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PKC signaling in CF/T43 cell line: regulation of NKCC1 by PKC-δ isotype

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Abstract

Cystic fibrosis (CF) airway epithelial cells have a reduced mass of ether-linked diacylglycerols which might alter protein kinase C (PKC)-regulated Cl secretion. PKC regulation of basolateral Na-K-2Cl cotransport (NKCC1) was investigated in CF nasal polyp epithelial cells and a CF/T43 cell line to ascertain whether PKC signaling was altered in CF. NKCC1 was detected as bumetanide-sensitive ^{86}Rb influx. Methoxamine, a α_1-adrenergic agonist, increased PKC activity in cytosol and a particulate fraction for a prolonged time period, as predicted from previous studies on the generation of diglycerides induced with methoxamine. Short-term stimulation of CF/T43 cells for 40 s promoted a shift in PKC-δ and -ζ to a particulate fraction, increased activity of immune complexes of cytosolic PKC-δ and of particulate PKC-ζ and increased activity of NKCC1. Pretreatment with antisense oligonucleotide to PKC-δ blocked methoxamine-stimulated PKC-δ activity, reduced PKC-δ mass by 61.4%, and prevented methoxamine-stimulated activity of NKCC1. Sense and missense oligonucleotide to PKC-δ and antisense oligonucleotide to PKC-ζ did not alter expression of PKC-δ or the effects of methoxamine. These results demonstrate that PKC-δ-dependent activation of NKCC1 is preserved in CF cells and suggest that regulation of NKCC1 is independent of low ether-linked diglyceride mass.

Keywords: Cotransport; Bumetanide; α-Adrenergic; Protein kinase C; Antisense oligonucleotide; Cystic fibrosis

1. Introduction

Cloning of mammalian cation-Cl cotransporters has revealed a high degree of homology based on deduced amino acid sequences [1,2]. One form of cation-Cl cotransporter is an epithelial secretory Na-K-2Cl isoform (NKCC1), which provides for the basolateral entry for Cl during fluid secretion. Large airway epithelium of trachea and bronchi express basolateral Na-K-2Cl cotransport which acts in concert with apical Cl channels and basolateral Na,K-ATPase and K channels to produce NaCl secretion [3]. It is presumed that tracheal epithelial Na-K-2Cl cotransport is the secretory form of cation-Cl cotransporters and, hence, will be referred to here as NKCC1 [3]. Cellular events leading to activation of NKCC1 during secretion are not well understood. In some cells, NKCC1 is activated by cAMP generating agents; however, in non-CF tracheal epithelial cells and cystic fibrosis (CF) nasal polyp epithelial cells, NKCC1 is activated by α_1-adrenergic agents through protein kinase C (PKC) [4–6]. Detailed studies of the

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mechanism of signal transduction demonstrated a coupling between α_1-adrenergic receptor and PtdIns(4,5)P_2-dependent PLC resulting in the generation of inositol tris- and bis-phosphates [7] and diacylglycerol [4]. The generation of inositol bis- and tris-phosphates was transient with peak levels attained before 1 min. The formation of diacylglycerol was biphasic with peak levels observed at less than 1 min and between 6 and 8 min. However, α_1-adrenergic stimulation of NKCC1 corresponded only to the initial diacylglycerol peak and not to the secondary peak. More importantly, quantitation of diacylglycerol flux showed differences between non-CF and CF cells in the molecular species of diglycerides generated during α_1-adrenergic stimulation [8]. Most striking was a reduction in the relative mass of ether-linked diglyceride species, which are thought to down-regulate PKC activity.

One suggestion from these studies was that CF cells compensated for defective CFTR, an apical Cl channel affected by the gene defect in CF, by augmenting PKC activity to maintain optimal Cl influx via NKCC1. Indeed, previous studies by this laboratory established that CF nasal polyp epithelial cells were strikingly similar to non-CF tracheal epithelial cells in the regulation of NKCC1 by α_1-adrenergic agonist, PKC inhibitors and protein phosphatase inhibitors [7]. In addition to activation of NKCC1, PKC has also been implicated in the regulation of CFTR [9–12]. More importantly, our recent studies provide evidence for PKC-ε regulation of cAMP-dependent activation of CFTR [13].

Molecular cloning has revealed that PKC is a multigene family consisting of at least 11 different isotypes that are classified as Ca^{2+}/diacylglycerol-dependent (α, βI, βII, γ), Ca^{2+}-independent but diacylglycerol-dependent (δ, ε, η, θ) and Ca^{2+}/diacylglycerol-independent (ζ, λ/ι) [14,15]. Recently, this laboratory reported that α_1-adrenergic stimulation increased the activity of PKC-δ and PKC-ζ [16]. Because long-term treatment of cells with phorbol ester did not diminish PKC mass or activity, we used antisense oligodeoxynucleotides specific for PKC-δ or PKC-ζ mRNA to gain further information on the role of each PKC isotype in the activation of NKCC1. We found that PKC-δ was necessary for activation of NKCC1 in non-CF human tracheal epithelial cells [6]. A similar approach also allowed us to link constitutively active PKC-ε to modulation of CFTR [13].

Because of its importance as a potential target for therapeutic intervention in CF airway epithelial cells, a PKC signaling pathway was further examined in CF cells. The goal of the present study was to determine whether PKC isotype(s) were regulated by α_1-adrenergic stimulation as in non-CF cells despite differences in profile of molecular species of diglycerides. Studies were performed on CF nasal polyp epithelial cells and CF/T43 cell line that is used extensively for studies on mutant CFTR. These studies are the first that examine PKC expression, activity, and function in this cell line.

2. Materials and methods

2.1. Materials

^{86}Rb (specific activity 154 Bq/g Rb, 4200 Ci/g Rb), [γ-^{32}P]ATP (specific activity 111 TBq/mmol, 3000 Ci/mol) and a enhanced chemiluminescence kit were purchased from Amersham Life Science (Arlington Heights, IL). Polyclonal anti-PKC isotype-specific antibodies, immunizing peptides, and recombinant PKC isotypes were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Methoxamine HCl was supplied by Burroughs Wellcome (Research Triangle Park, NC) and prazosine HCl by Pfizer Pharmaceutical (New York, NY). Calphostin was obtained from Calbiochem (La Jolla, CA) and chelerythrine Cl from Research Biochemicals International, (Natick, MA). A protein kinase C assay system and tissue culture supplies were obtained from Gibco-BRL, Grand Island, NY. All other chemicals were reagent grade.

2.2. Cell isolation and culture

Tissue was obtained from non-CF tracheas at the time of autopsy and from 18 nasal polyp specimens from CF patients at the time of polypectomy through the Cystic Fibrosis Center, Case Western Reserve University. Epithelial cells were isolated and grown in in vitro cell culture. Dr. J. Yankaskas (University of North Carolina at Chapel Hill) generously supplied a human airway epithelial cell line

CF/T43. The CF/T43 cell line was derived from airway epithelial cells of a homozygous ΔF508 CF patient using the SV40T gene [17] and was grown on tissue culture plastic dishes in Keratinocyte Growth Medium (Clonetics, San Diego, CA) supplemented with 2.0 μg/ml bovine pituitary extract, 10 ng/ml epidermal growth factor, 5 μg/ml insulin, and 0.5 μg/ml hydrocortisone. Culture medium was changed at 48-h intervals until confluence was reached. Monolayers expressed bumetanide-sensitive K uptake, indicating that drugs reached a basolateral surface.

2.3. Oligonucleotide treatment of cells

Oligonucleotides were purchased from Gibco-Life Technologies (Grand Island, NY). Antisense oligonucleotides were complementary to the translation initiation region of mRNA specific for mouse PKC-δ (AGGGTGCCATGATGGA, Genbank accession no. X60304) [18] and human PKC-ζ (GCTCCCTTCCATCTTGGG) [19]. Sense oligonucleotides to PKC-δ (TCGATCATGGCACCCT) and PKC-ζ (CCCAAGATGGAAGGGAGC) and three missense oligonucleotides to PKC-δ (TCGGAGCTAAGGAGGT, CGAGTAGTTAGAGCGG, AGGGCGTTACGACGGA) were used as controls. Oligodeoxynucleotides were dissolved in sterile deionized water to a final concentration of 1 mM and added to inside wells of cell cultures at a final concentration of 10 μM in serum-and antibiotic-free culture medium. Oligonucleotide incubation medium was replaced every 12 h for 48 h.

2.4. Western blot analysis of protein kinase C isotypes

After cell cultures reached confluence, culture medium was replaced with HEPES-buffered Hank's balanced salt, pH 7.5 (HPSS). Cells were treated with vehicle or drugs of interest at 37°C. Cells were immediately washed twice with ice-cold PBS, harvested and assayed for protein. Aliquots were solubilized in Laemmli buffer and subjected to 8% SDS-PAGE. Protein bands were transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA) and immunoblotted with polyclonal antibodies to specific PKC isotypes. Immunoreactive protein bands were detected using enhanced chemiluminescence and quantitated by laser densitometry.

2.5. Subcellular fractionation of primary cultures

Confluent cell cultures were washed twice with ice-cold PBS then harvested in 1.0 ml of ice-cold homogenization buffer consisting of 50 mM Tris-HCl, pH 7.5, 2 mM EGTA, 5 mM $MgCl_2$, 10 μM 4-(2-aminoethyl)benzenesulfonylfluoride hydrochloride (AEBSF), and 20 μg/ml leupeptin. The cells were homogenized by ultrasonic probe (3×10-s pulses) and cytosolic and particulate fractions recovered as described previously [16].

2.6. Immunoprecipitation of PKC isotypes

Cells were grown to confluence on 60 mm tissue culture plastic dishes then stimulated with vehicle or drug of interest. Reactions were stopped by rapid washing with ice-cold PBS. Cells were immediately lysed and PKC isotype immunoprecipitated as described [16].

2.7. PKC assay

PKC activity in subcellular fractions was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]-ATP into a PKC substrate, AcQKRPSQRSKYL (Ac-MBP(4–14)), in a protein kinase C assay system (Gibco-Life Technologies). Kinase activity of immune complexes of PKC isotype was measured by taking immunoprecipitates to a final volume of 50 μl in assay mixture (50 mM Tris-HCl, pH 7.5, 10 mM β-mercaptoethanol, 10 mM $MgSO_4$, 40 μg/ml phosphatidylserine, 0.1 μM PMA, 50 μM ATP, 10 μg/ml histone-III or 0.1 mM ε-peptide, 3 μCi [γ-^{32}P]ATP, 1 mM Na orthovanadate, 1 μM KN-93, and plus protease inhibitors) and incubating at 30°C for 15 min. Kinase activity of PKC-ε was measured using a synthetic peptide (ε-peptide) based on the pseudosubstrate region of PKC-ε. The reaction was terminated by addition of 30 μl glacial acetic acid. A 40-μl aliquot was spotted on P-81 phosphocellulose paper, washed, and counting for radioactivity by Cerenov counting.

2.8. K uptake

NKCC1 activity was measured as bumetanide-sensitive uptake of ^{86}Rb; a congener for K, into CF/T43

cells grown on six-well tissue culture plastic dishes. Cells were serum-deprived for 24 h prior to experiments and preincubated for 30 min at 32°C following addition of vehicle, 50 μM bumetanide, or drug of interest in 0.5 ml HEPES-buffered Ringers solution (HBR), consisting of 10 mM HEPES, 138 mM NaCl, 5 mM KCl, 2.5 mM Na_2HPO_4, 1.8 mM $CaCl_2$, 1 mM $MgSO_4$, and 10 mM glucose, pH 7.5. After preincubation, 5 μl HPR supplemented with 1 μCi ^{86}Rb plus drugs of interest was added to the cell monolayer to initiate radiotracer uptake. Uptake was terminated by rapidly removing the radioactive transport medium and washing the cell monolayer four times with ice-cold isotonic buffer consisting of 100 mM $MgSO_4$ and 137 mM sucrose [20]. Intracellular radioactivity was extracted by incubating cell monolayers in 0.1 N NaOH. Aliquots of extract were assayed for radioactive counts by liquid scintillation counting and for protein with a Bradford assay kit using bovine serum albumin as the standard. Intracellular radiotracer content was calculated as nmol K/mg protein.

2.9. Data analysis

Data are reported as mean ± S.E. To determine the level of significance, an unpaired *t*-test with Welch correction or analysis of variance followed by Bonferroni multiple comparison post-test was performed using a GraphPad Instat 3.0 computer program [21].

3. Results

To analyze the expression of PKC isotypes in a CF/T43 cell line and primary cultures of CF nasal polyp epithelial cells, identical amounts of protein from each cell type were applied to SDS-PAGE gels and analyzed by Western blot analysis. The amount of protein was within the linear range for quantitative analysis by chemiluminescence [6]. Immunoreactive protein bands to PKC-α, -βII, -δ, -ε, and -ζ that were found in non-CF human tracheal epithelial cells were also expressed in CF nasal polyp epithelial cells and in a CF/T43 cell line. PKC-βI, -γ and -η were not detected. Immunoblot analysis of subcellular fractions revealed differences in the pattern of distribution of PKC isotypes in each cell type. In non-CF tracheal epithelial cells, 7% of PKC-α and 12% of PKC-ζ localized to a particulate fraction and PKC-βII and -δ distributed approximately evenly between cytosolic and particulate fractions. However, in CF nasal polyp epithelial cells, 15% of PKC-δ was detected in a particulate fraction and, in a CF/T43 cell line, 22% of PKC-α, 94% of PKC-βII and 18% of PKC-ζ were localized to a particulate fraction. Although markedly different than non-CF tracheal epithelial cells, the unique distribution and relative level of expression of PKC isotypes in CF cells tested here might reflect differences in origin of each cell type and, in addition, transformation of CF/T43 cells with SV40T antigen.

In non-CF human tracheal epithelial cells, α_1-adrenergic agents stimulate NKCC1 [4] and increase PKC activity [6] within a short time frame of <1 min. When tested in both types of CF cells, methoxamine, a selective α_1-adrenergic agonist, increased PKC activity, after 40 s, in cytosolic and particulate fractions of CF nasal polyp epithelial cells and CF/T43 cells (Table 1). At this time point, the ratio of PKC activity in methoxamine-treated-cells to PKC activity in untreated cells was significantly greater than 1.0 ($P<0.03$ for each cell type). However, as

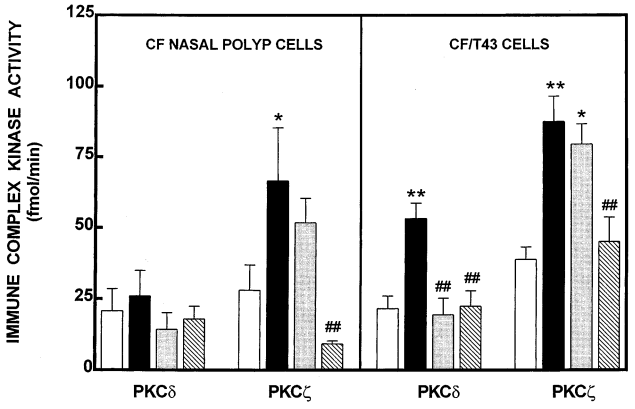

Fig. 1. Kinase activity of immune complexes of PKC-δ and -ζ from cells stimulated with methoxamine. Cells were incubated with vehicle (open bar), 10 μM methoxamine (solid bar), methoxamine plus 10 μM rottlerin (stippled bar) or 50 nM calphostin (crosshatch bar) then lysed with detergent buffer. PKC isotypes were immunoprecipitated and immediately assayed for kinase activity, as described in Section 2. Total kinase activity was calculated as fmol/min and is reported as mean ± S.E. for experiments on five CF nasal polyp epithelial cell preparations and on nine separate experiments with CF/T43 cells. Levels of significance: $^{*}P<0.05$, $^{**}P<0.001$ compared to vehicle; $^{\#}P<0.05$, $^{\#\#}P<0.001$ compared to methoxamine alone.

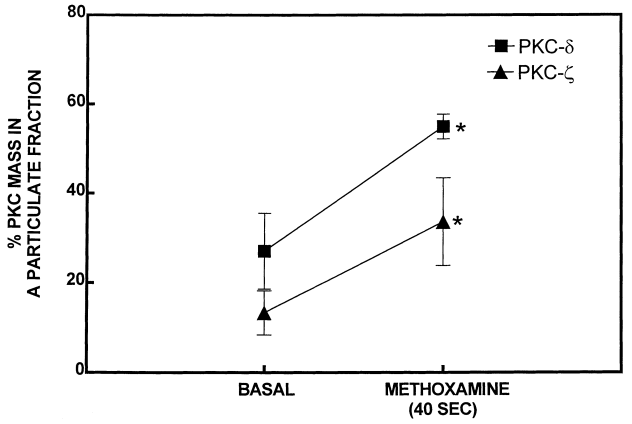

Fig. 2. α_1-Adrenergic agonist promotes translocation of PKC-δ and -ζ in CF/T43 cells. Cells were incubated with vehicle (basal) or stimulated for 40 s with 10 μM methoxamine, immediately washed with ice-cold homogenization buffer, and lysed. Cytosolic and particulate fractions were recovered by differential centrifugation. Aliquots of 20 μg protein were subjected to SDS-gel electrophoresis, as described in Section 2. Mass distribution in a particulate fraction is expressed as a percentage of total band intensity (cytosolic plus particulate). Data are reported as mean ± S.E. for experiments on three separate cell preparations. Levels of significance: $^*P < 0.01$ compared to basal unstimulated cells.

predicted, PKC activity remained elevated in CF cells after a long-term incubation for 6 min. At this time, PKC activity in non-CF tracheal epithelial cells was reduced by 90.0% in a cytosolic fraction ($P < 0.01$) and by 82.5% in a particulate fraction ($P < 0.01$).

The early response was studied in more detail by measuring kinase activity of immune complexes of PKC isotypes, using histone-III as the substrate for PKC-α, -βII, -δ and -ζ and ε-peptide for PKC-ε. In non-CF cells, methoxamine increased PKC-δ and PKC-ζ activities, but did not affect PKC-α or PKC-βII activities [16]. Fig. 1 illustrates results with CF nasal polyp epithelial cells and CF/T43 cells. In CF nasal polyp epithelial cells, methoxamine increased activity of PKC-ζ by 1.9 ± 0.3-fold ($n = 5$, $P < 0.05$) but did not significantly increase activity of PKC-δ (1.2 ± 0.1-fold, $n = 5$, n.s.). In addition, the data of Fig. 1 shows that, in CF/T43 cells, methoxamine significantly increased activity of PKC-δ by 2.9 ± 0.4-fold ($n = 8$, $P < 0.001$) and PKC-ζ activity by 2.4 ± 0.3-fold ($n = 8$, $P < 0.001$). The dual stimulation of two Ca^{2+}-independent PKC isotypes mimicked findings in non-CF tracheal epithelial cells [16]. Rottlerin, a selective inhibitor of PKC-δ [22], effectively blocked the increase in activity of PKC-δ in CF/T43 cells. Inhibition of activity of PKC-δ and -ζ by calphostin (Fig. 1) confirmed activation of PKC isotypes in the assay system. These data indicate that the CF/T43 cells, although a transformed

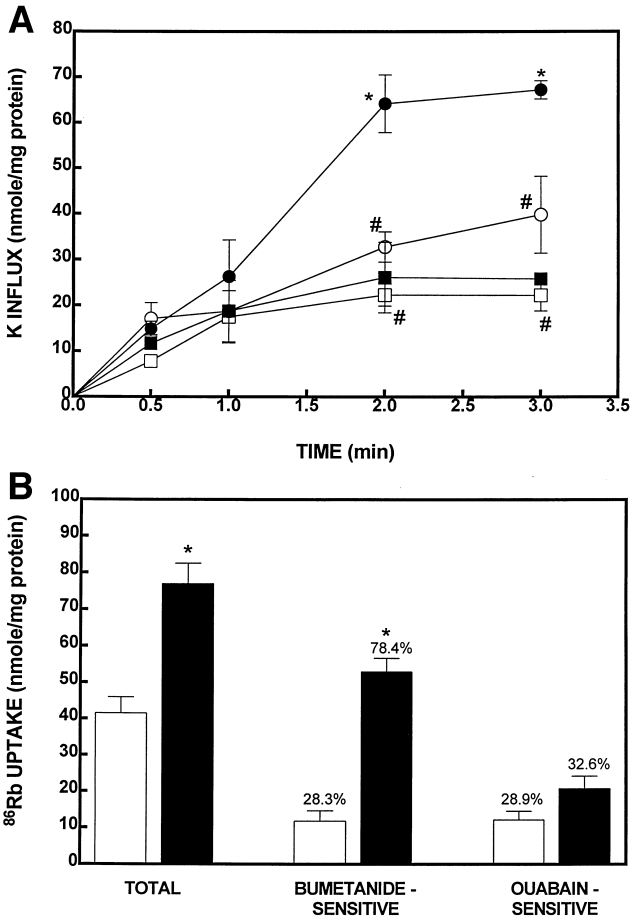

Fig. 3. Stimulation of NKCC1 by α_1-adrenergic agonist methoxamine in CF/T43 cells. (A) Time-dependent uptake of ^{86}Rb. Cells were unstimulated (solid square) or stimulated with 10 μM methoxamine in the absence (solid circle) or presence of 50 μM bumetanide (open squares) or 10 μM rottlerin (open circles). ^{86}Rb uptake was initiated by rapid addition of 1 μCi ^{86}Rb plus methoxamine. At indicated time intervals, uptake was stopped by washing the cell monolayer four times in ice-cold isotonic $MgSO_4$-sucrose buffer. Intracellular radioactivity was extracted, counted by Cerenkov counting and calculated as nmol K/mg protein. (B) Contribution of Na^+,K^+-ATPase to ^{86}Rb uptake. Cell monolayers were preincubated with 20 μM ouabain for 30 min. Uptake was terminated after 2 min incubation with vehicle (open bars) or with methoxamine (solid bars). Data represent mean of four to nine independent experiments. Bars represent 1 S.E. unit. $^*P < 0.001$ compared to vehicle; $^{\#}P < 0.005$ compared to methoxamine alone.

Table 1
Methoxamine induces a sustained increase in cytosolic and particulate PKC activity in CF cells

Incubation time	Relative PKC activity (experimental/vehicle)		
	Non-CF trachea	CF nasal polyp	CF/T43 cell line
Cytosolic fraction			
40 s	2.22 ± 0.34 (7)	1.56 ± 0.20 (6)	2.23 ± 0.21 (4)
6 min	1.11 ± 0.17 (9)[a]	1.55 ± 0.11 (6)	1.58 ± 0.14 (4)
Particulate fraction			
40 s	3.11 ± 0.37 (5)	2.28 ± 0.25 (5)	2.09 ± 0.22 (4)
6 min	1.37 ± 0.14 (9)[a]	2.33 ± 0.31 (7)	1.78 ± 0.21 (4)

PKC activity is expressed as a ratio of activity in methoxamine-stimulated cells divided by activity in control or untreated cells. The range of cytosolic PKC activity in unstimulated cells was 22.4 ± 11.6 (4) pmol/mg protein/min in CF nasal polyp epithelial cells to 45.6 ± 4.3 (4) in CF/T43 cells and of particulate PKC activity was 41.7 ± 11.6 (5) pmol/mg protein/min in CF nasal polyp cells to 42.4 ± 1.5 (4) in CF/T43 cells. These activities were not significantly different. Ratios are reported as mean ± S.E. for the number of determinations in parentheses. After incubation with 10 μM methoxamine or vehicle, the reaction was terminated and cytosolic and particulate fractions were obtained for assay of PKC activity, as described in Section 2.
[a] $P < 0.01$, compared to 40 s.

cell line, retain α_1-adrenergic regulation of PKC-δ activity.

In non-CF tracheal epithelial cells, short-term incubation with methoxamine promoted a small, but significant, shift in PKC-δ and -ζ to a particulate fraction, coincident with elevated diacylglycerol levels [4,8]. Redistribution of PKC isotypes was assessed in CF/T43 cells by densitometric analysis of immunoblots for PKC isotypes. Fig. 2 illustrates a summary of densitometric measurements from four experiments. Methoxamine induced a significant increase in the amount of PKC-δ and -ζ localized to a particulate fraction after a short-term incubation of 40 s. One paradigm for activation of PKC depicts increased activity with translocation of PKC mass from cytosol to a particulate fraction. However, we found that although activity of particulate PKC-ζ increased 1.5 ± 0.1-fold ($n = 5$, $P < 0.03$), activity of particulate PKC-δ did not increase (1.4 ± 0.5, $n = 6$). Rather, activity of cytosolic PKC-δ increased 2.6 ± 0.3-fold ($n = 5$, $P < 0.02$). Methoxamine produced a similar effect in non-CF tracheal epithelial cells [16]. The results suggest that elevated PKC activity reported in Table 1 in a cytosolic fraction cor-

Table 2
Effect of oligonucleotides on methoxamine-stimulated on activity of NKCC1 in CF/T43 cells

Oligonucleotide	Inhibitor treatment	
	Bumetanide (experimental/vehicle)	Rottlerin (% sensitive)
Untransfected	3.69 ± 0.7 (7)	49.4 ± 4.0 (8)
Antisense PKC-δ	1.06 ± 0.2 (3)[a]	13.6 ± 3.5 (5)
Sense PKC-δ	3.04 ± 0.8 (3)	35.6 ± 1.6 (3)
Missense PKC-δ	3.11 ± 0.5 (6)	44.8 ± 4.3 (6)
Antisense PKC-ζ	3.63 ± 0.6 (6)	44.9 ± 9.9 (5)

Values are mean ± S.E. for the number of determinations in parentheses. Cell monolayers were preincubated with 10 μM oligonucleotide for 48 h. Untransfected cells were incubated for the same time period in serum-free culture medium. Cells were preincubated with 50 μM bumetanide or with 10 μM rottlerin for 30 min just prior to measurement of ^{86}Rb uptake. ^{86}Rb uptake was measured at a fixed time interval of 2 min. For cells treated with bumetanide, relative activity represents bumetanide-sensitive uptake in methoxamine-treated cells divided by bumetanide-sensitive uptake in unstimulated cells. Bumetanide-sensitive ^{86}Rb uptake in untransfected control cells was 11.7 ± 2.9 ($n = 5$) nmol/mg protein and in methoxamine-treated cells was 39.7 ± 10.7 ($n = 8$, $P < 0.001$) nmol/mg protein. Rottlerin-sensitive uptake is calculated as the difference between methoxamine-stimulated ^{86}Rb uptake in the absence and the presence of rottlerin divided by methoxamine-stimulated-uptake in the presence of rottlerin times 100.
[a] $P < 0.05$, compared to untreated cells.

responded to PKC-δ activity and activity reported in a particulate fraction corresponded to PKC-ζ activity.

NKCC1 and its regulation by PKC were next investigated as bumetanide-sensitive K influx, using ^{86}Rb as a congener for K. ^{86}Rb uptake was rapid, attaining quasiequilibrium by 3 min (Fig. 3A). After a 30-min preincubation period, rottlerin caused a small insignificant decrease in radioisotopic uptake of $11.0 \pm 0.01\%$ ($n = 3$, $P = 0.09$). Methoxamine increased bumetanide-sensitive K uptake by 3.7-fold (Fig. 3B), indicating stimulation of NKCC1. Rottlerin significantly reduced methoxamine-stimulated ^{86}Rb uptake by $49.4 \pm 4.0\%$ ($n = 8$, $P < 0.005$) (Fig. 3A). Total ^{86}Rb uptake might include a contribution from Na^+,K^+-ATPase that was next assessed using ouabain. As shown in Fig. 3B, bumetanide-sensitive uptake in unstimulated cells accounted for 28.3% of total ^{86}Rb uptake and ouabain-sensitive uptake accounted for 28.9% of total ^{86}Rb uptake. Take together, 57.2% of ^{86}Rb uptake is accounted for as uptake by two major basolateral electrolyte transporters that mediate K uptake. Methoxamine increased bumetanide-sensitive uptake by 2.8-fold to 78.4% of total ^{86}Rb uptake. In contrast, the relative contribution of ouabain-sensitive uptake to total ^{86}Rb uptake did not significantly increase. Therefore, methoxamine selectively activated NKCC1 in CF/T43 cells.

To determine whether PKC-δ or -ζ or both were required for α_1-adrenergic stimulation of NKCCI, each PKC isotype was selectively downregulated using an antisense approach. CF/T43 cells were transfected with 10 μM antisense oligonucleotide or, as a control, sense or missense oligonucleotide to PKC-δ or antisense oligonucleotide to PKC-ζ for 48 h. As seen in Table 2, antisense oligonucleotide to PKC-δ decreased bumetanide-sensitive ^{86}Rb uptake by 71.3% and reduced rottlerin-sensitive uptake by 72.5%. Cells treated with sense or missense oligonucleotide to PKC-δ or with antisense oligonucleotide to PKC-ζ retained a bumetanide-sensitive stimulatory response to methoxamine. These results indicate that antisense oligonucleotide to PKC-δ blocked activity of NKCC1.

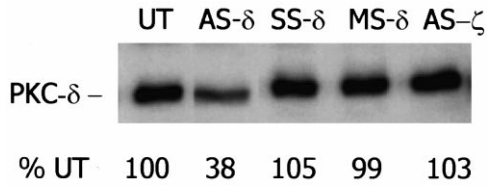

Fig. 4. Immunoblot analysis of PKC-δ. CF/T43 cell cultures were preincubated for 48 h in serum-free medium (UT) or with 10 μM sense (SS), missense (MS) or antisense (AS) oligonucleotide to PKC-δ or antisense oligonucleotide to PKC-ζ. Cell lysates were prepared and analyzed by immunoblot analysis, as described in Section 2. The figure represents results from one out of three separate experiments.

To determine whether treatment with antisense oligonucleotide to PKC-δ produced its effect by reducing PKC activity or PKC mass or both, kinase activity was measured in immune complexes of PKC-δ from cells treated with antisense oligonucleotides. As a control, kinase activity of PKC-ζ was measured in the same cells. The data of Table 3 shows that anti-

Table 3
Effect of oligonucleotides on methoxamine-stimulated activity of PKC isotypes

Isotype	Relative activity (experimental/vehicle)			
	Oligonucleotide pretreatment			
	None	Sense PKC-δ	Antisense PKC-δ	Antisense PKC-ζ
PKC-δ	2.71 ± 0.4 (4)[a]	2.16 ± 0.26 (5)[b]	1.18 ± 0.16 (5)	2.70 ± 0.61 (6)[a]
PKC-ζ	2.66 ± 0.4 (4)[b]	2.64 ± 0.02 (3)[b]	2.25 ± 0.22 (6)[c]	1.07 ± 0.25 (4)

Values are mean ± S.E. for the number of determinations in parentheses. CF/T43 cells were preincubated with 10 μM oligonucleotide for 48 h before stimulation with 10 μM methoxamine. Relative activity is calculated as (total activity in methoxamine-treated cells)/(activity in vehicle-treated cells). As a control for oligonucleotide treatment, cell monolayers were incubated for the same time period in serum-free culture medium.

[a] $P < 0.04$

[b] $P < 0.01$

[c] $P < 0.002$, compared to cells treated with HPSS.

sense oligonucleotide to PKC-δ decreased a methoxamine-stimulated activity of PKC-δ by 64.8%, but did not affect a robust increase in activity of PKC-ζ. Sense oligonucleotide to PKC-δ and antisense oligonucleotide to PKC-ζ did not block stimulation of PKC-δ. However, antisense oligonucleotide to PKC-ζ did block an increase in methoxamine-stimulated activity of PKC-ζ. Because loss of PKC-δ activity might result from loss of PKC-δ mass, we next assessed expression of PKC-δ by immunoblot analysis. As seen in Fig. 4, treatment with antisense oligonucleotide to PKC-δ reduced PKC-δ mass to 38.6 ± 3.0 ($n = 4$)% of levels in untreated cells, but did not affect amounts of PKC-α (83.0 ± 6.4%, $n = 6$), PKC-βII (93.7 ± 6.2%, $n = 5$), PKC-ε (95.8 ± 13.7% $n = 3$), and PKC-ζ (102.9 ± 5.0%, $n = 6$). Antisense oligonucleotide to PKC-ζ reduced PKC-ζ to 40.0 ± 5.0% ($n = 5$) of levels in untreated cells, but did not alter the abundance of PKC-δ (Fig. 4) or PKC-α, -βII, or -ε (data not shown). Additionally, sense and missense oligonucleotides did not significantly alter expression of PKC-δ.

4. Discussion

Antisense technology combined with a traditional biochemical approach provides compelling evidence, in this study, for a requirement for PKC-δ for rapid activation of NKCC1 in CF/T43 cells despite a lower ratio of ether/ester diglycerides compared to non-CF tracheal epithelial cells. Stimulation of CF cells with methoxamine, an α_1-adrenergic agonist, produced a rapid and sustained increase in activity of PKC; this was predicted from previous studies on lipid mediators generated during α_1-adrenergic stimulation [8]. In CF cells, the relative mass of ether-linked diglycerides species was reduced in the presence or absence of α_1-adrenergic stimulation. Thus, even though α_1-adrenergic stimulation increased the mass of ether- and ester-linked diglycerides, the ether/ester diglyceride ratio remained lower than non-CF cells. Because ether-linked diglycerides might modulate receptor-mediated activity of PKC [23,24], it was important to discern whether PKC signaling in CF cells was also altered.

These studies of the biochemical and physiological consequences of α_1-adrenergic stimulation indicate that CF/T43 cells retain at least the early signaling events of non-CF tracheal epithelial cells. Treatment of CF/T43 cells with methoxamine for a short time period of 40 s increased activity of PKC in cytosol and a particulate fraction (Table 1) and activity of PKC-δ and -ζ isotypes (Fig. 1), redistributed PKC-δ and -ζ to a particulate fraction (Fig. 2) and stimulated activity of NKCC1 (Fig. 3). Consistent with observations in non-CF tracheal epithelial cells [16], activity of cytosolic PKC-δ and particulate PKC-ζ also significantly increased.

An antisense approach was used to provide evidence for a role for a specific PKC isotype in the regulation of NKCC1 in CF/T43 cells. Preincubation with 10 μM antisense oligonucleotides to PKC-δ for 48 h decreased methoxamine-mediated activation of NKCC1 to 28.7% of untransfected cells (Table 2), decreased α_1-adrenergic-mediated increase in activity of PKC-δ to 35.8% of untransfected cells (Table 3) and reduced the amount of PKC-δ to 38.6% of untransfected cells. Downregulation of PKC-δ mass is in agreement with a half-life of ∼24 h for PKC in vitro [25] and corresponds to a reported 60% loss of PKC-δ in other cell lines treated with antisense oligonucleotide to PKC-δ [18]. At the concentrations used in this study, antisense oligonucleotide to PKC-δ was highly specific for PKC-δ. The expression and activities of PKC-α, -βII, -ε and -ζ were unaffected as was an increase in PKC-ζ activity by methoxamine. Moreover, responses to methoxamine were retained in cells treated with sense or missense oligonucleotide to PKC-δ (Tables 2 and 3). Antisense oligonucleotide to PKC-ζ blocked methoxamine-stimulated PKC-ζ activity (Table 3) and reduced PKC-ζ mass by 60%, but did not prevent activation NKCC1 by methoxamine (Table 2). Thus, CF/T43 cells retain a requirement for PKC-δ for activation of NKCC1 that was observed in non-CF tracheal epithelial cells [6].

Recent studies from this laboratory demonstrated intracellular signaling of α_1-adrenergic agents through a PtdIns(4,5)P_2-dependent PLC, subsequent biphasic generation of diacylglycerol and activation of basolateral NKCC1 in CF nasal polyp epithelial cells [4,7,8]. The studies reported here show that, in CF/T43 cells, elevated PKC activity after short-term stimulation with methoxamine coincides with an early generation of diacylglycerol and activation of

NKCC1. More importantly, PKC-δ differentially regulates NKCC1, a transporter that is crucial for a Cl secretory mechanism in airway epithelial cells, and offers a potential site for manipulation of Cl secretion in CF cells. Activity of PKC is, moreover, linked to cystic fibrosis transmembrane regulator, CFTR. The PKC activator, phorbol 12-myristate 13-acetate (PMA), a tumor-promoting phorbol ester, regulates Cl secretion in the large airways [26], in cell lines stably expressing the cystic fibrosis transmembrane regulator (CFTR) [27], in *Xenopus* oocytes expressing CFTR [28], and in epithelia expressing CFTR [9,12,26,29–32] by a mechanism that involves phosphorylation of channel protein [33,34]. Regulation of CFTR by PKC is less well understood. One report implies that PKC-mediated phosphorylation is essential for acute activation of recombinant CFTR channels by cAMP-dependent protein kinase [10]. In a recent study [13], we used oligonucleotides to PKC isotypes to provide evidence for PKC-ε regulation of cAMP-dependent activation of CFTR. These findings plus our new study reported here support a model depicting regulation of Cl secretion by PKC through targeting of specific PKC isotypes to electrolyte transporters that are required for fluid and Cl secretion. More importantly, the results indicate that CF airway epithelial cells retain PKC-δ regulation of basolateral NKCC1.

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