFTR can function in intracellular membrane compartments is supported by the finding that certain vesicle populations (for example, prelysosomes) are less acidic in airway epithelial cells from CF patients than those from normal individuals (*19*). Vesicular CFTR could be functioning as a Cl^- channel, stimulating acidification by shunting the voltage generated, by electrogenic H^+ entry (*20*). Whatever the mechanism by which CFTR regulates both Cl^- permeability and membrane recycling, it is clear that CF epithelial cells exhibit an altered regulation of endocytosis and exocytosis. Such alterations in the recycling of plasma membrane proteins could contribute significantly to the pleiotropic nature of cystic fibrosis.

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21. Polyadenylated mRNA isolated from CFPAC-PLJ-CFTR cells (Micro-Fast Track Kit, Invitrogen) was reverse-transcribed with random hexamers, and the resultant cDNA was amplified by 30 cycles of the polymerase chain reaction (PCR) (Gene Amp

RNA PCR Kit; Perkin-Elmer Cetus) with the following CFTR-specific primers: 5′-GCTGGATCCACTGGAGCAGGCAAG-3′ (exon 9) and 5′-AATTCTTGCTCGTTGACCTCCACT-3′ (exon 11). The size of the PCR product was identical to that predicted (300 bp). The PCR products were transferred to nylon membrane and hybridized with radiolabeled oligonucleotide probes, as described [E. J. Sorscher and Z. Huang, *Lancet* **337**, 1115 (1991)]. We could detect wild-type, but not mutant, mRNA in CFPAC-PLJ-CFTR cells after one round of PCR amplification, which implied that small amounts of the endogenous mutant gene were expressed, as previously reported (*3*).

22. Cell monolayers incorporated HRP by endocytosis as described (*12*). A membrane fraction enriched in endosomes was prepared by sucrose density gradient centrifugation of cell homogenates. We determined HRP activity specifically located within endosomes by monitoring latent peroxidase activity—that is, the increase in measured peroxidase activity that resulted from the release of HRP from endosomes after treatment with detergent. For fluorescent studies, cells were exposed to rhodamine-dextran (formula weight: 10,000), and the average fluorescent intensity of labeled vesicles (200 to 400 vesicles per image) was determined in five randomly selected fields with fluorescent microscopy as described (*12*). In contrast to the HRP enzyme assay, the fluorescent assay sampled the appearance of marker in relatively large, optically detectable compartments. This technical difference likely accounts for the differing degrees of endocytosis inhibited by forskolin observed with the two assays.
23. Cells grown on glass cover slips were exposed (2 hours at 37°C) to bWGA (1 μg/ml). After internalization of this marker, any remaining surface marker was saturated with unlabeled avidin (200 μg/ml for 15 min). Cells were then washed in avidin-free buffer and incubated under the appropriate conditions at 37°C for 30 min, during which they recycled the previously internalized bWGA back to the cell surface where it was detected by exposure of the cells to Texas Red–avidin (2.5 μg/ml for 30 min at 4°C). Fluorescent microscopic images were collected with a Zeiss IM-35 inverted microscope, ×40 objective lens, and cooled charge-coupled device video camera (Photometrics).
24. In each experiment, six images per cover slip were selected at random (one to two islands of cells or 40 to 200 cells per image). The focal plane was selected so that the apical cell surfaces (identified by the appearance of microvilli) were in focus. For each island, the fluorescence on the cell surface was quantified with interactive software that permitted tracing the edge of the island and calculating the mean fluorescence intensity within the traced island. Background fluorescence (mean fluorescent intensity of a measurement window located off the island) was subtracted to give a single value for each cover slip, which represented the mean intensity of several hundred cells within six randomly selected fields.
25. Of the two CFPAC-PLJ-CFTR clones that were assayed for membrane recycling as well as for endocytosis, clone 10 exhibited a 3.18 ± 1.66–fold increase in marker recycling over untreated controls in response to cAMP treatment (n = 2; compare to Fig. 2A). Clone 20 exhibited a 3.14 ± 0.84–fold increase after forskolin treatment (n = 5). None of the mock-transfected clones exhibited any cAMP responsiveness with any of the three assays of endocytosis or exocytosis.
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Regulation of Arterial Tone by Activation of Calcium-Dependent Potassium Channels

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Blood pressure and tissue perfusion are controlled in part by the level of intrinsic (myogenic) vascular tone. However, many of the molecular determinants of this response are unknown. Evidence is now presented that the degree of myogenic tone is regulated in part by the activation of large-conductance calcium-activated potassium channels in arterial smooth muscle. Tetraethylammonium ion (TEA^+) and charybdotoxin (CTX), at concentrations that block calcium-activated potassium channels in smooth muscle cells isolated from cerebral arteries, depolarized and constricted pressurized cerebral arteries with myogenic tone. Both TEA^+ and CTX had little effect on arteries when intracellular calcium was reduced by lowering intravascular pressure or by blocking calcium channels. Elevation of intravascular pressure through membrane depolarization and an increase in intracellular calcium may activate calcium-activated potassium channels. Thus, these channels may serve as a negative feedback pathway to control the degree of membrane depolarization and vasoconstriction.

Smooth muscle cells in small arteries depolarize and contract in response to increased intravascular pressure. The resulting vasoconstriction is independent of regional innervation and circulating hormones and has been termed myogenic (*1*). Myogenic tone is abolished by the removal of external Ca^{2+}, by Ca^{2+} channel blockers (*2*), and by membrane hyperpolarization (*3*), which suggests that myogenic tone depends on Ca^{2+} influx through voltage-dependent Ca^{2+} channels (*4*). The ability of arteries to grade myogenic tone and to sustain it at steady levels suggests the ability to grade depolarization and Ca^{2+} entry. One way this could be achieved is if Ca^{2+} entry and depolarization are opposed by hyperpolarizing mechanisms linked to Ca^{2+} accumulation in cells. Thus, a negative feedback (vasodilating) pathway might exist that is activated by pressure, intracellular free Ca^{2+}, or membrane depolarization. Interruption of this pathway could lead to poorly controlled and perhaps highly constricted arteries. The large-conductance Ca^{2+}-activated K^+ (K_{Ca}) channel is an ideal candidate for such a pathway because it is activated by both membrane depolarization and intracellular Ca^{2+} (*5*). However, although K_{Ca} channels exist in a variety of smooth muscle types, there is little evidence for any function of these channels in

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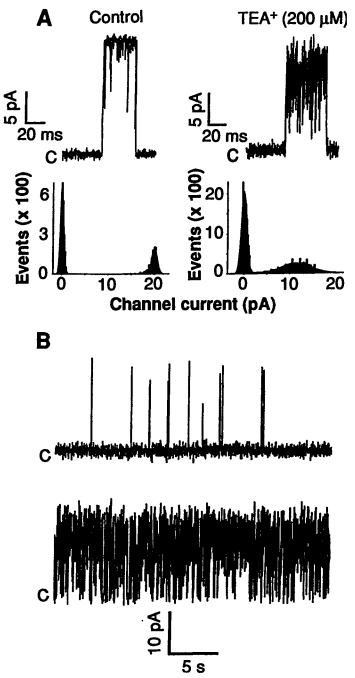

Fig. 1. Blockage of Ca^{2+}-activated (K_{Ca}) channels by external TEA^+. (**A**) Recordings and amplitude histograms from two inside-out patches from rabbit cerebral arteries held at +50 mV. According to the usual convention, membrane potentials are expressed inside relative to outside, and outward currents are given a positive sign. Pipette solutions contained no TEA^+ (left) and 200 μM TEA^+ (right). Gaussian curves were fitted to the peaks of the control amplitude histogram to yield a unitary current of 20.0 pA. The TEA^+ reduced the mean unitary current and broadened the open level peak. The mean amplitudes of unitary current before and after treatment with TEA^+ (200 μM) were 5.85 and 2.85 pA (n = 4) at 0 mV and 16.5 and 9.3 pA (n = 6) at +50 mV. (**B**) Ca^{2+}-activation of K_{Ca} channels. An inside-out patch was held at +50 mV in the presence of external TEA^+ (200 μM). Intracellular Ca^{2+} concentration was raised from approximately 1 nM (top) to 12.5 μM (bottom). The bath contained 136 mM KCl, 1 mM EGTA, 1 mM $MgCl_2$, and 15 mM Hepes (pH 7.2). The pipette solution contained 137 mM NaCl, 5.4 mM KCl, 0.55 mM KH_2PO_4, 2 mM $MgCl_2$, 4.17 mM $NaHCO_3$, 2.0 mM $CaCl_2$, 0.05 mM EGTA, and 10 mM Hepes (pH 7.4). The closed level of the channel is indicated by C; filter, 2.5 kHz; sample rate, 10 kHz.