Mechanisms of CFTR regulation by syntaxin 1A and PKA

Steven Y. Chang[1,*], Anke Di[2,*], Anjaparavanda P. Naren[3], H. Clive Palfrey[2], Kevin L. Kirk[3] and Deborah J. Nelson[2,‡]

[1]Department of Medicine, Section of Pulmonary and Critical Care Medicine, The University of Chicago Hospitals, 5841 S. Maryland Avenue, MC 6026, Chicago, IL 60637, USA
[2]Department of Neurobiology, Pharmacology and Physiology, The University of Chicago, 947 East 58th St, MC 0926, Chicago, IL 60637, USA
[3]Department of Physiology and Biophysics, Gregory Fleming James Cystic Fibrosis Research Center, University of Alabama at Birmingham, 1918 University Blvd, MCLM 985, Birmingham, AL 35294, USA
*These authors contributed equally to this work
‡Author for correspondence (e-mail: dnelson@drugs.bsd.uchicago.edu)

Accepted 4 November 2001
Journal of Cell Science 115, 783-791 (2002) © The Company of Biologists Ltd

Summary

Activation of the chloride selective anion channel CFTR is stimulated by cAMP-dependent phosphorylation and is regulated by the target membrane t-SNARE syntaxin 1A. The mechanism by which SNARE proteins modulate CFTR in secretory epithelia is controversial. In addition, controversy exists as to whether PKA activates CFTR-mediated Cl^- currents (I_{CFTR}) by increasing the number of channels in the plasma membrane and/or by stimulating membrane-resident channels. SNARE proteins play a well known role in exocytosis and have recently been implicated in the regulation of ion channels; therefore this investigation sought to resolve two related issues: (a) is PKA activation or SNARE protein modulation of CFTR linked to changes in membrane turnover and (b) does syntaxin 1A modulate CFTR via direct effects on the gating of channels residing in the plasma membrane versus alterations in membrane traffic. Our data demonstrate that syntaxin 1A inhibits CFTR as a result of direct protein-protein interactions that decrease channel open probability (P_o) and serves as a model for other SNARE protein-ion channel interactions. We also show that PKA activation can enhance membrane trafficking in some epithelial cell types, and this is independent from CFTR activation or syntaxin 1A association.

Key words: Membrane capacitance, FM1-43, Exocytosis, Endocytosis, SNARE proteins, Trafficking, Chloride channel, Voltage clamp

Introduction

It is not surprising that many layers of regulation exist for ubiquitous proteins such as ion channels as they play central roles in inter- and intracellular signaling in fluid and ion transport and in the development of memory. Ion channels and transporters are regulated by various mechanisms, including membrane potential, ligands, phosphorylation state, other ion channels, intracellular calcium concentration and second messengers (Hille, 1992). Syntaxin 1A is a target membrane t-SNARE that is classically thought to mediate vesicle trafficking along with other SNARE complex proteins including SNAP-25 and synaptobrevin (Wu et al., 1999). The first functional association observed for syntaxin 1A and ion channels/transporters was found in the brain where membrane-anchored syntaxin 1A modulates N-type calcium channel activity (Bennett et al., 1992). Regulation of both ion channels and transporters by syntaxin 1A has since been observed in a number of systems including CFTR, the amiloride-sensitive epithelial sodium channel, the GABA transporter and potassium channels (Bezprozvanny et al., 1995; Bezprozvanny et al., 2000; Deken et al., 2000; Fili et al., 2001; Naren et al., 2000; Naren et al., 1997; Naren et al., 1998; Peters et al., 1999; Qi et al., 1999; Rettig et al., 1996; Saxena et al., 1999; Sheng et al., 1996; Sheng et al., 1994; Yang et al., 1999). Despite the observations that syntaxin 1A functionally interacts with multiple ion channels, the mechanism of channel regulation by this t-SNARE is not known and may include indirect regulation by modulation of membrane trafficking or direct protein-protein interactions.

The problem of channel regulation has increased complexity in the case of CFTR, where cAMP not only activates the channel through protein-kinase-A-dependent phosphorylation but in some investigations appears to mobilize heterologously expressed CFTR into the plasma membranes of *Xenopus* oocytes (Takahashi et al., 1996). The question of whether an enhancement of membrane trafficking accompanies CFTR activation, independent of syntaxin modulation, remains controversial. For example, CFTR activation in Calu-3 cells (a human lung adenocarcinoma-derived line) as well as Madin Darby canine kidney (MDCK) cells is not accompanied by changes in membrane insertion and retrieval (Chen et al., 2001; Loffing et al., 1998; Moyer et al., 1998). However, a number of other investigations carried out in a variety of cell types demonstrated that cAMP facilitates translocation of CFTR from an intracellular compartment to the plasma membrane (Bradbury et al., 1992; Howard et al., 2000; Peters et al., 1999; Schwiebert et al., 1994; Takahashi et al., 1996).

Experiments carried out in this study sought to resolve two related issues: (a) is PKA activation or SNARE protein modulation of CFTR linked to changes in membrane turnover

and (b) does syntaxin 1A modulate CFTR via direct effects on the gating of channels residing in the plasma membrane versus alterations in membrane traffic. Using a combination of membrane capacitance measurements coupled with FM1-43 fluorescence we demonstrate that cAMP-dependent changes in membrane turnover that accompany CFTR activation are cell specific and can be revealed in some cell types only when dynamin-dependent endocytosis is inhibited. In addition, cAMP-dependent changes in membrane turnover are independent of the disruption of syntaxin 1A-CFTR interactions.

We have previously shown that syntaxin 1A and CFTR physically interact and that this interaction results in a negative modulation of cAMP-mediated CFTR function (Naren et al., 1997; Naren et al., 1998; Naren et al., 1999; Naren et al., 2000). Furthermore, three different reagents rescue CFTR from inhibition by plasma-membrane-anchored syntaxin 1A, namely, botulinum toxin C1, the syntaxin-binding protein Munc 18 and the cytosolic domain of syntaxin 1A (Naren et al., 1997). The interaction between CFTR and membrane-anchored syntaxin 1A is dynamic (reversible) and is dependent upon an intact actin cytoskeleton. Data in this investigation demonstrate that while changes in membrane turnover do not appear to be dependent upon the presence or absence of a direct interaction between membrane-anchored syntaxin 1A and CFTR, the interaction does result in a decrease in single channel open state probability. Disruption of the t-SNARE-channel interaction by the cytosolic domain of syntaxin 1A results in a significant increase in CFTR open-state probability in excised patches. These results indicate that binding of full length syntaxin 1A to CFTR inhibits PKA-dependent activation through direct protein-protein interaction. We speculate that second messenger pathways that regulate the physical interaction of these two proteins via alterations in the actin cytoskeleton or CFTR-syntaxin-Munc-18 interactions will fine tune channel activity through subtle changes in membrane-resident channel gating and not alterations in membrane turnover.

Materials and Methods

Immunoprecipitation and binding assay

Proteins, immunoprecipitated with 1 mg each of CFTR (Genzyme C-terminal monoclonal), non-immune serum (normal Mouse, Santa Cruz Biotech) or syntaxin 1A IgG immobilized on Protein A/G beads (Naren et al., 2000), were probed using an affinity-purified polyclonal antibody raised against nucleotide binding domain 1 (residues 430-580) of CFTR. Details have been published previously (Naren et al., 1997).

Electrophysiology

Whole-cell recordings were obtained from 16HBE14o- human airway epithelial and from HT29-CL19A human colonic epithelial cells using methods described previously (Hamill et al., 1981) and solutions published previously (Naren et al., 1997). The cAMP cocktail used to elicit CFTR activation in the whole-cell experiments contained 1 mM IBMX, 10 μM Forskolin and 400 μM cpt-cAMP. Peptides were added to the pipette solution at their specified concentrations. Single-channel recordings were obtained from 16HBE14o- cells using the inside-out configuration. The extracellular (pipette) solution contained 140 mM NMDG-Cl, 2 mM $MgCl_2$, 10 mM HEPES and 200 μM DIDS (to pharmacologically block non-CFTR anion channels) titrated to a pH of 7.4 with NMDG. The intracellular (bath) solution contained 140 mM NMDG-Cl, 10 mM EGTA, 2 mM $MgCl_2$, 8 mM Tris and 5 mM Mg-ATP adjusted to a pH of 7.4. Once the inside-out configuration was achieved, 75 U/ml of PKA catalytic subunit (Promega, Madison, WI) was added to activate CFTR. Subsequently, either 350 nM GST-Syn1AΔC or 350 nM GST-Syn1AΔH3 were added to the bath. In this manner, the same inside-out patch served as its own internal control. Single-channel currents were filtered at 1 kHz and sampled at 2 kHz. All experiments were conducted at room temperature (22-24°C) using an EPC-9 patch clamp amplifier (HEKA Electronik GmbH, Lambrecht, Germany) and using the Pulse V 8.31 acquisition program (HEKA Electronik GmbH, Lambrecht, Germany). Data analysis was performed using Tac V4.1.1 (Bruxton Corp., Seattle, WA, USA). Statistical significance of results was determined using the Student's *t*-test.

Simultaneous recording of membrane capacitance, conductance, and fluorescence

The EPC-9 includes a built-in data acquisition interface (ITC-16, Instrutech, Port Washington, NY, USA). The software package controlled the stimulus and data acquisition for the software lock-in amplifier in the 'sine + dc' mode as described (Gillis, 2000). The temporal resolution of the capacitance data was 40 mseconds per point using a 1 kHz, 20 mV sine wave. The holding potential in the capacitance experiments was –10 mV. The fluorescence intensity of FM1-43 excited at 470 nm was simultaneously measured with a photomultiplier system as described (Katnik and Nelson, 1993) using a Leitz inverted DM-IRB microscope. The photo-multiplier output was collected at 510 nm and averaged online. These data were stored together with the C_m and G_m measurements. Pipette and bath solutions were as described above. Peptides were added to the pipette solutions as in whole-cell experiments. All experiments were conducted at room temperature (22-24°C). Statistical significance of results was determined using the Student's *t*-test.

Results

Syntaxin 1A and CFTR associate in human airway epithelial cells

To determine if syntaxin 1A associates with CFTR in human airway epithelial cells as we have shown for canine tracheal epithelial cells (Naren et al., 2000), we performed a series of coimmunoprecipitation and whole-cell patch clamp studies on 16HBE14o- human airway epithelial cells. The mature form of CFTR (band 'C') could be coimmunoprecipitated from 16HBE14o- cells with a syntaxin 1A monoclonal antibody (Fig. 1A). Although it has been shown that CFTR binds to the H3 domain or SNARE-motif of syntaxin 1A (Jahn and Sudhof, 1999), it has also been shown that inhibition of I_{CFTR} is dependent on the presence of the C-terminal transmembrane domain (TMD) of syntaxin 1A (Naren et al., 1998). Both soluble syntaxin 1A lacking the C-terminal TMD (GST-Syn1AΔC) and the syntaxin-1A-binding protein Munc18 disrupt the regulatory interaction between CFTR and the membrane-anchored t-SNARE resulting in a marked enhancement of I_{CFTR} (Naren et al., 1997; Naren et al., 1998). This effect was also observed in human airway epithelial cells (Fig. 1B-D). Whole-cell I_{CFTR} was elicited by bath application of a cAMP cocktail (Fig. 1B). When either 350 nM GST-Syn1AΔC or 300 nM GST-Munc18 were included in the standard pipette solution in order to disrupt the syntaxin 1A-CFTR interaction, I_{CFTR} increased approximately threefold from approximately 30 to 90 pA/pF (Fig. 1D). Neither reagent affected basal currents in the absence of cAMP cocktail (Fig.

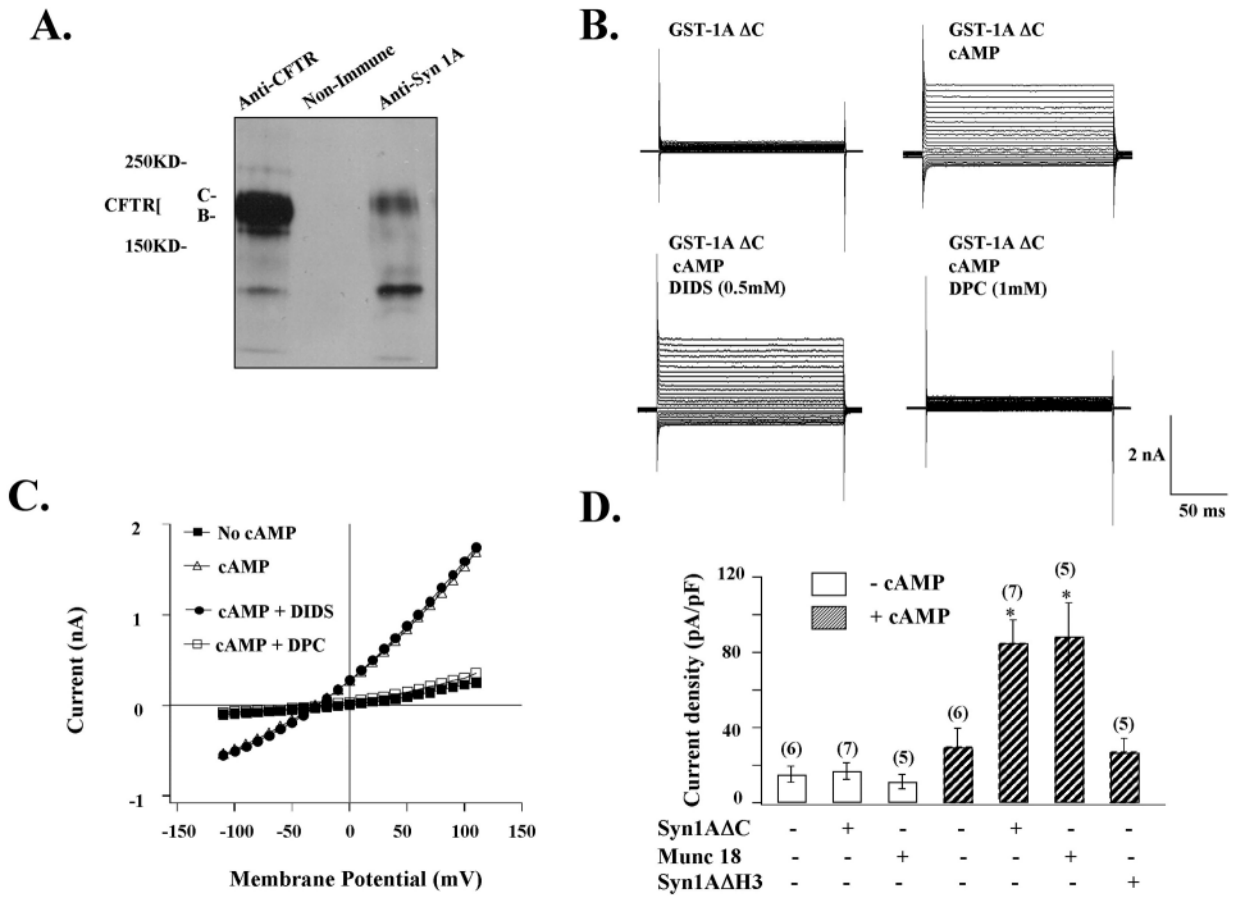

Fig. 1. Syntaxin 1A and CFTR physically and functionally interact in 16HBE14o- cells. (A) Syntaxin-1A-specific IgG (Naren et al., 2000) can coimmunoprecipitate CFTR from 16HBE14o- cells. (B) Representative whole-cell I_{CFTR} in 16HBE14o- cells maximally stimulated by addition of cAMP cocktail to the external bath with voltage steps ranging from –110 to +110 mV in increments of 10 mV in the absence and presence of 350 nM GST-Syn1AΔC. (C) Current-voltage relationship of I_{CFTR} from (B). The reversal potentials for the Cl^- current in control conditions and in the presence of GST-Syn1AΔC, GST-Munc18 and GST-Syn1AΔH3 were –18±1.5 mV (n=6), –15±2.7 mV (n=7), –10.2±1.2mV (n=5) and –14±1.5 mV (n=5), respectively. (D) The I_{CFTR} was similar irrespective of whether disruption of Syn1A-CFTR interaction was by GST-Syn1AΔC or by 300 nM GST-Munc18. Whole-cell I_{CFTR} in the presence of 350 nM GST-Syn1AΔH3 was not significantly different from the control currents. Current density for control I_{CFTR} was 29.6±10.0 pA/pF (n=6). Current densities in the presence of GST-Syn1AΔC and GST-Munc18 increased threefold to 85.0±12.3 pA/pF (n=7) and 88.4±17.9 pA/pF (n=5) respectively. When Syn1AΔH3 was included in the pipette solution, the current density was 26.9±7.4 pA/pF (n=5). Asterisk (*) indicates $p < 0.05$ when comparing the densities of either control currents or I_{CFTR} in the presence of GST-Syn1AΔH3 with currents in the presence of either GST-Syn1AΔC or GST-Munc18.

1D). To examine the specificity of this effect, we substituted a syntaxin 1A fusion protein lacking the H3 domain (GST-Syn1AΔH3) for GST-Syn1AΔC. As the H3 domain is necessary for syntaxin 1A binding to CFTR (Naren et al., 1998), GST-Syn1AΔH3 should not disrupt the syntaxin 1A-CFTR interaction and as expected, this construct did not rescue CFTR from membrane-anchored syntaxin 1A inhibition (data summarized in Fig. 1D). Characteristic of CFTR, currents under all conditions were insensitive to 500 μM 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS) but were inhibited by 1 mM diphenylamine-2-carboxylic acid (DPC) (Fig. 1C). Macroscopic current kinetics and current-voltage (IV) relationships remained unchanged by the disruption of the syntaxin 1A-CFTR interaction by either GST-Syn1AΔC (Fig. 1B,C) or GST-Munc 18.

Rescue of CFTR from inhibition by membrane-anchored syntaxin 1A by the cytosolic domain of syntaxin 1A was dependent on the actin cytoskeleton. If the 16HBE14o- cells were treated with cytochalasin D (0.5 μg/ml), which disrupts the actin cytoskeleton, cAMP-dependent current activation was not significantly different from that observed under control conditions (Fig. 2). However, the syntaxin 1A cytosolic domain failed to rescue CFTR from membrane-anchored syntaxin 1A inhibition in the cytochalasin-treated cells. Thus, the presence of an intact cytoskeleton appears to maintain the reversibility of the dynamic interaction between CFTR and membrane-anchored syntaxin 1A. This result suggested to us that CFTR current augmentation could involve a trafficking event mediated via cytoskeletal interactions.

Regulation of CFTR by syntaxin 1A and PKA does not involve membrane turnover

Reasoning that cytoskeleton-dependent vesicle insertion or inhibition of membrane endocytosis could account for the soluble syntaxin 1A rescue of CFTR inhibition by membrane

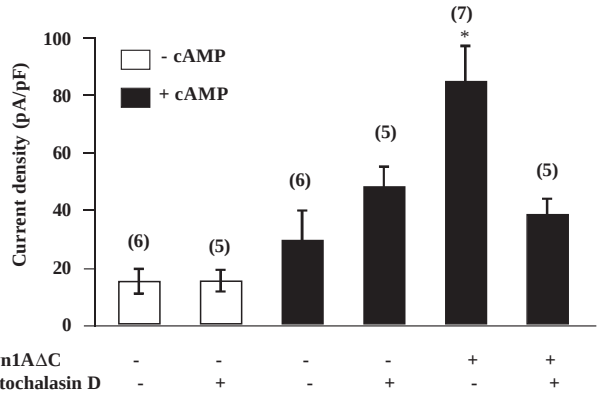

Fig. 2. Cytochalasin D prevents augmentation of I_{CFTR} by the cytosolic domain of syntaxin 1A. Whole-cell currents were obtained from 16HBE14o- cells as in Fig. 1. A summary of current density at 110 mV is given for cells under control conditions and cells stimulated with the cAMP cocktail added to the bath solution as indicated under the respective bars. Cells were treated with 0.5 μg/ml of cytochalasin D for 30 minutes prior to whole-cell voltage clamp in the presence and absence of soluble syntaxin 1AΔC (20 μg/ml) added to the pipette solution. There was no significant difference between ICFTR in the presence and absence of the cytoskeleton-disrupting reagent. ICFTR in the presence of the cytosolic domain of syntaxin 1A was not significantly different from that observed under control conditions.

anchored syntaxin 1A, we measured membrane turnover, I_{CFTR} activation and the regulation of these parameters by the cytoplasmic domain of syntaxin 1A in the 16HBE14o- cells. Membrane capacitance (C_m) is proportional to cell surface area, and the measurement of changes in C_m can give insight into the change in surface area resulting from exocytosis and endocytosis (Neher, 1988; Neher and Marty, 1982). Time resolved changes in membrane capacitance provide a direct and quantitative record of the time course of plasma membrane turnover in response to stimuli. Conductance (G_m) was markedly augmented by dissociation of the syntaxin 1A-CFTR interaction by either GST-Syn1AΔC (Fig. 3A and B) or GST-Munc18 (Fig. 3B). As expected, addition of 350 nM GST-Syn1AΔH3 to the pipette solution did not result in an increase in G_m. Under all conditions tested, the capacitance of the 16HBE14o- cells remained unchanged. Thus, neither cAMP activation alone nor cAMP activation in combination with GST-Syn1AΔC or GST-Munc18 had any detectable effect on C_m in human airway cells.

Studies of capacitance are limited by the fact that they measure net changes in cell surface area owing to simultaneous exocytosis and endocytosis. Examination of each process independently could lend valuable insight into the underlying membrane trafficking events. Exocytosis can be examined independently of endocytosis using the fluorescent styryl dye FM1-43, since it labels but does not permeate cell membranes.

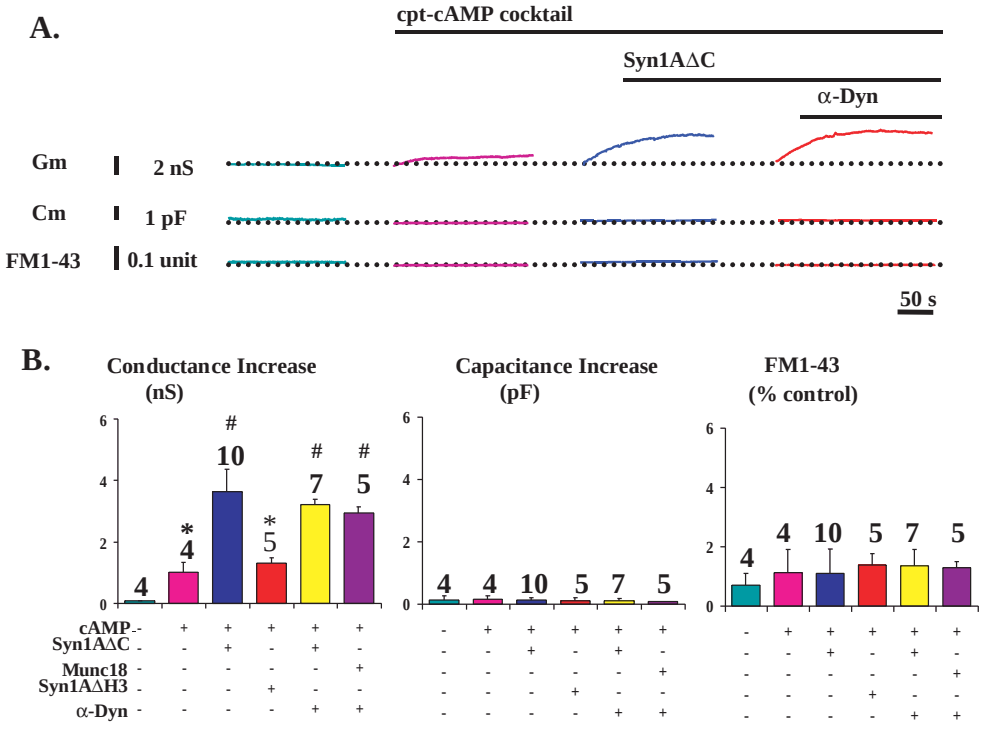

Fig. 3. cAMP and syntaxin 1A stimulate CFTR without affecting membrane turnover in human airway epithelial cells. (A) Representative simultaneous recordings of Gm, Cm and FM1-43 fluorescence intensity under various conditions in human airway epithelial cells. Horizontal bars indicate recording conditions in each representative cell. Each vertical column depicts a representative set of records from a single cell. While there is a progressive increase in Gm over the baseline with cAMP activation and then with addition of GST-Syn1AΔC, inhibition of endocytosis by 1 μg of α-Dyn had no additional effect on Gm. Activation of ICFTR in 16HBE14o- cells occurs without an apparent contribution from membrane trafficking as evidenced by a lack of change in either Cm or FM1-43 fluorescence intensity even when endocytosis is inhibited by α-Dyn. Inclusion of GST-Syn1AΔC or GST-Munc18 in the patch pipette resulted in similar increases in Gm, both in terms of magnitude and time course. (B) A summary of the changes in Gm, Cm and FM1-43 fluorescence intensity in 16HBE14o- cells on stimulation of current with cAMP under the various conditions indicated. When CFTR was activated by cAMP, a Gm of 1.03 (0.33 nS (n=4)) was measured. Dissociation of the Syn1A-CFTR interaction by GST-Syn1AΔC resulted in an approximately threefold increase in Gm to 3.57 (0.74 nS (n=10)). Gm increase by GST-Munc18 was also approximately threefold to 2.94 (0.20 (n=5)). No change in conductance was seen in the presence of GST-Syn1AΔH3 [Gm=1.32 (0.18 nS (n=5))]. For the 16HBE14o- cells, in all instances, disruption of the syntaxin 1A-CFTR interaction did not result in changes in measures of capacitance or FM1-43 fluorescent intensity. The asterisk (*) indicates P<0.05 for comparisons with baseline conditions (in the absence of cAMP stimulation). # indicates P<0.05 for comparisons with conditions in which the syntaxin 1A-CFTR interaction was not disrupted.

Fluorescence intensity of cells in the presence of FM1-43 remains unchanged during the process of endocytosis. As vesicles fuse with the plasma membrane, however, the newly exposed surface is labeled by the FM1-43 and fluorescence intensity increases. Thus, exocytosis can be measured without contamination from endocytotic processes (Smith and Betz, 1996). As summarized in Fig. 3B, we detected no changes in FM1-43 fluorescence upon activation of CFTR in the 16HBE14o- cells with or without GST-Syn1AΔC, GST-Munc18 or GST-Syn1AΔH3 (fluorescent intensity only increased 1.1 to 1.3% under the various conditions where n=4-10).

Dynamin mediates different types of endocytosis in various cell types (Hinshaw, 2000). Disruption of its function with anti-pan-dynamin IgG (α-Dyn) inhibits rapid endocytosis in chromaffin cells (Artalejo et al., 1995; Elhamdani et al., 2000). Therefore, we carried out a set of experiments to determine if we could detect effects of syntaxin-1A-CFTR disruptive reagents on C_m or G_m by blocking endocytosis with α-Dyn. Disruption of the syntaxin-1A-CFTR interaction with either GST-Syn1AΔC or GST-Munc18 did not change capacitance in 16HBE14o- human airway epithelial cells even in the presence of α-Dyn, suggesting again that membrane recycling is not involved in the augmentation of I_{CFTR} by the cytosolic domain of syntaxin 1A (Fig. 3). Capacitance changes were also not apparent in the presence of GST-Syn1AΔH3 plus α-Dyn. Thus, using two different assays, we could not detect an increase in exocytosis during the time course of the current augmentation response, whether or not the interaction between syntaxin 1A with CFTR was disrupted.

The possibility that SNARE-dependent membrane turnover contributes to the regulation of I_{CFTR} in other epithelial cell types lead us to examine the t-SNARE-channel interaction in a cell line of colonic origin. Support for these experiments came from reports that cAMP induces the insertion of heterologously expressed CFTR into the plasma membranes of *Xenopus* oocytes in the absence of syntaxin 1A (Peters et al., 1999). As it had been previously shown that cAMP stimulates apical protein secretion in CFTR-expressing HT29-CL19A human colonic epithelial cells (Jilling and Kirk, 1996), we assayed G_m, C_m and FM1-43 exocytosis in these cells in order to determine if membrane trafficking contributes to CFTR regulation by syntaxin 1A in cells of gastrointestinal origin. Results of these experiments are given in Fig. 4. We failed to observe an increase in C_m in response to cAMP in the presence or absence of GST-Syn1AΔC or GST-Munc18. However, there was a significant increase in the FM1-43 fluorescence following the addition of the cAMP cocktail, indicating that the simultaneity of the processes of exocytosis and endocytosis might result in an undetectable change in membrane capacitance. The cAMP-induced change

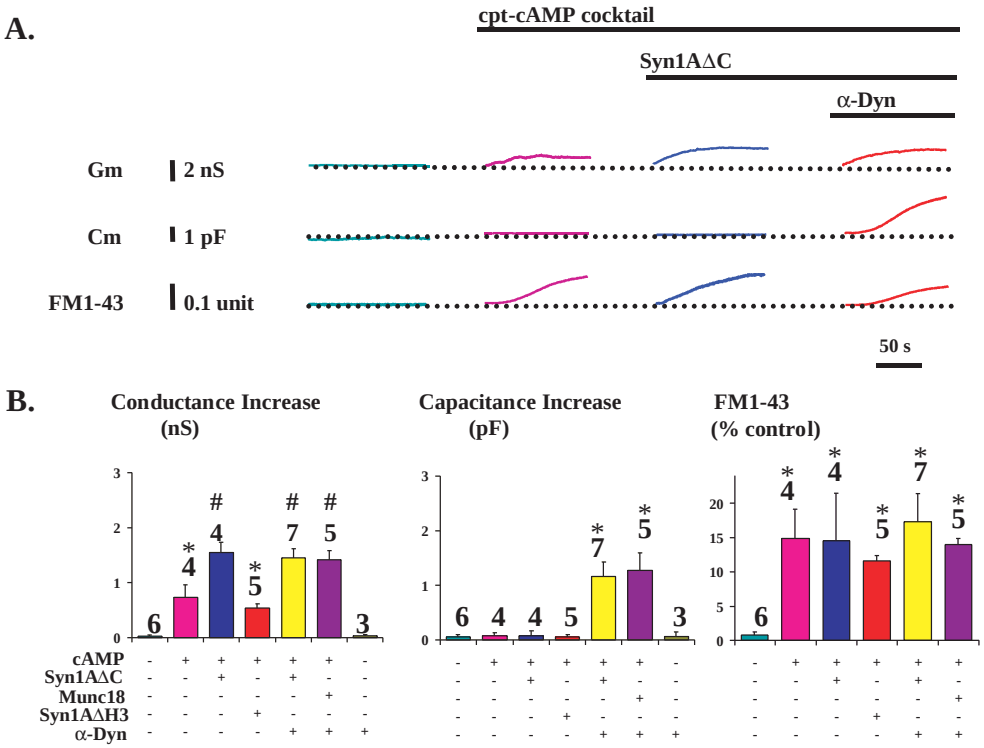

Fig. 4. cAMP increases membrane turnover in HT29-CL19A cells, but this effect is unrelated to CFTR activation or syntaxin 1A regulation. (A) Representative simultaneous recordings of Gm, Cm and FM1-43 intensity in HT29-CL19A cells. Disruption of the syntaxin 1A-CFTR interaction with GST-Syn1AΔC results in a large increase in Gm. Note that while FM1-43 intensity increases when Gm increases in response to cAMP activation, there is no further increase in intensity when GST-Syn1AΔC disrupts the syntaxin-1A-CFTR interaction. Also, whereas the addition of α-Dyn results in a large increase in C_m in the presence of GST-Syn1AΔC, G_m did not change, indicating that inhibition of endocytosis did not augment conductance. Changes induced by GST-Syn1AΔC were similar to those induced by GST-Munc18 (data not shown). (B) A summary of the changes in G_m, C_m and FM1-43 fluorescent intensity in HT29-CL19A cells on stimulation of current with cAMP under the various conditions indicated. When CFTR was activated by cAMP, a G_m of 0.73±0.23 nS (n=4) was measured. Dissociation of the syntaxin 1A-CFTR interaction by either GST-Syn1AΔC or GST-Munc18 resulted in an increase in G_m to 1.55±0.19 nS (n=4) or 1.41±0.16 nS (n=5) respectively, while conductance remained unchanged in the presence of GST-Syn1AΔH3 [(G_m=0.53 ± 0.08 nS (n=5))]. In the HT29-CL19A cells, fluorescence intensity increased to the same degree upon activation of I_{CFTR} regardless of whether or not the syntaxin-1A-CFTR interaction was interrupted (range of increases was 11.6±0.75% to 17.2±4.2% (n=4-7), no significant differences were detected on comparison of these groups). C_m did not change unless endocytosis was inhibited by α-Dyn. Measured capacitance in the absence of inhibition of endocytosis was consistently less than 0.075 pF. In the presence of α-Dyn, capacitance increased to 1.16±0.26 pF (n=7) and 1.27±0.32 pF (n=5) when interrupting the protein-protein interactions with GST-Syn1AΔC and GST-Munc18, respectively. The asterisk (*) indicates P<0.05 for comparisons with baseline conditions (in the absence of cAMP stimulation). # indicates P<0.05 for comparisons with conditions in which the syntaxin-1A-CFTR interaction was not disrupted.

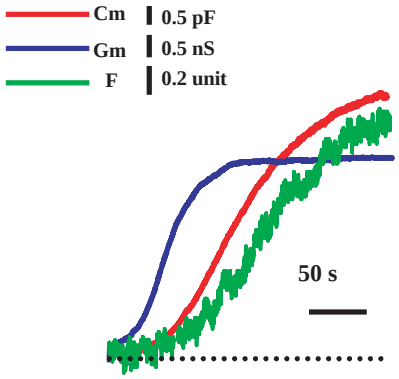

Fig. 5. Detailed time course of the changes in G_m, C_m and FM1-43 intensity in HT29-CL19A cells. It can be clearly seen that G_m activates before exocytosis occurs (as measured by increases in both C_m and FM1-43 intensity), suggesting that I_{CFTR} activation occurs independently of alterations in membrane turnover.

in C_m in the HT29-CL19A cells was not detectable unless dynamin-dependent endocytosis was inhibited. The addition of α-Dyn to the pipette in the presence of the cytosolic domain of syntaxin 1A resulted in a large increase in C_m, conductance and fluorescence. This increase in G_m was, however, not significantly different from that observed with GST-syn 1AΔC alone, indicating that inhibition of membrane retrieval did not contribute to G_m augmentation by either GST-Syn1AΔC or GST-Munc18 (Fig. 4B). Moreover, the increase in G_m induced by cAMP in the presence of α-Dyn was essentially complete before the increase in C_m was detectable, indicating that inhibition of membrane turnover was not causally linked to the conductance increase as seen in Fig. 5. There were no measurable changes in either capacitance or conductance induced by α-Dyn alone in the absence of activation with cAMP, as summarized in Fig. 6.

Syntaxin 1A decreases the open probability of CFTR

In order to examine the hypothesis that syntaxin 1A regulates CFTR by direct protein-protein interactions rather than through a modulation of membrane trafficking, we performed single channel studies using excised inside-out patches obtained from 16HBE14o- cells the results of which are illustrated in Fig. 7. The extracellular (pipette) solution contained 200 μM DIDS to pharmacologically block non-CFTR anion channels. In these experiments, the reversal potential for Cl^- was zero, and Cl^- was the only permeant ion in both bath and pipette solutions (see Methods). Once the inside-out configuration was achieved, 75 U/ml of PKA catalytic subunit was added to the bath solution to activate CFTR. Patches were exposed to the catalytic subunit only if background Cl^- currents were not observed during a 300 second baseline recording period. A channel with the characteristics of CFTR was associated with the addition of the catalytic subunit to the bath as seen in Fig. 7A. Channel activity was observed following exposure of the excised patch to the catalytic subunit of PKA and was determined to be CFTR on the basis of the following criteria: (1) current activation was observed in the presence of 200 μM DIDS to the pipette (extracellular solution), (2) currents had a linear I-V with a single channel conductance of 6 pS as expected for CFTR and (3) currents were not observed in the absence of the catalytic subunit. Patches with an open-state probability of greater than approximately 0.10 following PKA activation were discarded. Low open-state probability patches were subsequently exposed to either 350 nM GST-Syn1AΔC or 350 nM GST-Syn1AΔH3 added to the bath. Selection of patches with initial low levels of current activation allowed for an

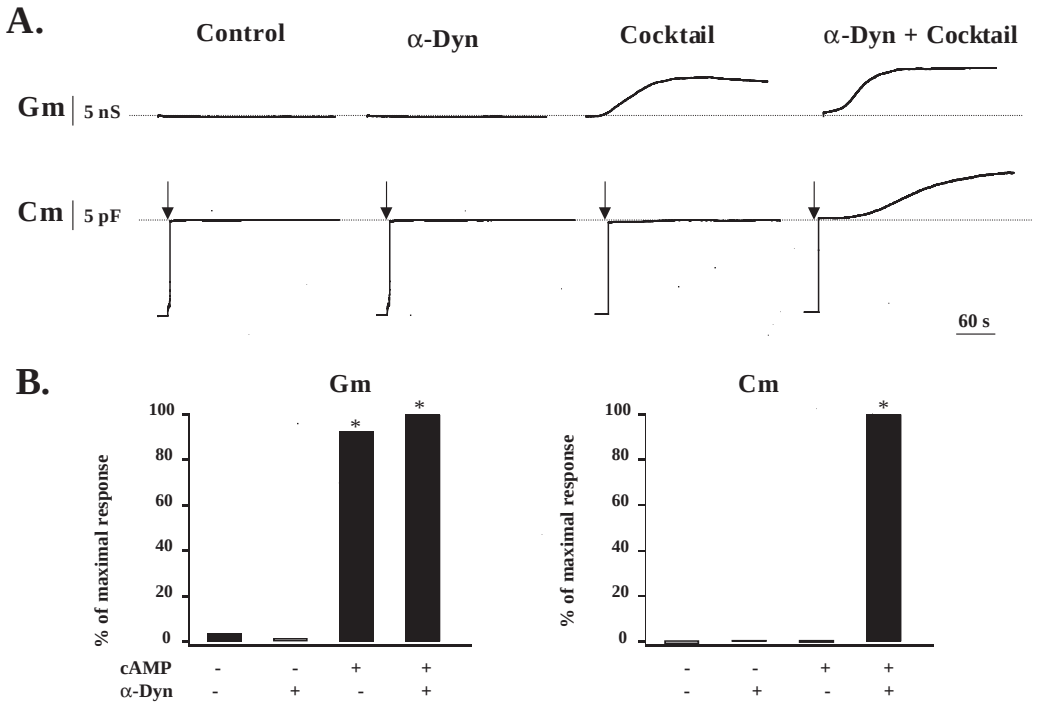

Fig. 6. Inhibition of rapid endocytosis by α-Dyn in the gastrointestinal HT29 cells does not change membrane capacitance or conductance in the absence of activation with cAMP. (A) Membrane capacitance experiments were carried out as in Fig. 3. Membrane capacitance and conductance is shown for four representative cells. Transition to the whole-cell configuration is marked by the arrow for each representative capacitance trace. (B) A summary of the capacitance and conductance increases for each of the conditions indicated below each bar. Significant increases over control conditions are indicated above each bar with an asterisk. Data are the average of five to six cells for each condition.

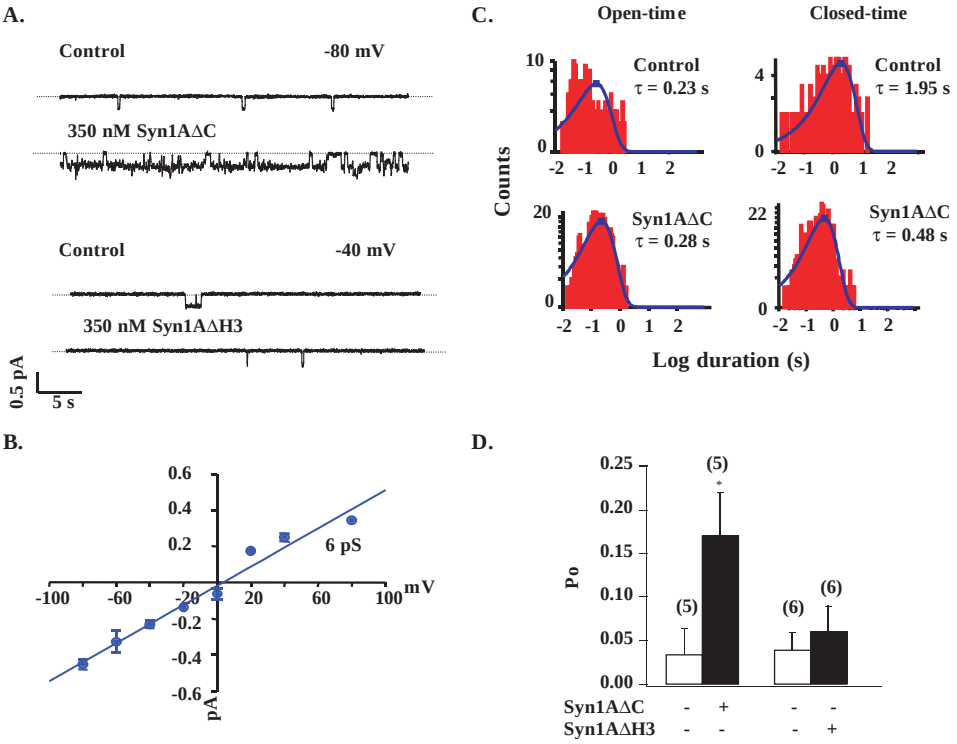

Fig. 7. Disruption of syntaxin 1A and CFTR interactions does not alter the biophysical properties of CFTR; however, open probability increases significantly. (A) Representative single-channel traces of CFTR in 16HBE14o- cells prior to and after addition of 350 nM GST-Syn1AΔC or 350 nM GST-Syn1AΔH3 to the cytoplasmic face of inside-out patches. (B) The single-channel conductance for currents in the presence or absence of GST-Syn1AΔC or GST-Syn1AΔH3 was 6 pS. The vertical bars are s.e.m. (n ranged from 1 to 8 at each measured potential). (C) Mean open time (τ_o) and mean closed times (τ_c) prior to and after disruption of syntaxin-1A-CFTR interaction by Syn1AΔC. τ_o (~250 mseconds) was unaffected whereas τ_c decreased from 1.95 seconds to 480 mseconds. (D) Open probability (P_o) in the absence and presence of GST-Syn1AΔC was 0.03±0.03 and 0.17±0.05, respectively (n=5 (P<0.05)). P_o remained unchanged from 0.07±0.03 to 0.04±0.02 following the addition of the negative control fusion peptide, GST-Syn1AΔH3 (n=6).

accurate quantitation of CFTR rescue from inhibition by membrane-anchored sytaxin 1A via the soluble reagents. We observed increases in channel activity in 5 of the 9 patches exposed to GST-Syn1AΔC as seen in Fig. 7A. The apparent level of patch variability was probably due to cytoskeletal disruption during patch excision that prevented the rescue of CFTR from inhibition by the cytosolic domain of syntaxin 1A in the whole cell experiments (Fig. 2). However, even if the cytoskeleton were intact, such variability in response to the soluble syntaxin 1A peptide would not be unexpected as the syntaxin 1A-CFTR interaction is reversible and likely to be dynamic such that not all channels would be expected to be bound to syntaxin 1A at a given time.

Analysis of open times (τ_o) for a representative patch revealed no change prior to or after disruption of the syntaxin-1A-CFTR interaction (Fig. 7C). Closed time analysis revealed that disrupting syntaxin 1A from CFTR resulted in a decreased closed time (τ_c) consistent with an increase in the likelihood of opening events. Open probability (P_o) increased threefold (similar to the increase seen in whole-cell current measurements) after addition of GST-Syn1AΔC from 0.06±0.03 to 0.19±0.04 (Fig. 7D). Channel activity, both prior to and after interruption of the syntaxin-1A-CFTR interaction, was stable for the duration of each 300 second segment of recording. The changes in P_o after addition of GST-Syn1AΔC were immediate. To examine the specificity of the response, we tested GST-Syn1AΔH3 for its effects on CFTR activity in excised membrane patches. P_o was unaffected by the soluble peptide lacking the CFTR binding domain as summarized in Fig. 7D.

Discussion

Although many ion channels and transporters associate with and are regulated by syntaxin 1A, we demonstrate that this t-SNARE inhibits the PKA-dependent Cl^- channel, CFTR, by decreasing the likelihood of channel opening. Our data are most consistent with a model in which syntaxin 1A directly regulates membrane-resident CFTR (and probably other ion channels and transporters) rather than one requiring alterations in plasma membrane turnover. In whole-cell experiments, partial disruption of the actin cytoskeleton with cytochalasin D prevented the rescue of membrane-anchored syntaxin 1A-induced inhibition of CFTR by the cytosolic domain of syntaxin 1A. Thus, our observation that the increase in open-probability with single-channel recordings was not always observed with the soluble syntaxin was not unexpected as we believe that although syntaxin 1A interacts directly with CFTR, it does so in a dynamic manner dependent on an intact cytoskeleton that may or may not be disrupted by patch excision. It is possible that the loss of cytoskeletal structure might lock the syntaxin-1A-CFTR complex together irreversibly. We speculate that the subset of patches in which augmentation could be detected occurred when the residual cytoskeletal elements accompanied the excised membrane. Finally, current augmentation ranged from small to large effects (as reflected in the s.e.m. values) under whole-cell recording conditions, demonstrating the variability of this response even in non-excised states.

In agreement with studies in Calu-3 cells that are derived from human airway submucosal glands and express high levels of CFTR (Shen et al., 1994), we found that I_{CFTR} activation in human airway epithelia did not involve membrane trafficking (Chen et al., 2001; Loffing et al., 1998). Rather, the increase in C_m occurred independently of the increase in current. This is not necessarily incongruous with studies that demonstrate that exocytosis of CFTR-containing vesicles are involved in

current activation (Lehrich et al., 1998; Takahashi et al., 1996). As has been suggested, the mechanism by which cAMP stimulates CFTR activation may be variously regulated in different cell lines and, furthermore, may be dependent on the state of tissue differentiation (Guggino, 1998; Moyer et al., 1998).

Endocytosis is the cellular process that serves the dual function of maintaining cell surface area constant while retrieving vesicular components for recycling. The severing activity that releases endocytotic vesicles from the plasma membrane is controlled by the dynamin family of proteins, which show high levels of GTPase activity. Dynamin is found in cells in both a soluble and membrane-associated form and is thought to oligomerize around the neck of retracting vesicular structures (Henley et al., 1999). Rapid endocytosis in neurons requires GTP hydrolysis, and the critical G protein regulating this process appears to be dynamin (Artalejo et al., 1995; Artalejo et al., 1997). The increase in exocytosis induced by cAMP and unmasked by α-Dyn that we observed for the HT-29 cells is normally balanced by a compensatory increase in endocytosis in a manner analogous to that in kidney epithelial cells expressing aquaporin 2 water channels (Katsura et al., 1995). Consistent with this interpretation, we were unable to observe an effect of α-Dyn on C_m unless exocytosis was stimulated by cAMP (Fig. 4B). There were no measurable changes in either capacitance or conductance in the presence of α-Dyn in the absence of activation with cAMP (Fig. 6). These results indicated that membrane-anchored syntaxin 1A inhibits CFTR via direct interactions rather than by modulation of membrane insertion in both human colonic and airway epithelial cells. In addition, cAMP activates two distinct processes in colonic epithelial cells: one involving activation of CFTR and the other involving membrane trafficking.

Understanding the complexities of CFTR regulation is an important prerequisite in the design of novel therapeutic strategies targeting cystic fibrosis and perhaps secretory diarrhea. We have previously shown that syntaxin 1A binds specifically and stoichiometrically to the N-terminal tail of CFTR (Naren et al., 1998). The N-terminal tail of CFTR modulates PKA-dependent channel gating, possibly by interacting with the cytoplasmic regulatory (R) domain (Naren et al., 1999). We hypothesize that membrane-bound syntaxin 1A sterically prevents the N-terminus from interacting with its R domain, thus resulting in a decrease in P_o. The present findings demonstrate that CFTR ion channel function can be stimulated directly by reagents that disrupt the association of syntaxin 1A with its N-terminal tail. While syntaxin 1A may also influence the intracellular traffic of CFTR in some systems (e.g. *Xenopus* oocytes) (Peters et al., 1999), this does not appear to be the case in epithelial cells. We show, in this study, that CFTR is modulated directly by SNARE interactions. A possible physiological role of such SNARE–ion-channel interactions may well link the activities of certain ion channels and transporters to membrane traffic events such as exocytosis in tissues for which such coordination is functionally advantageous. Given the growing body of evidence regarding direct SNARE-ion transport interactions, we speculate that SNAREs play roles both in modulation of ion channels and in trafficking of proteins and vesicles.

In summary, our data demonstrate that cAMP augments membrane turnover in a cell-specific manner. The cAMP-induced changes in membrane turnover, when observed, are not correlated with cAMP-induced changes in CFTR activation. In fact, the conductance change appears to be complete before the capacitance change is initiated. Finally, dissociation of syntaxin 1A from CFTR does not alter the cAMP-induced enhancement of membrane trafficking that we observed for the colonic secretory epithelial cells. Thus, in chloride transporting epithelia, there is a clear separation of the modulatory from the trafficking or anchoring functions of the syntaxins, and this is likely to be a generalized phenomenon for all membrane ion channels that interact with the ubiquitous SNARE proteins.

The authors thank Dieter Gruenert (University of Vermont) and Steven White (University of Chicago) for graciously supplying the 16HBE14o- cells and Harry Fozzard for critical review of the manuscript. This work was supported by NIH RO1 DK57882 (DJN) and a grant from the Cystic Fibrosis Foundation (DJN).

References

Artalejo, C. R., Henley, J. R., McNiven, M. A. and Palfrey, H. C. (1995). Rapid endocytosis coupled to exocytosis in adrenal chromaffin cells involves Ca^{2+}, GTP, and dynamin but not clathrin. *Proc. Natl. Acad. Sci. USA* **92**, 8328-8332.

Artalejo, C. R., Lemmon, M. A., Schlessinger, J. and Palfrey, H. C. (1997). Specific role for the PH domain of dynamin-1 in the regulation of rapid endocytosis in adrenal chromaffin cells. *EMBO J.l* **16**, 1565-1574.

Bennett, M. K., Calakos, N. and Scheller, R. H. (1992). Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. *Science* **257**, 255-259.

Bezprozvanny, I., Scheller, R. H. and Tsien, R. W. (1995). Functional impact of syntaxin on gating of N-type and Q-type calcium channels. *Nature* **378**, 623-626.

Bezprozvanny, I., Zhong, P., Scheller, R. H. and Tsien, R. W. (2000). Molecular determinants of the functional interaction between syntaxin and N-type Ca^{2+} channel gating. *Proc. Natl. Acad. Sci. USA* **97**, 13943-13948.

Bradbury, N., Jilling, T., Kirk, K. L. and Bridges, R. (1992). Regulated endocytosis in a Cl^-secretory epithelial cell line. *Am. J. Physiol.* **262**, C751-C759.

Chen, P., Hwang, T.-C. and Gillis, K. (2001). The relationship between cAMP, Ca^{2+}, and transport of CFTR to the plasma membrane. *J. General Physiol.* **118**, 135-144.

Deken, S. L., Beckman, M. L., Boos, L. and Quick, M. W. (2000). Transport rates of GABA transporters: regulation by the N-terminal domain and syntaxin 1A. *Nat. Neurosci.* **3**, 998-1003.

Elhamdani, A., Brown, M. E., Artalejo, C. R. and Palfrey, H. C. (2000). Enhancement of the dense-core vesicle secretory cycle by glucocorticoid differentiation of PC12 cells: characteristics of rapid exocytosis and endocytosis. *J. Neurosci.* **20**, 2495-2503.

Fili, O., Michaelevski, I., Bledi, Y., Chikvashvili, D., Singer-Lahat, D., Boshwitz, H., Linial, M. and Lotan, I. (2001). Direct interaction of a brain voltage-gated K+ channel with Syntaxin 1A: functional impact on channel gating. *J. Neurosci.* **21**, 1964-1974.

Gillis, K. D. (2000). Admittance-based measurement of membrane capacitance using the EPC-9 patch-clamp amplifier. *Pflugers Arch* **439**, 655-664.

Guggino, W. B. (1998). Focus on "exocytosis is not involved in activation of Cl- secretion via CFTR in Calu-3 airway epithelial cells". *Am. J. Physiol.* **275**, C911-C912.

Hamill, O. P., Marty, A., Neher, E. and Sakmann, B. (1981). Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. *Pflügers Archives Eur. J. Physiol.* **391**, 85-100.

Henley, J. R., Cao, H. and McNiven, M. A. (1999). Participation of dynamin in the biogenesis of cytoplasmic vesicles. *FASEB J.* **13**, S243-S2437.

Hille, B. (1992). Ionic channels of excitable membranes. Sunderland, Massachusetts: Sinauer Associates.

Hinshaw, J. E. (2000). Dynamin and its role in membrane fission. *Annu. Rev. Cell. Dev. Biol.* **16**, 483-519.

Howard, M., Jiang, X., Stolz, D. B., Hill, W. G., Johnson, J. A., Watkins,

S. C., Frizzell, R. A., Bruton, C. M., Robbins, P. D. and Weisz, O. A. (2000). Forskolin-induced apical membrane insertion of virally expressed, epitope-tagged CFTR in polarized MDCK cells. *Am. J. Physiol. Cell Physiol.* **279**, C375-C382.

Jahn, R. and Sudhof, T. C. (1999). Membrane fusion and exocytosis. *Annu. Rev. Biochem.* **68**, 863-911.

Jilling, T. and Kirk, K. L. (1996). Cyclic AMP and chloride-dependent regulation of the apical constitutive secretory pathway in colonic epithelial cells. *J. Biol. Chem.* **271**, 4381-4387.

Katnik, C. and Nelson, D. J. (1993). Platelet activating factor-induced increase in cytosolic calcium and transmembrane current in human macrophages. *J. Membr. Biol.* **134**, 213-224.

Katsura, T., Verbavatz, J. M., Farinas, J., Ma, T., Ausiello, D. A., Verkman, A. S. and Brown, D. (1995). Constitutive and regulated membrane expression of aquaporin 1 and aquaporin 2 water channels in stably transfected LLC-PK1 epithelial cells. *Proc. Natl. Acad. Sci. USA* **92**, 7212-7216.

Lehrich, R. W., Aller, S. G., Webster, P., Marino, C. R. and Forrest, J. N. Jr. (1998). Vasoactive intestinal peptide, forskolin, and genistein increase apical CFTR trafficking in the rectal gland of the spiny dogfish, Squalus acanthias. Acute regulation of CFTR trafficking in an intact epithelium. *J. Clin. Investigation* **101**, 737-745.

Loffing, J., Moyer, B. D., McCoy, D. and Stanton, B. A. (1998). Exocytosis is not involved in activation of Cl- secretion via CFTR in Calu-3 airway epithelial cells. *Am. J. Physiol.* **275**, C913-C920.

Moyer, B. D., Loffing, J., Schwiebert, E. M., Loffing-Cueni, D., Halpin, P. A., Karlson, K. H., Ismailov, I. I., Guggino, W. B., Langford, G. M. et al. (1998). Membrane trafficking of the cystic fibrosis gene product, cystic fibrosis transmembrane conductance regulator, tagged with green fluorescent protein in Madin-Darby canine kidney cells. *J. Biol. Chem.* **273**, 21759-21768.

Naren, A. P., Nelson, D. J., Xie, W., Jovov, B., Pevsner, J., Bennett, M. K., Benos, D. J., Quick, M. W. and Kirk, K. L. (1997). Regulation of CFTR chloride channels by syntaxin and Munc18 isoforms. *Nature* **390**, 302-305.

Naren, A. P., Quick, M. W., Collawn, J. F., Nelson, D. J. and Kirk, K. L. (1998). Syntaxin 1A inhibits CFTR chloride channels by means of domain-specific protein-protein interactions. *Proc. Natl. Acad. Sci. USA* **95**, 10972-10977.

Naren, A. P., Cormet-Boyaka, E., Fu, J., Villain, M., Blalock, J. E., Quick, M. W. and Kirk, K. L. (1999). CFTR chloride channel regulation by an interdomain interaction. *Science* **286**, 544-548.

Naren, A. P., Di, A., Cormet-Boyaka, E., Boyaka, P. N., McGhee, J. R., Zhou, W., Akagawa, K., Fujiwara, T., Thome, U., Engelhardt, J. F. et al. (2000). Syntaxin 1A is expressed in airway epithelial cells, where it modulates CFTR Cl(–) currents. *J. Clin. Investigation* **105**, 377-386.

Neher, E. (1988). The influence of intracellular calcium concentration on degranulation of dialysed mast cells from rat peritoneum. *J. Physiol. (London)* **395**, 193-214.

Neher, E. and Marty, A. (1982). Discrete changes of cell membrane capacitance observed under conditions of enhanced secretion in bovine adrenal chromaffin cells. *Proc. Natl. Acad. Sci. USA* **79**, 6712-6716.

Peters, K. W., Qi, J., Watkins, S. C. and Frizzell, R. A. (1999). Syntaxin 1A inhibits regulated CFTR trafficking in xenopus oocytes. *Am. J. Physiol.* **277**, C174-C180.

Qi, J., Peters, K. W., Liu, C., Wang, J. M., Edinger, R. S., Johnson, J. P., Watkins, S. C. and Frizzell, R. A. (1999). Regulation of the amiloride-sensitive epithelial sodium channel by syntaxin 1A. *J. Biol. Chem.* **274**, 30345-30348.

Rettig, J., Sheng, Z. H., Kim, D. K., Hodson, C. D., Snutch, T. P. and Catterall, W. A. (1996). Isoform-specific interaction of the alpha1A subunits of brain Ca2+ channels with the presynaptic proteins syntaxin and SNAP-25. *Proc. Natl. Acad. Sci. USA* **93**, 7363-7368.

Saxena, S., Quick, M. W., Tousson, A., Oh, Y. and Warnock, D. G. (1999). Interaction of syntaxins with the amiloride-sensitive epithelial sodium channel. *J.f Biol. Chem.* **274**, 20812-20817.

Schwiebert, E. M., Gesek, F., Ercolani, L., Wjasow, C., Gruenert, D. C., Karlson, K. and Stanton, B. A. (1994). Heterotrimeric G proteins, vesicle trafficking, and CFTR Cl- channels. *Am. J. Physiol.* **267**, C272-C281.

Shen, B. Q., Finkbeiner, W. E., Wine, J. J., Mrsny, R. J. and Widdicombe, J. H. (1994). Calu-3: a human airway epithelial cell line that shows cAMP-dependent Cl- secretion. *Am. J. Physiol.* **266**, L493-L501.

Sheng, Z. H., Rettig, J., Cook, T. and Catterall, W. A. (1996). Calcium-dependent interaction of N-type calcium channels with the synaptic core complex. *Nature* **379**, 451-454.

Sheng, Z. H., Rettig, J., Takahashi, M. and Catterall, W. A. (1994). Identification of a syntaxin-binding site on N-type calcium channels. *Neuron* **13**, 1303-1313.

Smith, C. B. and Betz, W. J. (1996). Simultaneous independent measurement of endocytosis and exocytosis. *Nature* **380**, 531-534.

Takahashi, A., Watkins, S. C., Howard, M. and Frizzell, R. A. (1996). CFTR-dependent membrane insertion is linked to stimulation of the CFTR chloride conductance. *Am. J. Physiol.* **271**, C1887-C1894.

Wu, M. N., Fergestad, T., Lloyd, T. E., He, Y., Broadie, K. and Bellen, H. J. (1999). Syntaxin 1A interacts with multiple exocytic proteins to regulate neurotransmitter release in vivo. *Neuron* **23**, 593-605.

Yang, S. N., Larsson, O., Branstrom, R., Bertorello, A. M., Leibiger, B., Leibiger, I. B., Moede, T., Kohler, M., Meister, B. and Berggren, P. O. (1999). Syntaxin 1 interacts with the L(D) subtype of voltage-gated Ca(2+) channels in pancreatic beta cells. *Proc. Natl. Acad. Sci. USA* **96**, 10164-10169.