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Activation of CFTR Trafficking and Gating by Vasoactive Intestinal Peptide in Human Bronchial Epithelial Cells

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ABSTRACT

Cystic fibrosis transmembrane conductance regulator (CFTR) is an apical membrane chloride channel critical to the regulation of fluid, chloride, and bicarbonate transport in epithelia and other cell types. The most common cause of cystic fibrosis (CF) is the abnormal trafficking of CFTR mutants. Therefore, understanding the cellular machineries that transit CFTR from the endoplasmic reticulum to the cell surface is important. Vasoactive intestinal polypeptide (VIP) plays an important role in CFTR-dependent chloride transport. The present study was designed to observe the affection of VIP on the trafficking of CFTR, and channel gating in human bronchial epithelium cells (HBEC). Confocal microscopy revealed CFTR immunofluorescence extending from the apical membrane deeply into the cell cytoplasm. After VIP treatment, apical extension of CFTR immunofluorescence into the cell was reduced and the peak intensity of CFTR fluorescence shifted towards the apical membrane. Western blot showed VIP increased cell surface and total CFTR. Compared with the augmented level of total CFTR, the surface CFTR increased more markedly. Immunoprecipitation founded that the mature form of CFTR had a marked increase in HBEC treated with VIP. VIP led to a threefold increase in Cl^- efflux in HBEC. Glibenclamide-sensitive and DIDS-insensitive CFTR Cl^- currents were consistently observed after stimulation with VIP (10^{-8} mol/L). The augmentation of CFTR Cl^- currents enhanced by VIP (10^{-8} mol/L) was reversed, at least in part, by the protein kinase A (PKA) inhibitor, H-89 and the protein kinase C (PKC) inhibitor, H-7, suggesting PKA and PKC participate in the VIP-promoted CFTR Cl^- currents. J. Cell. Biochem. 112: 902–908, 2011. © 2010 Wiley-Liss, Inc.

KEY WORDS: CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR (CFTR); TRAFFICKING; PKA; PKC; VASOACTIVE INTESTINAL POLYPEPTIDE

The cystic fibrosis transmembrane conductance regulator (CFTR) is an apical membrane chloride channel critical to the regulation of fluid, chloride, and bicarbonate transport 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 (PK) A and C phosphorylation [Riordan et al., 1989]. Mutations in the gene encoding CFTR result in the disease cystic fibrosis (CF) and are associated with the absence or dysfunction of CFTR on the apical membrane of epithelial cells, reduced chloride secretion across epithelia, viscous mucus secretions, chronic bacterial infections, and inflammation in the airways [Gadsby et al., 2006]. On the other hand, over stimulation of the secretary pathway and activation of luminal CFTR in the small intestine are implicated in the pathogenesis of toxigenic secretary diarrhea [Vaandrager et al., 1997; Thiagarajah and Verkman, 2003].

CFTR trafficking in the Golgi compartments involves a number of processes that ultimately regulate the levels of CFTR in the cell and at the plasma membrane [Takai et al., 2001]. After passing the ER quality control system, it eventually matures from the core-glycosylated ER form to the complex-glycosylated form [Yoo et al., 2002]. The exocytosis of CFTR from Golgi to cell surface can be constitutive or regulated in a cell type-specific manner. From the cell surface, CFTR is endocytosed via the clathrin adaptor

Fei Qu and Hui-Jun Liu equally contributed to this work.

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complex I [Weixel and Bradbury, 1999]. CFTR endocytosis requires the large GTPase dynamin activity and myosin VI [Swiatecka-Urban et al., 2004]. Cell surface CFTR uses a Rab5-dependent step to enter early endosomes. Endosomal CFTR then either recycles back to the surface through a Rab11-dependent step or moves to late endosomes via Rab7 or to the trans-Golgi network via Rab9-mediated processes. CFTR in these post-Golgi compartments is degraded in the lysosomes and proteasomes. Several groups have suggested that CFTR trafficking is regulated by cAMP-dependent secretagogues. Bradbury et al. [1992] found that elevated cAMP levels inhibited endocytotic activity and increased the exocytosis of membrane vesicles, while cells expressing mutant CFTR failed to exhibit these responses. They first proposed that cAMP promotes the exocytotic insertion of CFTR into apical membranes. Furthermore, CFTR protein can form functional chloride channels if expressed on the cell surface of Xenopus oocytes [Drumm et al., 1991] or mammalian cells [Howard et al., 2003], suggesting that augmented maturation and cell-surface expression could be of benefit to CF patients.

Although cAMP- and protein kinase A-dependent phosphorylation is recognized as a major signal transduction pathway for CFTR activation, other second messenger pathways including protein kinase (PKC), Ca^{2+}/calmodulin-dependent kinase, and GMP-dependent kinase signal CFTR activation may be participant [Gadsby and Nairn, 1999; Kunzelman and Mall, 2001]. Exposing the cytosolic aspect of CFTR channels to PKC enhances their subsequent responsiveness to PKA activation [Jia et al., 1997], in part through direct PKC phosphorylation of the R domain [Chappe et al., 2004]. PKC stimulation could increase the number of functional CFTR channels in apical membrane patches [Bajnath et al., 1992].

Vasoactive intestinal polypeptide (VIP) is a 28-amino-acid neuropeptide of the inhibitory nonadrenergic, noncholinergic nervous system in mammalian airways [Maggi et al., 1995]. A recent study demonstrated that VIP and forskolin stimulated sustained mucus secretion in pigs [Joo et al., 2002]. A study showed the stimulation of CFTR-dependent chloride secretion in HBECs was followed the activation of VIP and PACAP-27 of basolateral VPAC1 receptors [Dérand et al., 2004]. The density of VIP-positive nerves was found significantly higher in the glands of bronchitis than in nonbronchitic subjects [Lucchini et al., 1997]. Four main intracellular signal pathways are involved in the VIP-mediated intracellular signal transduction: PKA [Kajita et al., 2000; Corbitt et al., 2002]; PKC [Makhlouf and Murthy, 1997]; calmodulin (CaM) and MAPK [Fernández et al., 2005].

Previously, a novel oxidized cell model had been established in our laboratory by ozone stress [Tan et al., 2007]. And we found that ozone stress could down-regulate the expression and function of CFTR [Qu et al., 2009]. Because VIP is a physiological agonist that may act on multiple signaling pathways, we examined whether it promotes CFTR trafficking and channel function in human bronchial epithelium cells (HBECs).

MATERIALS AND METHODS

CHEMICALS

DMEM/F12 mediums, forskolin, glibenclamide, DIDS, H-7, H-89, and VIP were from Sigma Chemicals (St. Louis, MO). CFTR antibody was from Santa Cruz Biotech (CA). G-Sepharose was from Amersham Pharmacia Biotech (Sweden). The vehicles for all neuropeptides (water) and for other drugs (dimethyl sulfoxide, DMSO, final DMSO concentration: 0.1%) have no effect on transepithelial currents. The secondary antibody and protein marker were purchased from Beijing Dingguo Biotech (Beijing, China). ECL reagent was from Pierce Biotech (Rockford).

CELL CULTURE

HBEC (16HBE140-) [Qu et al., 2009] were cultured in a mixture medium of DMEM: F12 (1:1) containing 100 U/ml penicillin, 100 U/ml streptomycin, and 10% fetal bovine serum (FBS) and maintained at 37°C in a humidified environment with 5% CO_2 and 95% air.

IMMUNOFLUORESCENCE

HBEC were cultured on slides in DMEM/F12 medium with or without VIP. After 1 h, the cells were fixed in paraformaldehyde at room temperature for 20 min. The sections and cells were permeabilized with 0.05% Triton X-100 diluted in PBS, and washed with PBS several times to remove excess detergent. Nonspecific antibody bindings were blocked by incubating the samples with a 1:20 dilution of normal goat serum in PBS. Primary antibodies against CFTR in PBS were incubated overnight at 4°C. The cells were washed again and incubated with a FITC-Labeled secondary antibody for 1 h at room temperature. The cells were then examined by fluorescence microscopy. Images were captured at 400 magnifications on an Olympus IX170 inverted epifluorescence microscope with SenSys-cooled charge-coupled high-resolution camera. Quantitative image analyses were performed using MoticFluo1.0 software. Either the mean fluorescence per cell was determined or the ratio of total fluorescence associated with plasma membrane and intracellular compartment was calculated. For each image the background fluorescence was measured and subtracted.

IMMUNOPRECIPITATION

HBEC were cultured on plates in DMEM/F12 medium with or without VIP for 1 h, then replaced with fresh serum-free medium, and then attacked with or without 1.5 ppm ozone for 30 min. After 4 h, cell lysates were prepared with 1 ml of lysis buffer per 100 mm culture dish. In these experiments, 3 ml of cell lysates supplemented with 9 ml of NET buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.05% Nonidet P-40) were incubated overnight at 4°C with 2 μg of CFTR antibodies. To precipitate immune complexes, incubation with 3 μg of protein G-sepharose was conducted for 1 h at 4°C. Bead-bound complexes were washed three times with cold NET buffer and denatured in Laëmmli buffer for 15 min at room temperature. Samples were separated on SDS–PAGE and analyzed by Western blot.

WESTERN BLOT ANALYSIS

HBEC were cultured on plates in DMEM/F12 medium with or without VIP for 1 h, then replaced with fresh serum-free medium, and then attacked with or without 1.5 ppm ozone for 30 min. After 4 h, cells were homogenized by several passes through a 23-gauge syringe needle in RIPA buffer (50 mM Tris–HCl (pH 7.5), 1 mM EDTA, 100 mM NaCl, 1% Triton X-100) containing protease

inhibitors (20 μM leupeptin, 0.8 μM aprotinin, 10 μM pepstatin, and 1.25 mM phenylmethylsulfonyl fluoride). Cell lysates were incubated on ice for 30 min and clarified by centrifugation at 15,000*g* for 5 min at 4°C. Protein concentrations were estimated by Lowry assay with BSA as standard. Proteins were solubilized in sample buffer, separated by 7% polyacrylamide gel electrophoresis, and transferred onto polyvinylidene difluoride membranes. The membrane were then blocked with 5% milk in Tris-buffered saline (TBS)-Tween for 2 h at room temperature and then probed with CFTR antibody in TBS plus 5% milk, for overnight at 4°C. The membranes were washed three times in TBS-Tween for 5 min and once in TBS for 5 min and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody in TBS-Tween for 2 h at room temperature. The membranes were developed with chemiluminescence reagent for 1 min and exposed to chemiluminescence film for 1–5 min. The films were scanned and quantification of each band was performed using the ImageQuant program, then the relative intensity (the intensity of objective protein band/the intensity of refer protein band) was calculated and offered.

MEASUREMENTS OF CHLORIDE EFFLUX

HBEC were cultured on glass coverslips in DMEM/F12 medium with or without VIP for 1 h, then replaced with fresh serum-free medium, and then attacked with or without 1.5 ppm ozone for 30 min. Four hours later, cells were incubated with 10 mM N-(6-metioxyquinoyl)-acetoethylester (MQAE) for 2 h. They were rinsed with SR and the coverslips were placed at the bottom in a perfusion chamber on the stage of an inverted microscope (Nikon, Diaphot, Tokyo, Japan). The chamber volume was 70 μl and the perfusion rate was 1 ml/min. Temperature was kept at 37°C by heating the chamber holder and the objective separately. A monochromator, part of a Quanticell 700 image-processing system (VisiTech International, Sunderland, UK) provided excitation light at 353 nm (16 nm bandwidth). The emission was measured at 460 nm using CCD camera, with the initial value normalized to 100%.

PATCH-CLAMP EXPERIMENTS

HBEC were cultured on plates in DMEM/F12 medium with or without VIP for 1 h, then replaced with fresh serum-free medium, and then attacked with or without 1.5 ppm ozone for 30 min. Four hours later, whole cell recordings were performed on HBEC. Currents were recorded with a List EPC-10 patch-clamp amplifier. I–V relationships were built by clamping the membrane potential to −40 mV and by pulses from −100 mV to +100 mV by 20 mV increments. The pipette solution contained: 113 mM L-aspartic acid, 113 mM CsOH, 27 mM CsCl, 1 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 10 mM TES, 285 mOsm (pH 7.2). MgATP (3 mM) was added just before patch-clamp experiments were started. The external solution consisted of 145 mM NaCl, 4 mM CsCl, 1 mM $CaCl_2$, 5 mM glucose, 10 mM TES, 340 mOsm (pH 7.4). Results were analyzed with the pCLAMP6 package software (pCLAMP, Axon Instruments). Cells were stimulated with forskolin.

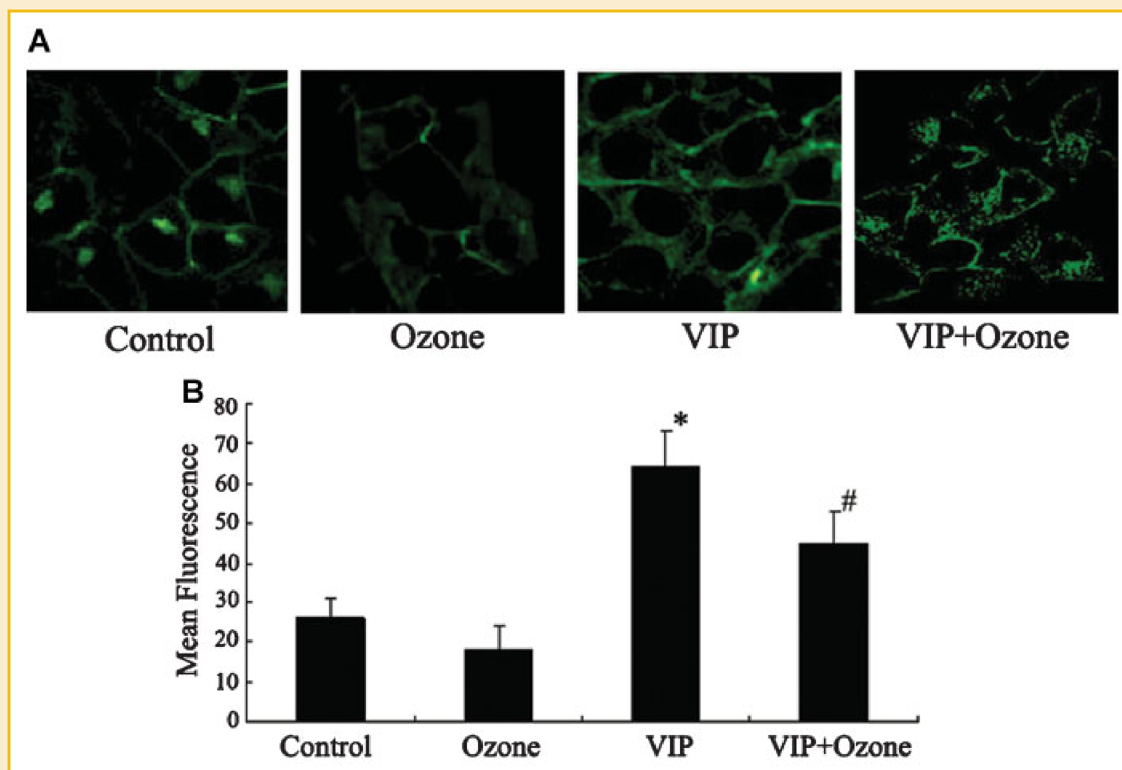

Fig. 1. The CFTR protein expression was assayed by immunofluorescence in HBECs. A: CFTR staining was detected either at the apical membrane surface or in the cytoplasm. B: Quantification of CFTR recycling to the plasma membrane. Mean green fluorescence per cell was determined as described in Materials and Methods Section by using MoticFluo1.0 software. Each point represents the average of at least 10 cells and standard deviations are indicated. $^{*}P < 0.05$ versus the control, $^{\#}P < 0.05$ versus ozone (n = 5).

STATISTICS

Results are expressed as means < remove-image > SE of n observations. Sets of data were compared with either an analysis of variance (ANOVA) or Student's *t*-test. Differences were considered statistically significant when $P < 0.05$.

RESULTS

VIP INCREASED THE AMOUNT OF CFTR IN THE APICAL MEMBRANE OF HBECs

To assess putative alterations in the CFTR protein expression in response to VIP (10^{-8} mol/L), the CFTR protein in HBECs was detected by immunofluorescence. As expected, normal HBEC showed weak CFTR staining at the apical membrane surface and cytoplasm. Ozone stress led to further reduce of CFTR expression level. CFTR staining was increased markedly in VIP treated epithelia (with or without ozone stress), especially at the apical membrane surface (Fig. 1).

VIP PROMOTED THE MATURATION OF CFTR

Cell lysates of HBEC were immunoprecipitated with CFTR antibody. Western blot revealed the presence of the ER (Band B, immature) and post-ER (Band C, mature) forms of CFTR in HBEC. Ozone stress could decrease the expression of mature CFTR, however VIP (10^{-8} mol/L) could increase the expression of mature CFTR (control group was 0.77 ± 0.05, ozone group was 0.52 ± 0.04, VIP group was 0.89 ± 0.08, ozone +VIP group was 0.64 ± 0.06, $^{**}P < 0.05$ versus control group, $^{\#\#}P < 0.05$ versus ozone group, n = 7) (Fig. 2).

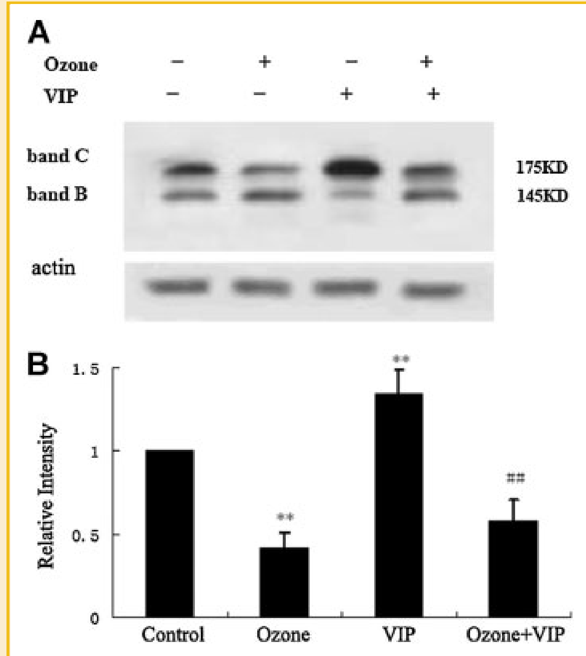

Fig. 2. VIP promoted the maturation of CFTR. A: Cell lysates were immunoprecipitated with anti-CFTR antibody and analyzed by SDS–PAGE. Bands B and C referred to partially and fully glycosylated CFTR, respectively. B: Graphic representation of means ± SE for densitometric values of CFTR protein band C obtained in the immunoprecipitated experiments. $^{**}P < 0.01$ versus the control, $^{\#\#}P < 0.01$ versus ozone (n = 7).

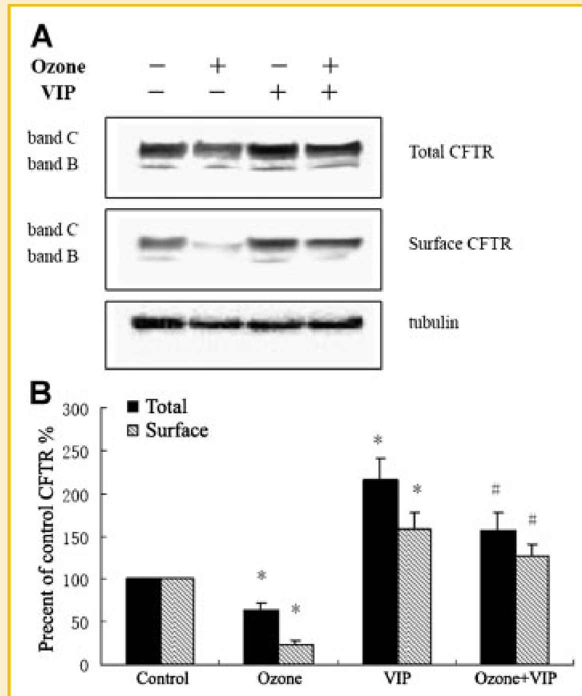

Fig. 3. VIP increased cell surface and total CFTR. A: CFTR was detected by Western blotting using an anti-CFTR polyclonal antibody. Tubulin was detected using an anti-tubulin monoclonal antibody to demonstrate equal loading of total cell lysates. B: Averages of the experiments described in (A). The open columns are values from total cellular CFTR. The solid columns are values from cell surface CFTR. The error bars are the standard deviations. $^{*}P < 0.05$ versus the control, $^{\#}P < 0.01$ versus ozone (n = 5).

VIP INDUCED THE TRAFFICKING OF CFTR

To determine whether the VIP-induced increase in CFTR maturation resulted in increased cell surface density, total and cell surface CFTR were assayed by Western blot. Both total and surface CFTR contents in the cell lysates were increased after the treatment of VIP (10^{-8} mol/L), but decreased after ozone stress, as monitored by Western blot (Fig. 3A). Meanwhile, the changes of the percentage of surface CFTR between control and VIP treatment or ozone stress were greater than that of total CFTR. For example, after VIP treatment, the total CFTR was $216.5 \pm 25.2\%$ of the controls, and the cell surface CFTR was $158.3 \pm 19.1\%$ of the controls (Fig. 3B). This suggested that the changes in apical CFTR occurred through redistribution of an existing pool, most likely by recruitment from the recycling endosome compartment.

VIP INCREASED THE FUNCTION OF CFTR

To examine the functional significance of the observed increase in apical CFTR protein, we compared control and stimulated chloride effluxes using cells that had been pretreated, or not, with VIP or ozone. Forskolin was used as a positive control. Ozone stress decreased the chloride efflux rate, while VIP treatment increased it (Fig. 4A). With concentrations of 0.01 nM up to 1,000 nM VIP (the highest concentration tested here), the chloride efflux was dramatically stimulated (Fig. 4B).

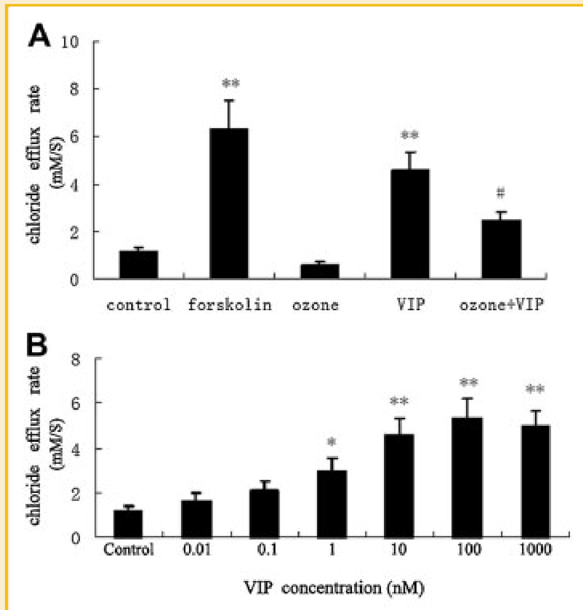

Fig. 4. VIP increased function of CFTR. A: Representative recording of the chloride efflux during an efflux experiment. $^{**}P < 0.01$, significant difference compared with control; $^{\#}P < 0.05$, compared with ozone group (n = 9). The number of experiments is given in each bar. B: The effect of different concentrations (in micromolar) of VIP on the HBECs. $^{*}P < 0.05$ or $^{**}P < 0.01$, < 0.01, compared with control (n = 9).

VIP-INDUCED ACTIVATION OF WHOLE-CELL CL$^-$ CURRENTS

The chloride current was completed by whole cell recordings to characterize the currents in HBEC. Figure 5 presented typical whole cell currents and associated current–voltage (I–V) plots in the presence or absence of activator in the bath. In control patch-clamp experiments (absence of any activator) on HBEC, no current was recorded. The addition of 5 μM forskolin stimulated a time-independent, nonrectifying conductance in HBEC, indicating the presence of a linear Cl$^-$-selective current typical of functional CFTR. Addition of 10^{-8} M VIP to the bath caused a statistically significant increase in Cl$^-$ current amplitude compared with basal current, and this Cl$^-$ current was glibenclamide-sensitive and DIDS-insensitive, which could due to CFTR activity (Fig. 5A). We also observed that VIP promoted the CFTR current with dose-dependence (Fig. 5B,C).

EFFECTS OF PROTEIN KINASE INHIBITORS ON VIP-INDUCED CFTR CURRENT

Because CFTR chloride channel regulation is critically dependent on both protein kinases A and C (PKA and PKC, respectively), we determined whether they are part of the signaling pathway to CFTR activity by using PKA inhibitor H-89 and the PKC inhibitor H-7. We found 60% inhibition of the forskolin-induced CFTR-mediated efflux in the presence of H-89 (10 μmol/L) and 55% inhibition with H7 (200 μmol/L) (when measured at +60 mV, VIP = 25.39 ± 3.78 pA/pF, H-89 = 10.32 ± 1.04 pA/pF and H-7 = 11.78 ± 1.35 pA/pF, n = 7) (Fig. 6A–C). The amplitude of the inhibition between either PKA or PKC inhibitor was not statistically significant, that is, both kinase inhibitors are equipotent in preventing the stimulation of VIP.

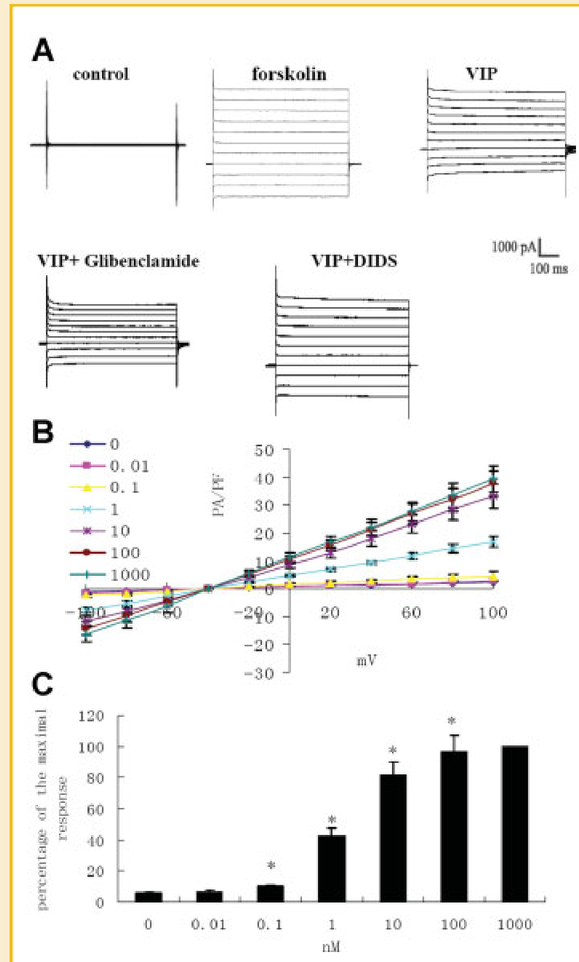

Fig. 5. Activation of CFTR current by VIP in HBEC. A: Representative current traces for CFTR are shown in three representative HBECs before and after application of activator: 5 μM forskolin, 10^{-8} M VIP. Step protocol consisted of 300-ms voltage steps from 80 to +80 mV from a holding potential of 40 mV. B: I–V curves for CFTR current. Cells were held at 40 mV and clamped with 300-ms voltage pulses from −80 to +80 mV. C: Dose response of VIP-activated CFTR current. Percentage of the maximal response expressed as current density (means ± SE) for a depolarizing step to +60 mV normalized to the maximum value obtained. $^{*}P < 0.05$, $^{**}P < 0.01$ (n = 5).

DISCUSSION

In the airway, three neural pathways are responsible for the innervation of secretory cells: sympathetic (adrenergic), parasympathetic (cholinergic), and nonadrenergic, noncholinergic (NANC) systems [Rogers, 2000]. The principal neurotransmitters of the NANC system are VIP/PACAP, substance P, neurokinin A, and calcitonin gene-related peptide. Both mucus and electrolyte secretion are part of the mucociliary transport that clear pathogens from the airway. Impairment of mucociliary clearance led to stagnation of mucus in the airways, which causes airway obstruction and provides an ideal environment for bacterial and fungal growth as it is diagnosed in diseases like cystic fibrosis, chronic bronchitis, and asthma [Rogers, 2000; Joo et al., 2002].

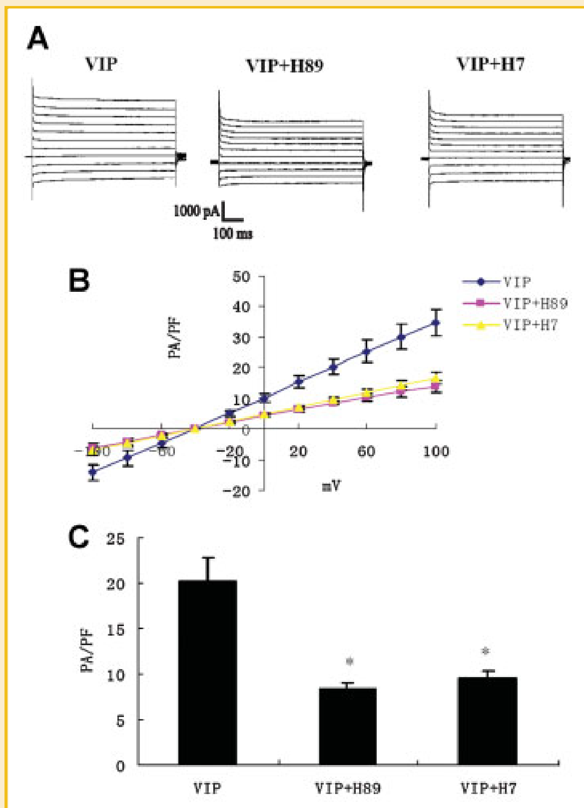

Fig. 6. Effects of protein kinase inhibitors on VIP-mediated CFTR current. A: Representative current traces in HBEC. VIP-activated currents were measured before and after exposure to H89 (10 μM) or H7 (200 μM). B: I–V curves for CFTR current. Cells were held at 40 mV and clamped with 300-ms voltage pulses from −80 to +80 mV. C: Histograms showed current densities measured at +60 mV for the different experimental conditions indicated at the bottom of each column. Data values are mean ± SE obtained from seven separate experiments. $^{*}P < 0.05$ versus VIP alone group.

In the airway, increasing evidence recognized submucosal glands as a major player in airway defense, mucus and electrolyte secretion as well as in airway diseases such as cystic fibrosis [Haws et al., 1994; Shen et al., 1994; Pilewski and Frizzell, 1999; Joo et al., 2002]. Ameen et al. [1999] found in 1999 that CFTR channel insertion to the apical surface in rat duodenal villus epithelial cells was upregulated by VIP in vivo. Recent studies demonstrated that VIP and forskolin stimulated sustained mucus secretion in pigs [Joo et al., 2002], VIP stimulated the efflux of CFTR [Robert et al., 2005], suggesting possible role for VIP in the modulation of airway and implications for therapeutic approaches to pulmonary diseases.

We observed that VIP increased the amount of CFTR in the apical membrane of HBECs, and found that the increase of CFTR at the cell surface in response to changes in CFTR activity, even VIP-induced increase in CFTR maturation resulted in increased cell surface density. Although the mechanism of CFTR trafficking has been studied in some details, its regulation by physiological agonists is much less well understood. By using internalization assays, Bradbury et al. [1992] found that VIP reduced the rate of CFTR endocytosis in Calu-3 cells, 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].

We also observed that VIP stimulated chloride effluxes. When chloride secretion was acutely stimulated with VIP, three quantitative changes were observed: (a) the extension of CFTR from the apical membrane into the cell decreased abruptly; (b) the peak intensity of CFTR fluorescence shifted towards the apical membrane; (c) after removal of VIP, both Cl^- secretion and CFTR trafficking extension reversed in parallel to basal values. We interpret these findings as compelling evidence that CFTR traffics to the apical membrane during hormonal stimulation of chloride secretion in the submucosal gland. Our data strongly support the concept that under physiological conditions, intracellular CFTR shifts to the apical membrane, and that VIP-stimulated insertion of CFTR-containing vesicles leads to an increase in chloride transport.

One of the novel and important findings of the present study was the observation that VIP stimulated ozone-repressed CFTR trafficking. In our previous research, we found that ozone stress down-regulated CFTR function, which could potentially contribute to CF-like symptoms in a variety of inflammatory airway diseases [Qu et al., 2009]. Indeed, we have shown that VIP mediated inflammation-induced suppression of responses to cAMP-dependent secretagogues in HBECs. Interestingly, CFTR trafficking and secretory function as measured returned to normal when tissues were treated with VIP.

The present patch-clamp study demonstrated VIP activated cAMP-dependent Cl^- channels characterized by the following macroscopic properties: (1) ohmic I–V relationship, (2) PKA dependence, (3) PKC dependence, (4) insensitivity to DIDS, (5) sensitivity to glibenclamide, and (6) extracellular Cl^- dependence of channel activity and inactivation gating. Because VIP stimulation of CFTR Cl^- current was abolished by pretreating with PKA and PKC inhibitors, our results support the hypothesis that CFTR activation by VIP is mediated by both PKA and PKC pathways in HBECs. PKC phosphorylation is known to weakly stimulate CFTR channel activity and dramatically enhance its response to PKA stimulation [Tabcharani et al., 1991; Jia et al., 1997; Chappe et al., 2003].

In summary, we present the potent evidence in an epithelial organ that CFTR translocates from intracellular sites to the apical membrane after VIP stimulation. These findings strongly support a dual role for secretagogues in stimulating chloride secretion: acute recruitment of CFTR from intracellular sites to the apical membrane, and PKA and PKC phosphorylation-mediated regulation of channels residing in the membrane.

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