Vasoactive Intestinal Peptide Increases Cystic Fibrosis Transmembrane Conductance Regulator Levels in the Apical Membrane of Calu-3 Cells through a Protein Kinase C-Dependent Mechanism

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ABSTRACT
Noncholinergic neurons contribute to innate airway defenses by releasing vasoactive intestinal peptides (VIP), which stimulates the submucosal glands to produce a bicarbonate-rich fluid containing mucins and antimicrobial factors. VIP elevates cAMP and activates cystic fibrosis transmembrane conductance regulator (CFTR) channels; however, its effects on surface expression have not been investigated. We studied CFTR levels in the apical membrane of polarized Calu-3 cell monolayers, a widely used model for submucosal gland serous cells. Biotinylation during VIP exposure revealed a significant increase in apical CFTR within 10 min, which reached a maximal 3.3-fold increase after 30 min. Total CFTR content of cell lysates was not altered during this time period; therefore, the increase in surface CFTR reflects redistribution from intracellular pools. Internalization assays revealed that apical accumulation was due, at least in part, to a reduction in the rate of CFTR endocytosis. VIP-induced accumulation of apical CFTR was mimicked by phorbol ester but not by forskolin, and it was blocked by the protein kinase (PK)C inhibitors bisindolylmaleimide X (BisX) or chelerythrine chloride but not by the PKA inhibitor N-[2-(p-bromocinnamylamino)ethyl]-5-isouquinolinesulfonamide dihydrochloride (H89). Increases in surface expression were paralleled by enhanced iodide effluxes during cAMP stimulation. BisX inhibition of VIP responses was abrogated when monolayers were pretreated with tannic acid to inhibit endosome recycling. Thus, PKC increases the surface expression of CFTR channels in addition to potentiating their responsiveness to PKA phosphorylation. Integrated regulation through multiple signaling pathways may be a common feature of VIP and other physiological secretagogues.

The cystic fibrosis transmembrane conductance regulator (CFTR) is a tightly regulated ion channel that mediates cAMP-stimulated anion conductance in epithelia and other cell types. It contains 12 membrane-spanning regions (TM1–TM12), which may surround the channel pore, two nucleotide binding domains (NBD1 and NBD2), which control ATP-dependent gating, and a highly charged regulatory (R) domain, which mediates channel activation by protein kinase (PKA and C phosphorylation (Riordan et al., 1989). Mutations in the CFTR gene that reduce its channel function or cause CFTR retention in the endoplasmic reticulum lead to cystic fibrosis, the most common lethal genetic disease in Caucasian populations (Gadsby et al., 2006). Cystic fibrosis is characterized by reduced chloride secretion across epithelia, viscous mucus secretions, chronic bacterial infections, and inflammation in the airways. In contrast, hyperstimulation of CFTR channels during bacterial infection of the intestine

ABBREVIATIONS: CFTR, cystic fibrosis transmembrane conductance regulator; R, regulatory; PK, protein kinase; VIP, vasoactive intestinal peptide; sulfo-NHS-SS-biotin, sulfo-2-(biotinamido)-ethyl-1,3-dithiopropionate; BisX, bisindolylmaleimide X; ChCl, chelerythrine chloride; PMMA, phorbol 12-myristate 13-acetate; H89, N-[2-(p-bromocinnamylamino)ethyl]-5-isouquinolinesulfonamide dihydrochloride; cpt-cAMP, 8-(4-chlorophenylthio) adenosine-3',5'-cyclic monophosphate; IBMX, 3-isobutyl-1-methylxanthine; CFTRinh172, 3’-(3-trifluoromethyl)[phenyl]-5’-[(4-carboxyphenyl)ethyl]-2-thioxo-4-thiazolidinone; G68976, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indololo(2,3-e)-pyrrole(3,4-c)-carbazole.
by *Vibrio cholerae* may result in secretory diarrhea, dehydration, acidosis, and death (Thiagarajah and Verkman, 2003).

CFTR is activated by PKA-dependent phosphorylation. Exposing the cytosolic aspect of CFTR channels to PKC enhances their subsequent responsiveness to PKA activation (Tabcharani et al., 1991; Jia et al., 1997), in part through direct PKC phosphorylation of the R domain (Chappe et al., 2003, 2004). Moreover, phorbol esters potentiate cAMP-stimulated chloride conductance in *Xenopus* oocytes heterologously expressing CFTR (Sullivan et al., 1991), and similar effects have been reported in the human colonic HT-29 cell line (Bajnath et al., 1993) and in isolated rat pancreatic duct cells (Winpenny et al., 1995). Although mutagenesis studies indicate that these responses depend, at least in part, on direct phosphorylation of CFTR (Chappe et al., 2003, 2004), PKC could also act through other mechanisms. For example, studies of HT-29 cells suggest that PKC stimulation can increase the number of functional CFTR channels in apical membrane patches (Bajnath et al., 1993).

To date, most studies of CFTR regulation by PKC have used purified kinases or artificial activators rather than physiological secretagogues. Vasoactive intestinal peptide (VIP) is the major physiological stimulus for CFTR-dependent secretion by serous cells of the airway submucosal glands (Wine and Joo, 2004; Ianowski et al., 2007). Although VIP was initially considered as a cAMP-mediated secretagogue, it has recently been shown to regulate CFTR through both PKA- and PKC-dependent signaling pathways (Dérand et al., 2004; Laburthe et al., 2007). Because VIP is a physiological agonist that may act on multiple signaling pathways, we examined whether it elevates the surface expression of CFTR channels at the apical membrane in polarized Calu-3 cell monolayers, a widely used model for serous cells in human airway submucosal glands.

**Materials and Methods**

**Chemicals**

Cell culture media and supplements were purchased from Invitrogen (Carlsbad, CA); sulfo-NHS-SS-biotin was obtained from Pierce (Madison, WI); streptavidin-coated beads were from Novagen/EMD Chemicals Inc. (La Jolla, CA); M3A7 monoclonal anti-CFTR antibody was from Upstate (Billerica, MA); goat anti-mouse secondary antibody conjugated to peroxidase was from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA); ECL chemiluminescence detection kits were from GE Healthcare (Chalfont St. Giles, UK); and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) and were of the highest grade available.

**Cell Culture**

Cells were cultured in α-minimum essential medium supplemented with 15% fetal bovine serum, 1% penicillin-streptomycin, 1% nonessential amino acids, and 1% sodium pyruvate with 5% CO₂ at 37°C. For apical biotinylation experiments, Calu-3 cells were cultured in Transwells (24-mm diameter with 0.4-µM diameter pores;
Corning Life Sciences, Acton, MA) at the air-liquid interface until they formed tight monolayers. For short-circuit current studies, Calu-3 cells were seeded on clear polyester Snapwells (1-cm² diameter, 0.4-µm pores; Corning Life Sciences) at a density of 2 × 10⁶ cells/per well. After 7 days, apical medium was removed and monolayers were maintained at the air-liquid interface for 14 days. The apical surface was washed with phosphate-buffered saline, and the basolateral medium was changed every 4 days.

**Cell Surface Biotinylation**

Calu-3 cells were cultured on porous supports at the air-liquid interface until they formed tight monolayers, and no medium leakage to the apical side was observed. Biotinylation experiments were performed as described previously (Chappe et al., 2005). Biotinylated proteins were eluted from streptavidin beads using 5× sample buffer containing dithiothreitol to cleave the NHS-SS-biotin and Western blotted with the monoclonal anti-CFTR antibody M3A7.

**Experimental Design.** VIP or (other drugs) was added directly into complete culture medium in the basolateral compartment for 5 min to 2 h (or as indicated on individual figures) at 37°C, and proteins in the apical membrane were labeled at 4°C with the cleavable and membrane-impermeant sulfo-NHS-SS-biotin, then pulled down as described previously (Chappe et al., 2005). To assess the role of PKC or PKA in mediating the VIP-stimulated increase in apical CFTR expression, PKC or PKA activators/inhibitors were added in the apical compartment filled with complete culture medium for the indicated periods (at 37°C) before biotinylation. Inhibitors were added 30 min before VIP. Apical CFTR was quantified by densitometry of scanned Western blots, and results (in arbitrary units) were corrected for nonspecific CFTR binding to the beads by subtraction of the background signal when biotin was omitted from the reaction mix. Corrected densities were then normalized to the corresponding controls.

**CFTR Internalization Assays**

Apical CFTR internalization was measured according to Ganeshan et al. (2007). In brief, cells were biotinylated on ice as described above, returned to 37°C for the indicated periods, placed on ice again, and membranes were stripped of biotin by incubation with a membrane-impermeant stripping solution as follows: 100 mM mercaptoethane-sulfonic acid, 50 mM Tris, 100 mM NaCl, 1 mM EDTA, 0.2% bovine serum albumin, pH 8.0, for 15 min (repeated three times). Cells were then harvested by scraping and lysed in radioimmunoprecipitation assay buffer on ice for 30 min. Remaining (intracellular) biotinylated proteins were pulled down on streptavidin-coated beads and visualized on Western blots as described previously.

![Fig. 2](image) PKC inhibitors abolish the VIP-induced increase in apical CFTR. Polarized Calu-3 cells were treated with 500 nM BisX or 10 µM ChCl for 30 min before adding 300 nM VIP to the basolateral compartment for 5 to 30 min. Representative Western blots of biotinylated apical CFTR are shown in A and B; C, representative Western blot showing total CFTR present in 100 µg of lysate from control cells (1), and cells treated for 30 min with 300 nM VIP (2), 300 nM VIP + 500 nM BisX (3), or 300 nM VIP + 10 µM chelerythrine (4). D and E, changes in apical CFTR levels estimated by densitometry of scanned Western blots and normalized to the control level of apical CFTR. Values are the means ± S.E.M. for n = 4 to 6 independent experiments. Student’s t test was used to evaluate significant differences versus control, versus VIP + BisX, or VIP + ChCl; ns, not significant; p > 0.05; **, p < 0.01.
Experimental Design. Cells were incubated with VIP for 30 min (or not: control) before biotinylation at 4°C then returned to 37°C to enable the labeled protein to be internalized for 0, 5, 15, or 30 min, in the absence (control) or presence of VIP for the indicated periods. After this timed incubation at 37°C, cells were placed on ice and any biotin remaining on the apical surface was cleaved by exposure to stripping solution. The amount of internalized CFTR present in streptavidin pull downs, after stripping biotin from the cell surface, was estimated by densitometry of scanned Western blots (probed with the M3A7 anti-CFTR antibody), and densities were normalized to total apical CFTR biotinylated (no incubation at 37°C and no membrane stripping). The amount of labeled CFTR that was precipitated immediately after biotinylation at 4°C was taken as a measure of total surface CFTR (100%) when calculating the percentage of CFTR that had been internalized. The absence of CFTR in pull downs from cells in which membranes had been stripped immediately after biotinylation at 0°C confirmed the efficiency of biotin cleavage by the stripping solution, and also the specificity of cell surface biotinylation.

Iodide Effluxes

Cells were cultured in six-well plates for 3 to 7 days. Confluent monolayers were used, and iodide effluxes were assayed as described previously (Chappe et al., 2003). The iodide concentration of each aliquot was measured using an iodide-sensitive electrode (Thermo Fisher Scientific, Waltham, MA), and the I⁻ efflux rate constant k (min⁻¹) was calculated according to Becq et al. (2003).

Experimental Design. Activators or inhibitors were included in the efflux buffer from time 0, and collection continued at 1-min intervals for an additional 12 min in the continued presence of the tested compounds. The first three samples (time = −2–0 min) were used to establish a stable baseline of efflux. To test the effect of VIP exposure on halide permeability, control iodide efflux experiments were performed using Calu-3 cell monolayers that had been incubated with 300 nM VIP for 30 min to 2 h before the efflux experiment. We then measured basal iodide release during the first minute after removing extracellular iodide loading buffer; or calculated efflux rate for the entire efflux experiment without any acute stimulation.

Immunoblotting

Membrane proteins were solubilized on ice for 30 min in radioimmunoprecipitation assay buffer, and 100 μg of the proteins were subjected to 7.5% SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and probed with M3A7 monoclonal anti-CFTR antibody as reported previously (Chappe et al., 2003). Densitometry of scanned Western blots, using ImageJ software (http://rsb.info.nih.gov/ij/), was used to estimate CFTR density in each condition. Only the complex glycosylated, band C form of CFTR can be readily detected in Calu-3 cells that are cultured on permeable supports (J. Liao, unpublished observation).

Short-Circuit Current Measurements

Snapwells with differentiated Calu-3 cells monolayers were mounted in modified Ussing chambers (Easymount, Physiologic Instruments, San Diego, CA) and equilibrated for 20 min in standard Krebs bicarbonate-Ringer’s solution containing 10 mM glucose. The epithelium was voltage clamped at 0 mV, and 10 mV pulses were used to monitor resistance before and after adding agonists. The short-circuit current needed to clamp the transepithelial potential at 0 mV was recorded using a PowerLab (ADInstruments, Colorado Springs, CO) analog-to-digital data acquisition system.

Statistics

Results are reported as the means ± S.E.M., n = number of independent experiments. Differences were assessed using the Student’s t test, with p < 0.05 considered significant (*, p < 0.05; **, p < 0.01; and ***, p < 0.001).
could increase due to activation of its gating (i.e., an increase in open probability) and/or to an increase in its surface expression (i.e., the number of channels in the membrane). To test the latter possibility, we used biotinylation to quantify surface CFTR in polarized Calu-3 cells after adding 300 nM VIP to the basolateral side, where the VPAC1 receptor for VIP has been identified (Derand et al., 2004). As shown in Fig. 1B, apical CFTR increased during VIP exposure to 169.8 ± 22.2% after 10 min (n = 4, p < 0.037), reaching a peak of 334.4 ± 79.3% (n = 7, p < 0.0023) above control levels after 30 min, and remained more than 2-fold above control levels during >2 h of continuous VIP exposure (227.0 ± 68.8%; n = 6, p < 0.01).

Role of PKC in VIP-Induced Elevation of CFTR Surface Expression. Previous studies have suggested that PKC may influence apical CFTR trafficking in addition to increasing channel open probability (Barthelson et al., 1987; Bajnath et al., 1995). Pretreating cells with the PKC inhibi-
tor bisindolylmaleimide X (BisX; 500 nM) for 30 min did not change the level of apical CFTR expression under control conditions (BisX alone: 118.2 ± 13.1% of control, p > 0.55) but abolished the VIP-stimulated increase in surface CFTR (VIP 20 min + BisX: 90.0 ± 27.1% of control, p > 0.51; VIP 30 min + BisX: 118.0 ± 19.0% of control, p > 0.35) (Fig. 2, A and D). VIP-stimulated increases in apical membrane CFTR were also strongly inhibited by pretreatment with 10 μM chelerythrine chloride (ChCl), another PKC inhibitor that is structurally distinct from BisX (VIP 30 min + ChCl: 111.2 ± 11.6% of control, p > 0.45) (Fig. 2, B and E). To further investigate the role of PKC in the VIP response, we examined the effect of a phorbol ester (phorbol 12-myristate 13-acetate; PMA) on apical CFTR expression (Fig. 3). Exposure to 20 nM PMA for 30 or 120 min caused striking increases in surface CFTR (185.1 ± 58.9% of control, n = 4, p < 0.04 and 190.4 ± 35.1% of control, n = 4, p < 0.0025, respectively), and this stimulation was inhibited by pretreating cells with the PKC inhibitor BisX (500 nM). With prolonged (24 h) PMA treatment to down-regulate PKC, the amount of apical membrane CFTR declined to control levels (96 ± 13.67%, n = 4, p > 0.68).

PKC, but Not PKA, Signaling Increases Apical CFTR Surface Expression. The contribution of increased channel density to cAMP-stimulated secretion seems to vary with cell type. Because VIP raises intracellular [cAMP] in secretory epithelia, which might affect apical CFTR density transiently, we carried out time course experiments with forskolin. In marked contrast to the results obtained with VIP or PMA, the amount of surface CFTR was not affected by 5 to 30-min forskolin stimulation (Fig. 4; A and C; n = 4, p > 0.097). Likewise, the VIP-dependent increase in apical CFTR protein was unaffected by the PKA inhibitor H89 (Fig. 4, B and D).

**VIP Reduces CFTR Endocytosis Rate.** Total CFTR content in the cell lysates as monitored by Western blotting was not altered under any of the conditions used in our experiments (Tables 1; Figs. 1, 3, and 4). This suggests that the increase in apical CFTR occurs through redistribution of an existing pool, most likely by recruitment from the recycling endosome compartment. To investigate the effect of VIP on endocytosis, we performed internalization assays using polarized Calu-3 cells monolayers. Figure 5 shows that 42.6 ± 5.7% of the apical CFTR protein was internalized in 5 min at 37°C, and approximately 40% remained at the surface after 15 to 30 min of internalization. However, on cells pretreated with 300 nM VIP for 30 min, the amount of biotinylated CFTR internalized was markedly reduced compared with untreated cells, with only 12.5 ± 2.7% of apical CFTR internalized in 5 min. This slow rate of retrieval from the surface remained constant for 30 min.

Functional Effects of Acute and Short-Term VIP Exposure. To examine the functional significance of the observed increase in apical CFTR protein, we compared control and stimulated iodide effluxes using cells that had been pretreated, or not, with VIP or PMA. Acute stimulation of CFTR with VIP (100 nM) or 250 μM cpt-cAMP + 1 mM IBMX produced a large, transient stimulation that was detectable after 3 min and maximal after 5 min (Fig. 6). VIP stimulation was abolished by pretreating cell monolayers with the PKA inhibitor H89 (10 μM) or with the PKC inhibitor BisX (500 nM) for 30 min. The control efflux rate was not affected by including either inhibitor in the iodide loading solution for 30 min before the efflux (p > 0.2). Preincubating the monolayers with BisX also caused a modest but significant inhibition of the acute response to cpt-cAMP + IBMX (Fig. 6D). Pretreating cells with PMA alone for 1 h did not affect the control iodide efflux rate, and acute stimulation by PMA + VIP (added at time 0 of the efflux) was similar to the response to VIP alone (Fig. 7, A and B) as expected. By contrast, pretreatment with PMA significantly enhanced subsequent responses to cpt-cAMP + IBMX or to VIP. The peak efflux rate during VIP stimulation was 0.37 ± 0.04 min⁻¹ after cells had been pretreated with PMA for 1 h, compared with 0.26 ± 0.06 min⁻¹ without PMA (Fig. 7). Likewise, the maximal efflux rate (peak) during cAMP + IBMX stimulation was elevated ~2-fold to 0.81 ± 0.06 min⁻¹ by PMA pretreatment. In addition, preincubation with VIP for 30 min or 1 h significantly enhanced halide permeability, such that efflux rates during the first 5 min were higher than during acute activation of CFTR by VIP (compare Fig. 8, B and C, with Fig. 6A). After 2 h of preincubation with VIP, the efflux rate was still higher than untreated controls, although there was some decline compared with shorter treatments (data not shown). On cells pretreated with VIP for 30 min, iodide released during the first minute (t = –2; see Materials and Methods) was ~1.7 times higher compared with untreated controls (Fig. 8A). Moreover, the iodide efflux elicited by 250 μM cpt-cAMP + 1 mM IBMX became maximum within 1 min and remained elevated for 5 min (Fig. 8E) when cell monolayers were preincubated with VIP for 30 min. The effect of VIP pretreatment was abolished by 10 μM of CFTR inhibitor CFTBR-ab172, which confirms that the increase in halide permeability was mediated by CFTR channels.

**Effects of Inhibiting PKC Activity and Membrane Recycling on Short-Circuit Current Responses to VIP.** The functional consequences of inhibiting PKC and the role of CFTR recycling in polarized epithelial cells were explored by measuring short-circuit current responses to VIP, Calu-3 monolayers that had been cultured at the air-liquid interface were mounted in modified Ussing chambers and equilibrated for 20 min in standard Krebs bicarbonate-Ringer’s solution with 10 mM glucose. Exposure to 500 nM BisX for 25 min reduced the subsequent short-circuit current response to VIP by approximately half compared with control monolayers.
Fig. 5. VIP slows the rate at which apical CFTR is internalized. Endocytosis was quantified by measuring the retrieval of biotinylated CFTR into polarized Calu-3 cell monolayers. A, representative Western blot showing total surface CFTR immediately after biotinylation (control or VIP) and internalized CFTR after 0, 5, 15, and 30 min in control monolayers (top panel) or monolayers pretreated with VIP (300 nM) for 30 min before biotinylation (bottom panel). B, the percentage of CFTR remaining on the cell surface after incubation at 37°C are plotted versus incubation time for monolayers pretreated (dark squares) or not (open triangles) with 300 nM VIP. Values are mean ± S.E.M. for n = 5 independent experiments. Student’s t test was used to evaluate significant difference versus control; ns, not significant; p > 0.05; ***, p < 0.001.

Discussion

VIP is released from intrinsic airway neurons and serves as a physiological stimulus of CFTR-mediated secretion in airway submucosal glands. Although originally thought to signal exclusively through [cAMP], we have confirmed that at least part of the VIP response in polarized Calu-3 cell monolayers is mediated by activation of PKC (Dérand et al., 2004), and we have explored the mechanism of this PKC regulation. VIP and phorbol ester both increased the surface expression of CFTR by severalfold without increasing the total amount of CFTR present in cell lysates. Because CFTR is rapidly internalized by highly efficient, clathrin-mediated endocytosis (Bradbury et al., 1994; Prince et al., 1994), the rate of endocytosis must be a determinant of steady-state surface expression level.

The carboxyl terminus of CFTR contains tyrosine and dileucine endocytic motifs (see review by Ameen and Apodaca, 2007). Binding to the activator protein-2 adaptor complex drives the clathrin-dependent endocytosis of CFTR, and the amino terminus of CFTR also modulates its surface stability through interaction with filamins and the actin cytoskeleton (Thelin et al., 2007). Although the mechanism of CFTR endocytosis has been studied in some detail, its regulation by physiological agonists is much less well understood. By using internalization assays, we found that VIP reduces the rate of CFTR endocytosis in Calu-3 cells, as shown previously in the intestinal T84 cell line (Bradbury et al., 1992), whereas control internalization rate was consistent with values reported for apical CFTR endocytosis in Calu-3 and other polarized epithelial cells (Loffing et al., 1998; Varga et al., 2004). However, in contrast to T84 cells where the action of VIP (Ameen and Apodaca, 2007) was mediated by cAMP, we found that inhibition of PKA by H89 did not block the increase in surface CFTR induced by VIP, nor did elevating cAMP with forskolin mimic the effect of VIP on surface CFTR expression. These negative results are consistent with previous studies of Calu-3 (Loffing et al., 1998; Chen et al., 2001) and airway epithelial cells more generally (Bertrand and Frizzell, 2003); thus, airway cells may differ from other cell types with respect to their regulation of CFTR surface expression by cAMP.

Although VIP control over CFTR surface expression did not require PKA phosphorylation, it was critically dependent on PKC because two different PKC inhibitors blocked the VIP-induced increase in surface expression, and PMA mimicked the effect of VIP. There is a precedent for PKC inhibi-
tion of CFTR endocytosis in nonepithelial cells (Lukacs et al., 1997); however, its effects have not been explored in airway epithelial cells, and the site of PKC phosphorylation and mechanism by which endocytosis is inhibited remains to be determined. In addition, it will be important to learn whether nucleotides and other physiological agonists that stimulate phospholipase C (and hence PKC) up-regulate the surface expression of CFTR as reported here. Stimulation of CFTR exocytosis and inhibition of its endocytosis may be linked with mucus release in Calu-3 and some other cells that express both CFTR and mucins (Berger et al., 1999). Apical P2Y2 receptors in the SPOC1 cell line, a model for rat airway goblet cells, trigger exocytosis and mucus release by activating PKC6 (Abdullah et al., 2003).

It is important to note that no evidence for cAMP-stimulated exocytosis was obtained in two previous studies of Calu-3 cells that used very different methodologies (Loffing et al., 1998; Chen et al., 2001). The regulation of surface CFTR levels by PKC has not been studied previously in Calu-3 cells, and we report here the first evidence that stimulating PKC increases apical CFTR surface expression in these cells. There is electrophysiological evidence for a phorbol ester-induced increase in apical CFTR channel density in the human colon cell line HT29 (Bajnath et al., 1993). PMA

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**Fig. 6.** Role of protein kinases A and C during acute stimulation of CFTR channel activity by VIP and cAMP. A, iodide efflux from Calu-3 cells under control conditions (squares) and after addition of 100 nM VIP (triangles) at time 0. B, iodide efflux responses to 100 nM VIP (added at time 0) on untreated cells (triangles) or after pretreatment with 10 μM H89 for 30 min (circles). C, effect of pretreating cells with 500 nM BisX for 30 min on control (circles) or VIP-stimulated iodide efflux (triangles). For comparison, control efflux without pretreatment is shown (squares). D, iodide efflux responses to 250 μM cpt-cAMP + 1 nM IBMX (added at time 0) on untreated cells (triangles) or after pretreatment with 500 nM BisX for 30 min (circles). Values are the means ± S.E.M. for n = 3 to 6 independent experiments, each performed in duplicate. Student’s t test was used to evaluate significant difference versus control or versus cpt-cAMP + IBMX (D); ns, not significant; p < 0.05; *, p < 0.05; ***, p < 0.001.
stimulates exocytosis in primary cultures of rat pancreatic duct by approximately 2.7-fold, and this is blocked by bisindolylmaleimide I (500 nM), although effects on chloride conductance and CFTR surface expression have not been examined (Koh et al., 2000). There is also a precedent for PKC inhibition of CFTR endocytosis in nonepithelial Chinese hamster ovary cells (Lukacs et al., 1997), although PKC did not affect CFTR internalization in T84 intestinal epithelial cells (Bradbury and Bridges, 1992). Its effects have not been explored in airway epithelial cells, and the site of PKC phosphorylation and mechanism by which endocytosis is inhibited remains to be determined.

In addition to PKC, phorbol esters can activate a family of “nonprotein kinase C” phorbol ester/diacylglycerol receptors called chimerins, protein kinase D, RasGRPs, diacylglycerol kinase γ, and Munc13 (Kazanietz, 2002). Munc13-1 is partic-

Fig. 7. Effect of PMA pretreatment on CFTR activation by VIP or cAMP. A, I− efflux was measured under control conditions on cell monolayers pretreated (diamond) or not (squares) with 20 nM PMA for 1 h. B, iodide efflux responses to 20 nM PMA + 100 nM VIP added at time 0 (open circles) or 100 nM VIP alone (triangles) on control cells compared with the stimulation by 100 nM VIP of cell monolayers pretreated with PMA for 1 h (filled circles). C, iodide efflux responses to stimulation at time 0 with 250 μM cpt-cAMP + 1 mM IBMX of control cells (triangles) or PMA-pretreated cells (circles). Values are means ± S.E.M. for n = 3 to 4 independent experiments performed in duplicate. Student’s t test was used to evaluate significant difference versus PMA-pretreated cells: ns (not significant) = p > 0.05; **, p < 0.01; ***, p < 0.001.
Fig. 8. Increased halide permeability of cell monolayers after pretreatment with VIP. A, spontaneous iodide release, during the first minute of efflux, by cell monolayers pretreated with VIP for 30 min – 2 h. B and C, control efflux rates calculated from untreated cell monolayers (squares) or cell monolayers pretreated with 300 nM VIP (triangles) for 30 min (B) or 1 h (C). D, control efflux rates calculated from cell monolayers pretreated with 300 nM VIP for 1 h in presence (open circles) or absence (triangles) of 10 μM CFTR inhibitor CFTRinh-172. E, iodide efflux responses to 250 μM cpt-cAMP + 1 mM IBMX (added at time 0) on cell monolayers pretreated (circles) or not (triangles) with 300 nM VIP for 30 min. Values are means ± S.E.M. for n = 3 independent experiments performed in duplicate. Student’s t test was used to evaluate significant difference versus control (A–C), versus CFTRinh-172 (D) or versus cAMP + IBMX on untreated cells (E). *, p < 0.05; **, p < 0.01; ***, p < 0.001.
ularly relevant because it, rather than PKC, mediates phorbol ester-stimulated exocytosis in hippocampal neurons (Rhee et al., 2002), and high concentrations of PMA have been proposed to activate Munc13-2 in airway epithelial cells (Abdullah et al., 2003). For this reason, we examined the sensitivity of both VIP- and PMA-induced surface expression of CFTR to the bisindolylmaleimide BisX. The bisindolylmaleimide PKC inhibitor BisX does not affect PKA at concentrations normally used to inhibit PKC. BisX and the structurally unrelated PKC inhibitor chelerythrine gave similar results, strongly suggesting that PKC mediates the VIP-induced increase in CFTR surface expression.

At the functional level, BisX (500 nM) abolished VIP-stimulated iodide efflux, whereas chelerythrine (1 μM) caused <50% inhibition in a previous study (Dérand et al., 2004). However, the stronger effect of BisX is not surprising because it directly inhibits conventional (α, β, γ) PKC isozymes by binding at the ATP site ($K_i = 16–20$ nM), whereas chelerythrine disrupts substrate targeting through competition with the phosphaacceptor. Moreover, chelerythrine has ~10-fold

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**Fig. 9.** Role of PKC during VIP stimulation of short-circuit current across polarized Calu-3 monolayers. A, effect of 500 nM BisX on the short-circuit current induced by 100 nM VIP. Control, $n = 18$; BisX, $n = 18$; means ± S.E.M.; *, difference significant at $p < 0.05$. B, inhibiting vesicle trafficking with 0.05% tannic acid (pH = 7.4 in Krebs bicarbonate-Ringer) eliminated the inhibition of VIP-induced short-circuit current by 500 nM BisX. C, tannic acid alone caused a small decrease in short-circuit current when added to unstimulated monolayers. Control, $n = 13$; BisX, $n = 13$; ± S.E.M.; difference not significant. D, representative short-circuit current traces of VIP-stimulated short-circuit current, and the effect of pretreatment with 500 nM BisX. E, representative traces showing that the VIP-induced short-circuit was not BisX sensitive after exposure to tannic acid, consistent with the proposed role of PKC in CFTR endocytosis.
lower potency ($K_e = 660 \text{ nM}$) compared with bisindolylmaleimide and causes little inhibition in vitro (Davies et al., 2000), presumably because it mainly affects targeting. Preincubating Calu-3 cell monolayers with BioX caused a modest but significant inhibition of the acute response to ctp-cAMP + IBMX. This is consistent with the role of PKC in potentiating CFTR channel activation by PKA in Chinese hamster ovary and BHK cells, where inhibition of cAMP responses by G6976 and chelerythrine have been reported previously (Jia et al., 1997; Chappe et al., 2003). Because VIP stimulation of iodide efflux was abolished by pretreating cell monolayers with PKA or PKC inhibitors, our results support the hypothesis that CFTR activation by VIP is mediated by both PKA and PKC pathways in Calu-3 cells. PKC phosphorylation is known to weakly stimulate CFTR channel activity and dramatically enhance its response to PKA stimulation (Tabacharani et al., 1991, Jia et al., 1997; Chappe et al., 2003). To examine the functional significance of CFTR accumulation at the apical membrane, we also activated PKC directly using PMA and compared the response to that obtained by maximal concentrations of VIP (Dérand et al., 2004) or cAMP + IBMX. PMA pretreatment significantly enhanced subsequent responses to VIP or ctp-cAMP + IBMX, and VIP preincubation significantly enhanced halide permeability. This elevated activity after stimulation with high VIP concentrations or PKC would be consistent with an increase in the number of active CFTR channels at the plasma membrane. Short-circuit current responses in the presence of tannic acid (to inhibit recycling) are consistent with the biotinylation and iodide efflux data, but do not exclude the possibility that PKC enhances apical CFTR insertion, in addition to inhibiting its retrieval from the cell surface. Taken together, these results strongly suggest that direct activation of CFTR gating by phosphorylation and channel accumulation at the apical membrane have additive effects on macroscopic halide permeability.

In summary, we report here that a physiological agonist, VIP, increases the number of active CFTR channels in the apical membrane of the airway epithelial cell line Calu-3. This response is independent of PKA and is mediated by PKC because it is blocked by two structurally unrelated PKC inhibitors and mimicked by PMA exposure. The increase in surface expression is due, at least in part, to inhibition of constitutive endocytosis. Thus, VIP, can act through converging pathways to increase the secretory response. Such regulation by coordinated signaling may also occur in vivo during stimulation by multiple peptide hormones and transmitters that activate distinct signaling pathways, as reported for the VIP and carbachol synergistic effect on mucus secretion (Choi et al., 2007). This emphasizes the importance of studying physiological secretagogues in addition to selective activators of particular signaling pathways.

References


Jia Y, Mathews CJ, and Hanrahan JW (1997) Phosphorylation by protein kinase C (to inhibit recycling) are consistent with the biotinylation and iodide efflux data, but do not exclude the possibility that PKC enhances apical CFTR insertion, in addition to inhibiting its retrieval from the cell surface. Taken together, these results strongly suggest that direct activation of CFTR gating by phosphorylation and channel accumulation at the apical membrane have additive effects on macroscopic halide permeability.

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References


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