Antisense oligonucleotide to PKC-ε alters cAMP-dependent stimulation of CFTR in Calu-3 cells

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Liedtke, Carole M., and Thomas S. Cole. Antisense oligonucleotide to PKC-ε alters cAMP-dependent stimulation of CFTR in Calu-3 cells. *Am. J. Physiol.* 275 (*Cell Physiol.* 44): C1357–C1364, 1998.—Protein kinase C (PKC) regulates cystic fibrosis transmembrane conductance regulator (CFTR) channel activity but the PKC signaling mechanism is not yet known. The goal of these studies was to identify PKC isotype(s) required for control of CFTR function. CFTR activity was measured as ^{36}Cl efflux in a Chinese hamster ovary cell line stably expressing wild-type CFTR (CHO-wtCFTR) and in a Calu-3 cell line. Chelerythrine, a PKC inhibitor, delayed increased CFTR activity induced with phorbol 12-myristate 13-acetate or with the cAMP-generating agents (−)-epinephrine or forskolin plus 8-(4-chlorophenylthio)adenosine 3′,5′- cyclic monophosphate. Immunoblot analysis of Calu-3 cells revealed that PKC-α, -β$_{II}$, -δ, -ε, and -ζ were expressed in confluent cell cultures. Pretreatment of cell monolayers with Lipofectin plus antisense oligonucleotide to PKC-ε for 48 h prevented stimulation of CFTR with (−)-epinephrine, reduced PKC-ε activity in unstimulated cells by 52.1%, and decreased PKC-ε mass by 76.1% but did not affect hormone-activated protein kinase A activity. Sense oligonucleotide to PKC-ε and antisense oligonucleotide to PKC-δ and -ζ did not alter (−)-epinephrine-stimulated CFTR activity. These results demonstrate the selective regulation of CFTR function by constitutively active PKC-ε.

Chinese hamster ovary cell line; chelerythrine; chloride efflux; epinephrine; protein kinase A; protein kinase C; cystic fibrosis transmembrane conductance regulator

CYSTIC FIBROSIS (CF) is a disease of electrolyte transport abnormalities that is characterized by the production of dehydrated viscous secretions in lungs, pancreatic duct, and intestinal tract. The genetic basis for CF is mutation of the CF transmembrane conductance regulator (CFTR), a secretory Cl channel and conductance regulator. CFTR is a 1,480-amino acid protein with a unique structure characterized by three cytoplasmic domains, two nucleotide binding folds, and a regulatory (R) domain that contains consensus sequences for phosphorylation by protein kinase A (PKA) and by protein kinase C (PKC). There is direct evidence for phosphorylation of serine residues in the R domain by PKA (5, 24) and by PKC (24). Firm evidence for PKA-mediated phosphorylation of CFTR as the primary regulator of CFTR activity comes from studies showing that altering or removing sites of in vivo phosphorylation in the R domain reduces, but does not eliminate, PKA stimulation of CFTR in intact cells and excised patches (4, 27, 33). However, the role of PKC in regulating the CFTR channel is less clear.

Addition of phorbol ester potentiated cAMP responses in *Xenopus* oocytes expressing wild-type CFTR (28) and in HT-29 colonic cells (1), T84 cells (6), C127 cells (7), and pancreatic duct cells (34). In membrane patches excised from cells expressing CFTR, addition of exogenous PKC caused a modest increase in CFTR channel activity and enhanced the rate and magnitude of subsequent PKA stimulation of open probability (29). Nevertheless, the interpretation of effects of phorbol 12-myristate 13-acetate (PMA) is not uniform and, instead, varies from direct PKC regulation of CFTR channel (6, 7, 29) to PMA-mediated increase in cell membrane area (34) and also PKC-mediated de novo insertion of channels into the plasma membrane (1). Trying to pinpoint how PKC regulates CFTR is complicated, however, by the use of PMA, which also influences CFTR expression (30) and degradation (3). Moreover, although PKC is considered to be the major receptor of PMA, its interactions with other enzymes might obscure a specific role for PKC in CFTR function.

Our studies on PKC regulation of a Cl secretory pathway in tracheal epithelial cells (20, 22) led us to test the effects of a potent PKC inhibitor, chelerythrine, on CFTR function. Preliminary data showed that chelerythrine delayed efflux of ^{36}Cl in Calu-3 lung cells stimulated by (−)-epinephrine, a cAMP-generating agent (see Fig. 1), suggesting that constitutive PKC activity in unstimulated cells is necessary for maximal activation of CFTR. Similar findings were reported by Jia et al. (14), from patch-clamp studies of Chinese hamster ovary (CHO) and baby hamster kidney (BHK) cells expressing wild-type CFTR. The question of how PKC regulates CFTR is still unanswered. One step in understanding the regulation of CFTR by PKC is to identify PKC isotype(s) that are required for CFTR function. That is the goal of these studies.

MATERIALS AND METHODS

Materials. ^{36}Cl (specific activity 260 MBq/g, 7.5 mCi/g) was purchased from ICN Radiochemical (Irvine, CA) and [γ-^{32}P]ATP (specific activity 111 TBq/mmol, 3,000 Ci/mol) was purchased from Amersham Life Science (Arlington Heights, IL). An enhanced chemiluminescence kit was purchased from Amersham, and the PKC assay system, PKA assay system, and Lipofectin reagent were from GIBCO BRL Life Technologies (Gaithersburg, MD). KN-93 and chelerythrine chloride were purchased from Calbiochem (La Jolla, CA), PMA and forskolin were obtained from Research Biochemicals International (Natick, MA), and 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate (CPT-cAMP) and (−)-epinephrine were from Sigma (St. Louis, MO). Rabbit polyclonal anti-PKC

isotype-specific antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and monoclonal mouse anti-human CFTR (epitope 729–736 of the R domain) was purchased from Genzyme (Cambridge, MA). Tissue culture supplies were obtained from GIBCO BRL (Grand Island, NY) and Sigma. All other chemicals were reagent grade.

Cell isolation and culture. The Calu-3 cell line (American Type Culture Collection HTB-55; Ref. 26) was grown submerged in Eagle's medium with Earle's balanced salt solution and 10% fetal bovine serum (FBS) at 37°C in a humidified CO_2 incubator. For experiments, cells were seeded onto six-well tissue culture plastic dishes and grown to confluence, typically 7–8 days after subculture. A CHO cell line stably expressing wild-type CFTR (CHO-wtCFTR) was generously given to us by Dr. J. Riordan (Mayo Clinic, Scottsdale, AZ). The CHO-wtCFTR cell line was maintained in culture medium consisting of αMEM, 8% FBS, 200 μM methotrexate, and 1% penicillin-streptomycin. CHO cells were grown to confluence in a 5% CO_2 incubator at 37°C.

Measurement of CFTR activity as Cl efflux. CFTR activity was assayed by measuring the rate of ^{36}Cl efflux (32). Cell cultures were grown to confluence in six-well tissue culture dishes and preincubated in serum-free medium for 24 h before use. Cells were preincubated for 1 h at 35°C with 3.5 μCi ^{36}Cl in 10 mM HEPES-buffered Ringer (HBR; pH 7.5) solution that contained (in mM) 136.9 NaCl, 5.4 KCl, 0.4 KH_2PO_4, 0.3 $NaHPO_4$, 4.2 $NaHCO_3$, 1.3 $CaCl_2$, 0.5 $MgCl_2$, 0.4 $MgSO_4$, and 5.6 D-glucose. Medium with radioactive tracer was removed, and cells were washed four times with HBR to remove extracellular ^{36}Cl. After the wash, 0.5-ml aliquots of isotope-free HBR were added and sequentially removed every 60 s for up to 11 min. The first three aliquots were used to establish a stable baseline in efflux buffer only. Agonists were added after the third aliquot was removed. Inhibitors were present in the bathing medium for the last 30 min of the ^{36}Cl loading period and during the efflux period. Radioactive counts remaining in the cells were extracted with 0.1 N NaOH. The fraction of intracellular ^{36}Cl remaining in the cell layer during each time point was calculated from the sample and extract counts. Time-dependent rates of ^{36}Cl efflux were calculated as $\ln(^{36}\text{Cl}_{t=1}/^{36}\text{Cl}_{t=2})/(t_1 - t_2)$, where ^{36}Cl is the percent intracellular Cl at time t and t_1 and t_2 are successive time points.

Oligonucleotide treatment of cells. Phosphodiester oligonucleotides were purchased from GIBCO BRL. Antisense oligonucleotides to PKC isotypes were complementary to the translation initiation region of mRNA specific for the animal species delineated in Table 1. Sense oligonucleotides were used as controls. Oligonucleotides were dissolved in sterile deionized water to a final concentration of 1 mM, aliquoted, and stored at −20°C until ready for use.

Oligonucleotides were added to inside wells of confluent cell cultures in combination with the cationic lipid *N*-[1,(2,3-dioleyloxy)propyl]-*n*,*n*,*n*-trimethylammonium chloride and

dioleoyl phosphatidylethanolamine lipid (Lipofectin) in serum- and antibiotic-free culture medium, as previously described (20). Oligonucleotide incubation medium was replaced every 12 h for 48 h.

Immunoblot analysis of CFTR and PKC isotypes. Culture medium was replaced with Hanks' balanced salt solution supplemented with 10 mM HEPES (pH 7.5). Cells were treated with vehicle or drugs of interest at 35°C for times indicated. Cell cultures were immediately washed twice with ice-cold PBS and then harvested in 1 ml 100 mM NaCl, 50 mM NaF, 50 mM Tris·HCl (pH 7.55), 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate, 100 μM leupeptin, 1 μg/ml aprotinin, and 1 mM 4-(2-aminoethyl)benzenesulfonylfluoride hydrochloride. Immunoblot analysis of cell proteins for PKC isotypes was performed as described previously (22). Protein bands immunoreactive to specific antibodies were detected using enhanced chemiluminescence and analyzed by laser densitometry in a Sciscan 5000 (United States Biochemical), using the OS-Scan image analysis system software package (Oberlin Scientific).

To confirm the expression of wtCFTR in transfected cells and CFTR in Calu-3 cells, immunoblot analysis for CFTR was performed on lysates of cells. Lysis buffer was supplemented with 0.1% SDS. Lysates were incubated at 30°C for 30 min in 50 mM Tris (pH 6.8), 100 mM dithiothreitol, 5% glycerol, 4% SDS (wt/vol), and 0.1 β-mercaptoethanol (vol/vol) and centrifuged at 12,500 g for 2 min to pellet particulate material. Immunoblot analysis was performed on proteins separated on 6% SDS-polyacrylamide gels for CFTR using monoclonal antibody to the R domain of CFTR. CFTR was detected in Calu-3 cells and in CHO-wtCFTR cells (data not shown).

Measurement of PKC and PKA activity. Cell cultures grown on 60-mm tissue culture plastic dishes were treated with vehicle or the drug of interest and then harvested in 0.5 ml of lysis buffer. Lysates were clarified and incubated with antiserum against a specific PKC isotype, as previously described (22). Kinase activity of immune complexes of PKC isotypes was measured using histone III as the substrate for PKC-α, -β$_{II}$, -δ, and -ζ and a peptide derived from the pseudosubstrate region of PKC-ε as the substrate for PKC-ε (25). PKA activity of clarified lysates was measured using a commercially available assay system (GIBCO BRL Life Technologies).

Data analysis. Protein levels were determined with a Bradford assay kit (Bio-Rad, Hercules, CA) using BSA as the standard. Data were analyzed by ANOVA followed by Bonferroni multiple comparison tests or by Student's *t*-tests for unpaired samples. Data are reported as means ± SE for the number of cell monolayers tested (*n*).

RESULTS

Effect of PKC inhibitor on cAMP-stimulated efflux. CFTR function has been assessed in CHO-wtCFTR and Calu-3 cells using iodide efflux and whole cell and patch-clamp recordings of cell-attached and cell-excised patches (13, 29). These methods detected a cAMP-regulated Cl permeability that was indistinguishable from CFTR. For the studies reported here, ^{36}Cl efflux was used as an indicator of CFTR function. Figure 1 illustrates the effect of chelerythrine, a general PKC inhibitor, on CFTR function in CHO-wtCFTR and Calu-3 cells. Addition of the combination of forskolin and CPT-cAMP to CHO-wtCFTR cells rapidly increased the rate of ^{36}Cl efflux, with peak rates at 2 min after addition of stimulatory agents (Fig. 1*A*). Efflux

Table 1. *Oligonucleotide sequences of PKC isotypes*

PKC Isotype	Species	Antisense	Sense
PKC-δ	Mouse	AGGGTGCCATGATGGA	TCGATCATGGCACCCT
PKC-ε	Human	GGCTGGTACCATCACAAG	CCGACCATGGTAGTGTTC
PKC-ζ	Human	GCTCCCTTCCATCTTGGG	CCCAAGATGGAAGGGAGC

Oligonucleotides that hybridize to region of AUG initiation codon were selected for these studies. Sequences were complementary to translation initiation region as nucleotides −6 to 10 for protein kinase C (PKC)-δ, −6 to 12 for PKC-ε, and −6 to 12 for PKC-ζ. Sense oligonucleotides were used as controls.

rates subsequently declined to the steady-state level observed just before addition of stimulatory agents. Calu-3 cells gave a similar response to 3 μM (−)-epinephrine, an endogenous hormone that occupies α- and β-adrenergic receptors and increases cAMP levels (Fig. 1B). Maximal rates of ^{36}Cl efflux occurred 1–2 min after addition of hormone and declined afterward to prestimulatory steady-state levels. Figure 2 summarizes the sensitivity of CFTR activity at peak rates to cAMP-generating agents. The combination of forskolin and CPT-cAMP significantly increased the rate of efflux in CHO-wtCFTR to 0.78 ± 0.11 min^{-1} ($n = 9$; Fig. 2A) and in Calu-3 cells to 0.80 ± 0.16 min^{-1} ($n = 5$; Fig. 2B).
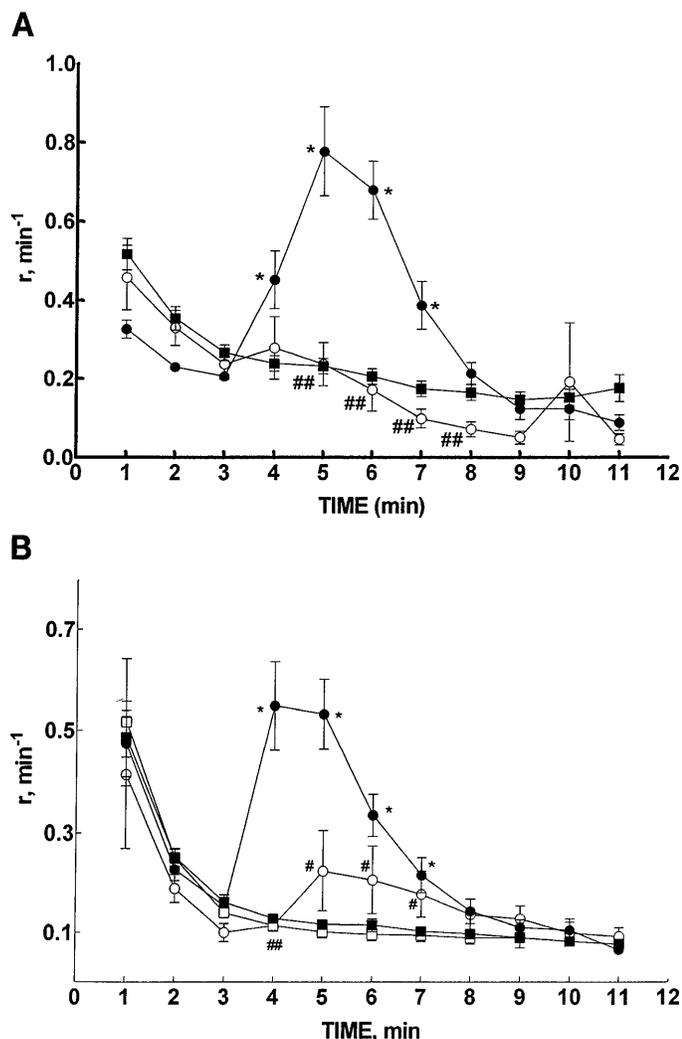

Fig. 1. Detection of cystic fibrosis transmembrane conductance regulator (CFTR) activity in CHO-wtCFTR (A) and Calu-3 (B) cell lines. Rate of ^{36}Cl efflux (r) is plotted as function of time. Chelerythrine chloride, at final concentration of 10 μM, was added to cells 30 min before start of radioisotopic efflux. Stimulatory agents were added immediately after sampling medium at 3 min and were present during remainder of efflux period. A: ■, vehicle ($n = 10$); ●, 10 μM forskolin and 50 μM CPT-cAMP ($n = 9$); ○, forskolin and CPT-cAMP + chelerythrine ($n = 5$). B: ■, vehicle ($n = 25$); □, chelerythrine ($n = 5$); ●, 3 μM (−)-epinephrine ($n = 6$); ○, (−)-epinephrine + chelerythrine ($n = 4$). Error bars are SE. * $P < 0.001$ compared with vehicle; # $P < 0.02$, ## $P < 0.005$ compared with cells treated with cAMP-generating agent.
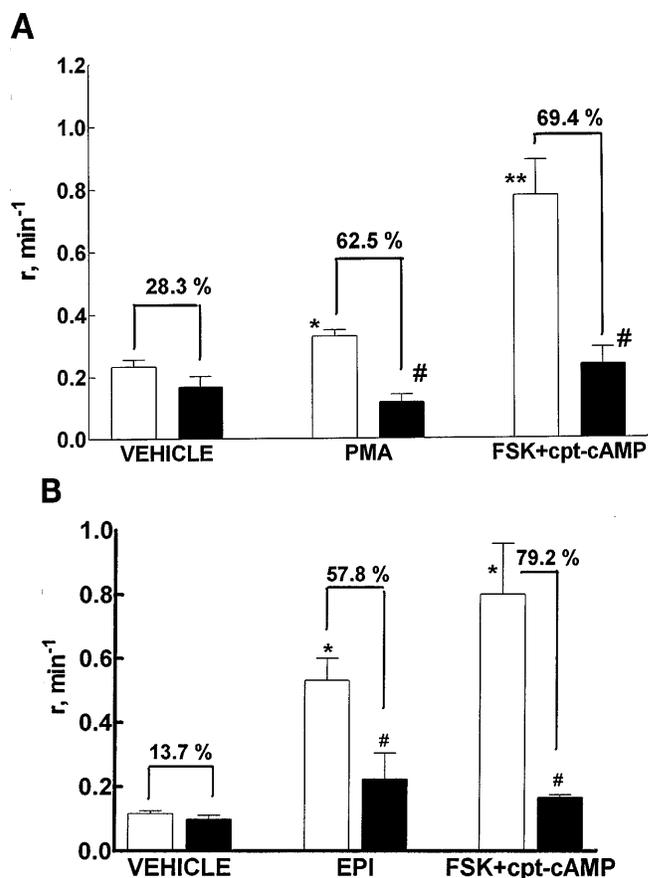

Fig. 2. Sensitivity of ^{36}Cl efflux from CHO-wtCFTR (A) and Calu-3 (B) cells to stimulatory agents. Data are taken from Fig. 1 at a time point 2 min after addition of vehicle or stimulatory agent. Percentages indicate component of radioisotopic efflux that was sensitive to 10 μM chelerythrine. Final concentrations were 1 μM phorbol 12-myristate 13-acetate (PMA), 10 μM forskolin (FSK), 50 μM CPT-cAMP, and 3 μM (−)-epinephrine (EPI). Error bars are SE for 4–10 replicate experiments for each condition. * $P < 0.03$, ** $P < 0.001$ compared with cells treated with vehicle; # $P < 0.02$, compared with cells treated with stimulatory agent alone.

As seen in Fig. 2B, forskolin and CPT-cAMP mimicked the effect of (−)-epinephrine in Calu-3 cells. A signaling mechanism for the effects of forskolin plus CPT-cAMP focuses on the elevation of cAMP levels by bypassing membrane receptors, which leads to activation of PKA and subsequent phosphorylation of CFTR and increased channel activity. However, the data of Fig. 1 suggest a role for PKC in cAMP-dependent activation of CFTR. Figure 1 shows that pretreatment with 10 μM chelerythrine, a general PKC inhibitor, abolished the stimulatory effects of forskolin and CPT-cAMP on CHO-wtCFTR cells and of (−)-epinephrine on Calu-3 cells, suggesting that PKC activity in unstimulated cells regulates CFTR function. As seen in Fig. 2A, chelerythrine significantly reduced peak rates elicited with cAMP-generating agents and, more importantly, with the phorbol ester PMA, an activator of PKC, in CHO-wtCFTR cells. The sensitivity of PMA-induced CFTR activity to chelerythrine indicates that inhibition of PKC blocks PKC-induced CFTR activity.

In Calu-3 cells, responses to forskolin plus CPT-cAMP were also markedly sensitive to preincubation

with chelerythrine (Fig. 2B). A comparison of the two cell lines shows that chelerythrine reduced peak Cl efflux to rates observed in cells treated only with vehicle. Inhibition by chelerythrine affected 69.4% of peak Cl efflux in CHO-wtCFTR cells and 79.2% of peak Cl efflux in Calu-3 cells. The data of Fig. 2 also show that the two cell lines differed in the rates of baseline Cl efflux in cells exposed only to vehicle. CHO-wtCFTR cells consistently displayed a 2.0-fold higher rate compared with Calu-3 cells ($P < 0.001$). This might indicate that, in cells grown in this laboratory, there is constitutive CFTR activity in unstimulated, stably transfected CHO-wtCFTR cells. $(-)$-Epinephrine induced a smaller increase in Cl efflux than did forskolin plus CPT-cAMP. One possible explanation for this response is activation of intracellular signaling pathways in addition to cAMP-generating pathways through α_1-adrenergic receptors (19), leading to increased activity of PKC-δ and -ζ with subsequent modulation of cAMP-increased CFTR function in addition to basolateral Na-K-2Cl cotransport (22).

Effect of antisense oligonucleotides on cAMP-dependent CFTR activation. The approach of antisense technology to reduce PKC isotype mass and activity was used by this laboratory to identify a critical role for PKC-δ in the regulation of Na-K-2Cl cotransport in human tracheal epithelial cells (20) and in CF/T43 cells (21). Calu-3 cells were used in the next series of experiments, in which an antisense approach was used to mimic the effect of inhibition of PKC by chelerythrine on CFTR function. First, PKC isotypes expressed by Calu-3 cells were identified by immunoblot analysis. Calu-3 cells were immunoreactive with polyclonal antibodies to PKC-α, -β$_{II}$, -δ, -ε, and -ζ (Fig. 3A). PKC-β$_I$, -γ, and -η were not detected. Proteins immunoreactive to PKC isotypes corresponded closely in apparent molecular weight to recombinant PKC isotypes and to calculated PKC isotype molecular weights. Immunoreactive protein bands to PKC isotypes that were found in Calu-3 cells were also found in normal human tracheal epithelial cells (22) and in CF/T43 cells (21). The relative distribution of PKC isotypes to cytosol and a particulate fraction in Calu-3 cells (Fig. 3A) shared similarities with normal human tracheal epithelial cells (22) and with CF/T43 cells (21), with PKC-α and -ζ localized predominantly in cytosol and PKC-β$_{II}$ distributed approximately evenly between cytosol and a particulate fraction. PKC-δ and -ε were distributed predominantly to cytosol.

Next, cells were cultured in the presence of antisense oligonucleotide to PKC-δ, -ε, or -ζ for 48 h. Optimal concentrations of oligonucleotide and Lipofectin were determined from dose-response curves. Antisense oligonucleotide to PKC-δ or -ζ did not alter $(-)$-epinephrine-stimulated ^{36}Cl efflux (Table 2), even though PKC-δ and -ζ mass were reduced by 73.7 ± 1.5 ($n = 3$) and $86.1 \pm 0.3\%$ ($n = 3$), respectively, without affecting PKC-α, -β$_{II}$, or -ε (Fig. 3B). Antisense oligonucleotide to PKC-ε, on the other hand, abolished stimulation of CFTR by
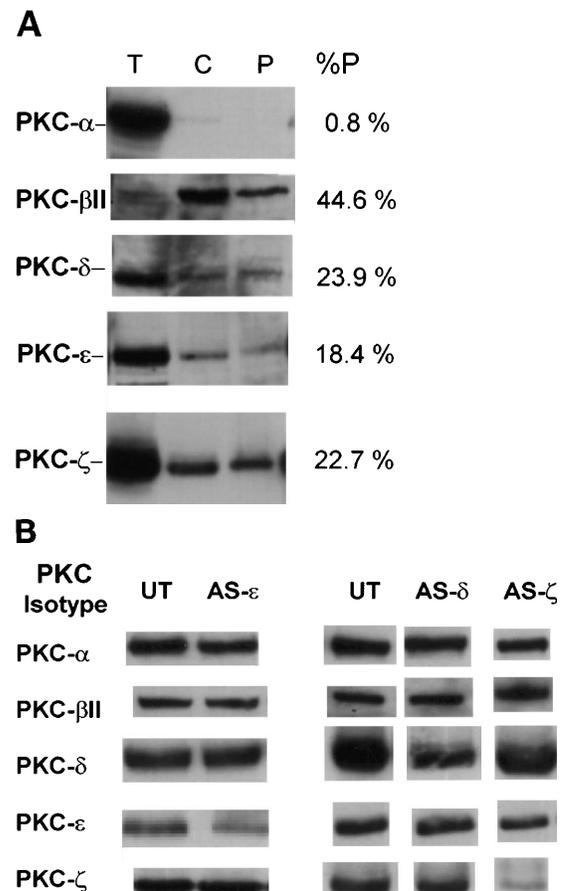
Fig. 3. Immunoblot analysis of protein kinase C (PKC) isotypes in Calu-3 cells. A: Calu-3 cells were lysed in hypotonic buffer and fractionated into cytosol (C) and a particulate (P) fraction, as previously described (22); 20 μg of homogenate (T), cytosol, and particulate fraction were subjected to electrophoresis on 8% SDS-PAGE gels. Protein bands were transferred to Immobilon papers and probed with polyclonal antibody to PKC isotypes, as previously described (22). Laser densitometry of bands detected in cytosol and particulate fractions was performed to obtain portion of total PKC isotype mass in cytosol and particulate fraction that was detected in particulate fraction (%P). B: cells were untreated (UT) or incubated with Lipofectin + antisense oligonucleotide (AS) to PKC-δ, -ε, or -ζ as described in MATERIALS AND METHODS; 20 μg of whole cell lysate were electrophoresed and analyzed as described for A.

$(-)$-epinephrine (Table 2 and Fig. 4). Baseline CFTR function was not significantly affected by treatment with Lipofectin or oligonucleotides (Fig. 4B). Cells pretreated with sense oligonucleotide to PKC-ε retained the stimulatory response to $(-)$-epinephrine and a chelerythrine-sensitive component of Cl flux similar to untreated and Lipofectin-treated cells (Fig. 4B). These results indicate that PKC-ε regulates CFTR function.

Effect of antisense oligonucleotide on PKC-ε expression and activity. Antisense oligonucleotide to PKC-ε could block activation of CFTR by cAMP by diminishing PKC-ε activity in unstimulated cells, decreasing the amount of PKC-ε, or both. The first possibility was investigated by measuring kinase activity of immune complexes of PKC isotypes in untreated cells or in cells preincubated for 24 h with 1 μg/ml Lipofectin or with Lipofectin plus antisense oligonucleotide to PKC-ε.

Table 2. *Effect of antisense oligonucleotides to PKC isotypes on CFTR function*

Condition	Rate of ^{36}Cl Efflux, min^{-1}	
	Vehicle	(−)-Epinephrine
Untransfected	0.117 ± 0.01 (25)	$0.528 \pm 0.07^*$ (6)
Lipofectin	0.122 ± 0.01 (13)	$0.524 \pm 0.04^*$ (14)
Antisense PKC-δ	0.111 ± 0.01 (6)	$0.528 \pm 0.01^*$ (6)
Antisense PKC-ϵ	0.154 ± 0.02 (5)	0.206 ± 0.09 (5)
Antisense PKC-ζ	0.099 ± 0.01 (6)	$0.443 \pm 0.03^*$ (14)

Values are means $\pm$ SE for the no. of experiments in parentheses. CFTR, cystic fibrosis transmembrane conductance regulator. Each experiment was performed on cells grown to confluence on 6-well tissue culture dishes. Cells were incubated in serum- and antibiotic-free medium in absence (untransfected) or in presence of 1 µg/ml Lipofectin or 1 µg/ml Lipofectin + 1 µM antisense oligonucleotides to indicated PKC isotype. Medium was changed every 12 h for 48 h. Data are rates of ^{36}Cl efflux measured 2 min after addition of (−)-epinephrine to a final concentration 3 µM. $^* P < 0.0001$ compared with vehicle-treated cells.

Incubation of Calu-3 cells with antisense oligonucleotide reduced total PKC-ϵ activity by 52.1% (Table 3). Moreover, PKC-ϵ activity per unit protein also significantly decreased, indicating a loss of PKC-ϵ activity. Treatment of cells with Lipofectin did not significantly alter baseline PKC-ϵ activity (Table 3). PKC-ϵ expres-

Table 3. *Effect of antisense oligonucleotides on activity of PKC-ϵ in Calu-3 cells*

Condition	Kinase Activity	
	Total activity, fmol/min	Specific activity, fmol·min^{-1}·mg protein^{-1}
Untransfected	305.1 ± 38.4 (5)	169.1 ± 8.5 (5)
Lipofectin	433.1 ± 95.2 (4)	184.4 ± 47.5 (3)
Antisense PKC-ϵ	$146.2 \pm 16.9^*$ (3)	$95.1 \pm 6.1^*$ (3)
Sense PKC-ϵ	249.7 ± 15.5 (4)	140.8 ± 16.5 (3)

Values are means $\pm$ SE for no. of experiments in parentheses. Each experiment was performed on confluent cells grown on 60-mm tissue culture dishes. Cells were incubated in serum- and antibiotic-free medium in absence (untransfected) or in presence of Lipofectin or Lipofectin + 1 µM antisense oligonucleotide to PKC-ϵ. Culture medium was discarded, and cell monolayers were washed twice in ice-cold PBS and then solubilized in lysate buffer. PKC-ϵ was immunoprecipitated using polyclonal antibody to PKC-ϵ, and kinase activity of immune complexes of PKC-ϵ was measured, as described in MATERIALS AND METHODS. Aliquots of lysate were subjected to immunoblot analysis for PKC-ϵ and analyzed by laser densitometry. $^* P < 0.02$ compared with untransfected cells.

sion was next evaluated in cells treated with antisense oligonucleotide to PKC-ϵ and, as a control, in untransfected cells (Fig. 3B). Antisense oligonucleotide reduced PKC-ϵ by $76.1 \pm 4.5\%$ ($n = 6$) and did not affect PKC-α,
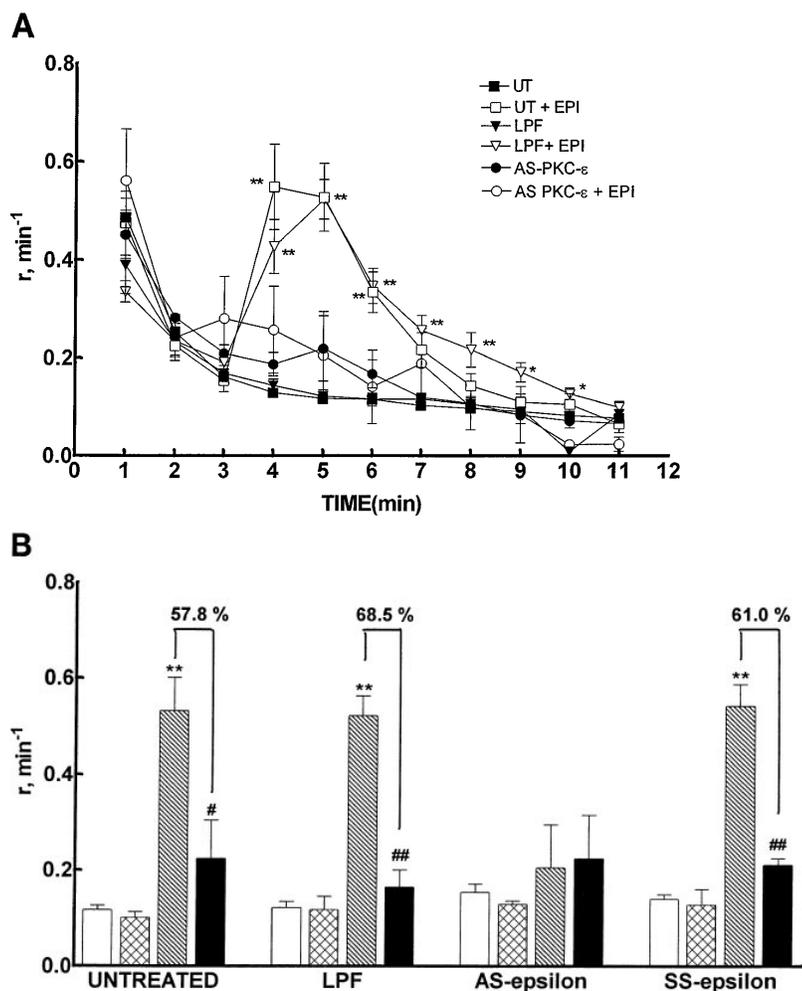

Fig. 4. Inhibition of cAMP-stimulated CFTR activity by antisense oligonucleotide to PKC-ϵ. *A*: rate of ^{36}Cl efflux is plotted as function of time. Cell monolayers were incubated without lipids or nucleotide (UT; $n = 25$), with 1 µg/ml Lipofectin (LPF; $n = 12$), or with Lipofectin + antisense oligonucleotide to PKC-ϵ (AS-PKC-ϵ; $n = 12$). Cells were stimulated with vehicle or with 3 µM (−)-epinephrine. Error bars are SE. *B*: plot of peak rates of ^{36}Cl efflux. Cell monolayers were incubated with 1 µM antisense oligonucleotide to PKC-ϵ (AS-epsilon; $n = 8$) or 1 µM sense oligonucleotide to PKC-ϵ (SS-epsilon; $n = 8$) for 48 h. Cells were treated with vehicle (open bar), 10 µM chelerythrine (crosshatched bar), 3 µM (−)-epinephrine (hatched bar), or epinephrine + chelerythrine (solid bar). Percentages refer to component of rate of efflux attributed to PKC-sensitive mechanism. Error bars are SE. $^* P < 0.05$, $^{**} P < 0.001$ compared with cells treated with vehicle; $^\# P < 0.03$, $^{\#\#} P < 0.0001$ compared with cells treated with (−)-epinephrine alone.

Table 4. *(−)-Epinephrine-stimulated PKA activity is unaffected by antisense oligonucleotide to PKC-ε*

Treatment	Additive	Kinase Activity	
		Total activity, pmol/min	%Activated
None	Vehicle	204.4 ± 11.4 (4)	18.0 ± 3.5 (3)
None	(−)-Epinephrine	191.9 ± 5.0 (3)	$61.0 \pm 3.2^*$ (3)
Lipofectin	Vehicle	246.9 ± 7.8 (4)	29.9 ± 2.9 (4)
Lipofectin	(−)-Epinephrine	205.4 ± 48.4 (4)	$58.2 \pm 9.5^*$ (4)
Antisense PKC-ε	Vehicle	188.8 ± 29.9 (4)	15.5 ± 4.5 (5)
Antisense PKC-ε	(−)-Epinephrine	197.8 ± 50.2 (4)	$52.4 \pm 6.4^*$ (4)

Values are means $\pm$ SE for no. of experiments in parentheses. Each experiment was performed on confluent cells grown on 30-mm tissue culture dishes. Cells were incubated in serum- and antibiotic-free medium as described for Table 2. Culture medium was discarded, and cell monolayers were washed twice with HEPES-buffered Hanks' balanced salt solution, incubated 2 min with indicated additive, and then extracted. Final concentration of (−)-epinephrine was 3 μM. Extract was clarified before assay for protein kinase A activity, as described in MATERIALS AND METHODS. $^*P < 0.005$ compared with vehicle-treated cells.

-β_{II}, -δ, or -ζ mass (Fig. 3*B*). These results indicate that antisense oligonucleotide to PKC-ε blocks CFTR function by decreasing PKC-ε mass and activity in Calu-3 cells.

Effect of antisense oligonucleotide on PKA activity. Regulation of CFTR function by PKC-ε could be due to modulation of PKA to alter PKA-dependent phosphorylation of CFTR. To test this possibility, PKA activity was quantitated in untransfected cells, Lipofectin-treated cells, and cells transfected with antisense oligonucleotide to PKC-ε. As seen in Table 4, antisense oligonucleotide to PKC-ε did not affect (−)-epinephrine-stimulated PKA activity, indicating the independence of PKA from PKC-ε. Therefore, PKC-ε regulates CFTR function at a different site.

DISCUSSION

Regulation of CFTR Cl channel by PKC has been inferred from studies of CFTR expressed in a variety of epithelial cell lines and in heterologous expression systems (1, 6, 7, 14, 28, 34), yet few studies provide detailed information about PKC regulation of CFTR. The current study provides the first report of regulation of CFTR channel function by a Ca-independent PKC isotype, PKC-ε. The cAMP-generating agent forskolin in combination with CPT-cAMP increased CFTR Cl channel activity, measured as ^{36}Cl efflux, from 0.23 ± 0.02 min^{-1} ($n = 10$) to 0.78 ± 0.11 min^{-1} ($n = 9$) in CHO-wtCFTR cells and from 0.12 ± 0.01 min^{-1} ($n = 25$) to 0.80 ± 0.16 min^{-1} ($n = 5$) in Calu-3 cells. Pretreatment of both cell lines with the general PKC inhibitor chelerythrine blocked cAMP-induced CFTR Cl channel activity (Figs. 1 and 2), suggesting that baseline, constitutive PKC activity is necessary for acute stimulation of CFTR. Jia et al. (14) came to a similar conclusion from a study of two mammalian expression systems, CHO-wtCFTR and BHK-wtCFTR, using an electrophysiological approach. The studies reported here also show that elevated CFTR function induced by PMA in CHO-wtCFTR cells (Fig. 2*A*) was blocked by pretreatment with chelerythrine, indicating

that chelerythrine blocks the effects of PMA by preventing an increase in PKC activity.

Previous studies by this laboratory on basolateral Na-K-2Cl cotransport showed that, in the absence of maximal Cl secretion, activation of cotransport by α_1-adrenergic agents requires a PKC signaling mechanism and that increased PKC-δ activity in a cytosolic fraction is necessary for activation (20). The identity of a PKC isotype necessary for cAMP-dependent CFTR Cl channel activity was investigated first by establishing which PKC isotypes were expressed in Calu-3 cells and then by mimicking the effects of inhibition of PKC enzyme activity by individually downregulating PKC isotypes. Calu-3 cells express five PKC isotypes representative of three major types of PKC isotypes (Fig. 3). Conventional PKC isotypes α and β_{II}, novel PKC isotypes δ and ϵ, and atypical PKC isotype ζ were identified by immunoblot analysis. The same PKC isotypes were also found in human tracheal epithelial cell cultures and in CF/T43 cells, indicating that this panel of PKC isotypes is a signature of tracheal epithelial cells and cell lines derived from primary tracheal epithelial cell cultures (20, 21).

Downregulation of PKC isotypes was accomplished using an antisense approach. Using this approach, this laboratory obtained convincing evidence for a role for PKC-δ in the activation of Na-K-2Cl cotransport. A major reason for using this approach is that long-term treatment with the phorbol ester PMA for 18 h did not deplete PKC isotype activity or abundance in CF/T43 cells (21) or in primary cultures of human tracheal epithelial cells (22). Treatment of Calu-3 cells with antisense oligonucleotide to PKC-δ or -ζ for 48 h reduced PKC-δ by 73.7% and PKC-ζ by 81.1%, respectively, but failed to prevent cAMP-dependent CFTR Cl channel function (Table 2) and did not affect Cl loading. Previous studies established that antisense oligonucleotide to PKC-δ prevents α_1-adrenergic activation of Na-K-2Cl cotransport but not baseline cotransport activity in unstimulated cells. Thus there may be sufficient cotransport activity in cells treated with antisense oligonucleotide to PKC-δ to support Cl loading. Alternatively, activity of other Cl transport pathways (e.g., Cl/HCO$_3$ exchange) might mediate Cl loading. A major finding of this study is that antisense oligonucleotide to PKC-ε potently blocked cAMP-dependent activation of CFTR Cl channel function (Table 3 and Fig. 4). Antisense oligonucleotide to PKC-ε also reduced PKC-ε by 76.1%, a finding consistent with the half-life of ~24 h for PKC in vitro, and significantly reduced baseline activity of PKC-ε (Table 3). The latter finding provides evidence for constitutive PKC-ε activity that was also suggested by Jia et al. (14) to explain their results.

A PKC-ε signaling mechanism involved in regulation of CFTR function was next investigated by asking whether PKC-ε modulated activity of PKA, a protein kinase that is specifically activated by cAMP and is necessary for increased CFTR Cl channel activity. Antisense oligonucleotide to PKC-ε did not affect a

(−)-epinephrine-mediated increase in PKA activity (Table 4) despite blocking (−)-epinephrine-induced CFTR Cl channel activity (Fig. 4). Hence, PKC-ε regulates CFTR Cl channel function at a site other than PKA. The phorbol ester PMA stimulates CFTR Cl channel function, apparently by increasing PKC phosphorylation of CFTR; however, more definitive studies are needed to determine whether phosphorylation of CFTR by PKC-ε in unstimulated cells is necessary to achieve maximal CFTR channel activity when cAMP levels are elevated.

Downregulation of PKC-ε by antisense oligonucleotides or by long-term phorbol ester treatment has implicated PKC-ε in a number of cellular functions, including regulation of electrolyte and nonelectrolyte transporters. In a human liver epithelial BC1 cell line, long-term PMA treatment reduced CFTR mRNA, with a concomitant inhibition of CFTR Cl channel activity, and induced a cytosol-to-membrane translocation of PKC-α and -ε (16), indicating a role for these PKC isotypes in previously observed PMA-sensitive CFTR expression (30). PKC-ε was also found to be necessary for inhibition of vasopressin-stimulated Na transport in rabbit cortical collecting duct cells, suggesting cAMP-mediated activation of PKC-ε (8), and for amino acid transport in cultured human fibroblasts (11). In rat cardiac muscle, PKC-ε translocation to cross-striated structures after stimulation with PMA or with α_1-adrenergic agonists (10) can be blocked with chelerythrine or the V1 fragment of PKC-ε (10), resulting in loss of contractility (9, 15) and protection from ischemic injury (12). PKC isotypes, either alone or in combination with other PKC isotypes, play major roles in cross talk among signal transduction pathways, as demonstrated in recent studies. For example, mechanosensitive signal transduction in endothelial cells involves PKC-ε and extracellular signal-regulated kinase-1/2 (31), and tumor necrosis factor-α-regulated insulin signaling in HEK-293 cells involves PKC-ε (17). However, selective activation of PKC-α or -ε in rat adrenal glomerulosa cells is stimulus dependent (23), and the level of expression of human endothelial nitric oxide synthase increases with PMA-stimulated PKC-α and -ε (18).

Identification of a Ca-independent PKC isotype in the regulation of CFTR agrees with the phosphorylation studies of Picciotto et al. (24), who report that PKC phosphorylated CFTR in a Ca-independent manner. Phosphopeptide mapping localized PKC phosphorylation sites at serines 686 and 790 of the R domain; however, in vivo PKC phosphorylation using PMA as a PKC activator revealed additional sites outside the R domain. The role of PKC phosphorylation sites is still not clear, although recent studies by Wilkinson et al. (33) suggest an interaction between phosphorylated amino acid residues to explain roles for inhibitory and stimulatory phosphorylation sites (16).

The finding that PKC-ε regulates CFTR function is novel and clearly differentiates PKC-ε regulation of CFTR from PKC-δ modulation of Na-K-2Cl cotransport. Although both PKC isotypes are diacylglycerol dependent and Ca²⁺ independent, cotransport activation requires increased PKC-δ activity as opposed to an apparently constitutive PKC-ε activity linked to CFTR. These results indicate specific roles for selective PKC isotypes in airway epithelial cells and, furthermore, suggest that a critical component of a PKC signaling mechanism is the targeting of a PKC isotype to its substrate protein, which can be localized to apical or basolateral membranes.

REFERENCES

1. **Bajnath, R. B., J. A. Groot, H. R. DeJonge, M. Kansen, and J. Bijman.** Synergistic activation of non-rectifying small-conductance chloride channels by forskolin and phorbol esters in cell-attached patches of the human colon carcinoma cell line HT-29cl.19A. *Pflügers Arch.* 425: 100–106, 1993.

2. **Basta, P., M. B. Strickland, W. Holmes, C. R. Loomis, L. M. Ballas, and D. J. Burns.** Sequence and expression of human protein kinase C-epsilon. *Biochim. Biophys. Acta* 1132: 154–160, 1992.

3. **Breuer, W., H. Glickstein, N. Kartner, J. R. Riordan, D. A. Ausiello, and I. Z. Cabantchik.** Protein kinase C mediates down-regulation of cystic fibrosis transmembrane regulator levels in epithelial cells. *J. Biol. Chem.* 268: 13935–13939, 1993.

4. **Chang, X.-B., J. A. Tabcharani, Y.-X. Hou, T. J. Jensen, N. Kartner, N. Alon, J. W. Hanrahan, and J. R. Riordan.** Protein kinase A (PKA) still activates CFTR chloride channel after mutagenesis of all 10 PKA consensus phosphorylation sites. *J. Biol. Chem.* 268: 11304–11311, 1993.

5. **Cohn, J. A., A. C. Nairn, C. R. Marino, O. Melhus, and J. Kole.** Characterization of the cystic fibrosis transmembrane conductance regulator in a colonocyte cell line. *Proc. Natl. Acad. Sci. USA* 89: 2340–2344, 1992.

6. **Dechecchi, M. C., R. Rolfini, A. Tamanini, C. Gamberi, G. Berton, and G. Cabrini.** Effect of modulation of protein kinase C on the cAMP-dependent chloride conductance in T84 cells. *FEBS Lett.* 311: 25–28, 1992.

7. **Dechecchi, M. C., A. Tamanini, G. Berton, and G. Cabrini.** Protein kinase C activates chloride conductance in C127 cells stably expressing the cystic fibrosis gene. *J. Biol. Chem.* 268: 11321–11325, 1993.

8. **DeCoy, D. L., J. R. Snapper, and M. D. Breyer.** Antisense DNA down-regulates protein kinase C-ε and enhances vasopressin-stimulated Na⁺ absorption in rabbit cortical collecting duct. *J. Clin. Invest.* 95: 2749–2756, 1995.

9. **Deng, X.-F., S. Mulay, and D. R. Varma.** Role of Ca²⁺-independent PKC in α_1-adrenoreceptor-mediated inotropic responses of neonatal rat hearts. *Am. J. Physiol.* 273 (*Heart Circ. Physiol.* 42): H1113–H1118, 1997.

10. **Disatnik, M.-H., G. Buraggi, and D. Mochly-Rosen.** Localization of protein kinase C isozymes in cardiac myocytes. *Exp. Cell Res.* 210: 287–297, 1994.

11. **Franchi-Gazzola, R., R. Visigalli, O. Bussolati, and G. C. Gazzola.** Involvement of protein kinase Cε in the stimulation of anionic amino acid transport in cultured human fibroblasts. *J. Biol. Chem.* 271: 26124–26130, 1996.

12. **Gray, M. O., J. S. Karliner, and D. Mochly-Rosen.** A selective ε-protein kinase C antagonist inhibits protection of cardiac myocytes from hypoxia-induced cell death. *J. Biol. Chem.* 272: 30945–30951, 1997.

13. **Haws, C., W. E. Finkbeiner, J. H. Widdicombe, and J. J. Wine.** CFTR in Calu-3 human airway cells: channel properties and role in cAMP-activated Cl⁻ conductance. *Am. J. Physiol.* 266 (*Lung Cell. Mol. Physiol.* 10): L502–L512, 1994.

14. **Jia, Y., C. J. Mathews, and J. W. Hanrahan.** Phosphorylation by protein kinase C is required for acute activation of cystic fibrosis transmembrane conductance regulator by protein kinase A. *J. Biol. Chem.* 272: 4978–4984, 1997.

15. **Johnson, J. A., M. O. Gray, C.-H. Chen, and D. Mochly-Rosen.** A protein kinase C translocation inhibitor as an isozyme-selective antagonist of cardiac function. *J. Biol. Chem.* 271: 24962–24966, 1966.

16. **Kang-Park, S., N. Dray-Charier, A. Munier, C. Brahimi-Horn, D. Veissiere, J. Picard, J. Capeau, G. Cherqui, and O. Lascols.** Role for PKC alpha and PKC epsilon in down-regulation of CFTR mRNA in a human epithelial liver cell line. *J. Hepatol.* 28: 250–262, 1998.

17. **Kellerer, M., J. Mushack, H. Mischak, and H. U. Haring.** Protein kinase C (PKC) epsilon enhances the inhibitory effect of TNF alpha on insulin signaling in HEK293 cells. *FEBS Lett.* 418: 119–122, 1997.

18. **Li, H., S. A. Oehrlein, T. Wallerath, I. Ihrig-Biedert, P. Wohlfart, T. Ulshofer, T. Jessen, T. Herget, U. Forstermann, and H. Kleinert.** Activation of protein kinase C alpha and/or epsilon enhances transcription of the human endothelial nitric oxide synthase gene. *Mol. Pharmacol.* 53: 630–637, 1998.

19. **Liedtke, C. M.** The role of protein kinase C in α-adrenergic regulation of NaCl(K) cotransport in human airway epithelial cells. *Am. J. Physiol.* 268 (*Lung Cell. Mol. Physiol.* 12): L414–L423, 1995.

20. **Liedtke, C. M., and T. Cole.** Antisense oligodeoxynucleotide to PKC-δ blocks α₁-adrenergic activation of Na-K-2Cl cotransport. *Am. J. Physiol.* 273 (*Cell Physiol.* 42): C1632–C1640, 1997.

21. **Liedtke, C. M., and T. S. Cole.** Antisense oligodeoxynucleotide to PKCδ downregulates PKCδ mass and activity and suppresses α₁-adrenergic activation of Na-Cl-K cotransport in human tracheal epithelial cells. *Pediatr. Pulmonol. Suppl.* 14: 243, 1997.

22. **Liedtke, C. M., T. Cole, and M. Ikebe.** Differential activation of PKC-δ and -ζ by α₁-adrenergic stimulation in human airway epithelial cells. *Am. J. Physiol.* 273 (*Cell Physiol.* 42): C937–C943, 1997.

23. **Natarajan, R., L. Lanting, L. Xu, and J. Nadler.** Role of specific isoforms of protein kinase C in angiotensin II and lipoxygenase action in rat adrenal glomerulosa cells. *Mol. Cell. Endocrinol.* 101: 59–66, 1994.

24. **Picciotto, M. R., J. A. Cohn, G. Bertuzzi, P. Greengard, and A. C. Nairn.** Phosphorylation of the cystic fibrosis transmembrane conductance regulator. *J. Biol. Chem.* 267: 12742–12752, 1992.

25. **Schaap, D., P. J. Parker, A. Bristol, R. Kriz, and J. Knopf.** Unique substrate specificity and regulatory properties of PKC-ε: a rationale for diversity. *FEBS Lett.* 243: 351–357, 1989.

26. **Shen, B.-Q., W. E. Finkbeiner, J. J. Wine, R. J. Mrsny, and J. H. Widdicombe.** Calu-3: a human airway epithelial cell line that shows cAMP-dependent Cl⁻ secretion. *Am. J. Physiol.* 266 (*Lung Cell. Mol. Physiol.* 10): L493–L501, 1994.

27. **Siebert, F. S., J. A. Tabcharani, X.-B. Chang, A. M. Dulhanty, C. Mathews, J. W. Hanrahan, and J. R. Riordan.** cAMP-dependent protein kinase-mediated phosphorylation of cystic fibrosis transmembrane conductance regulator residue Ser-753 and its role in channel activation. *J. Biol. Chem.* 270: 2158–2163, 1995.

28. **Sullivan, S. K., K. Swamy, and M. Field.** cAMP-activated Cl conductance is expressed in *Xenopus* oocytes by injection of shark rectal gland mRNA. *Am. J. Physiol.* 260 (*Cell Physiol.* 29): C664–C669, 1991.

29. **Tabcharani, J. A., X.-B. Chang, J. R. Riordan, and J. W. Hanrahan.** Phosphorylation-regulated Cl⁻ channel in Cho cells stably expressing the cystic fibrosis gene. *Nature* 352: 628–631, 1991.

30. **Trapnell, B. C., P. L. Zeitlin, C.-S. Chu, K. Yoshimura, H. Nakamura, W. B. Guggino, J. Bargon, T. C. Banks, W. Dalemans, A. Pavirani, J.-P. Lecocq, and R. G. Crystal.** Down-regulation of cystic fibrosis gene mRNA transcript levels and induction of the cystic fibrosis chloride secretory phenotype in epithelial cells by phorbol ester. *J. Biol. Chem.* 266: 10319–10323, 1991.

31. **Traub, O., B. P. Monia, N. M. Dean, and B. C. Berk.** PKC-ε is required for mechano-sensitive activation of ERK1/2 in endothelial cells. *J. Biol. Chem.* 272: 31251–31257, 1997.

32. **Vanglarik, C. J., R. J. Bridges, and R. A. Frizzell.** A simple assay for agonist-regulated Cl and K conductances in salt-secreting cells. *Am. J. Physiol.* 259 (*Cell Physiol.* 28): C358–C364, 1990.

33. **Wilkinson, D. J., T. V. Strong, M. K. Mansoura, D. L. Wood, S. S. Smith, F. S. Collins, and D. C. Dawson.** CFTR activation: additive effects of stimulatory and inhibitory phosphorylation sites in the R domain. *Am. J. Physiol.* 273 (*Lung Cell. Mol. Physiol.* 17): L127–L133, 1997.

34. **Winpenny, J. P., H. L. McAlroy, M. A. Gray, and B. E. Argent.** Protein kinase C regulates the magnitude and stability of CFTR currents in pancreatic duct cells. *Am. J. Physiol.* 268 (*Cell Physiol.* 37): C823–C828, 1995.