Chloride Secretory Response of Cystic Fibrosis Human Airway Epithelia

Preservation of Calcium but Not Protein Kinase C- and A-dependent Mechanisms

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Abstract

Because the defect in Cl^- secretion exhibited by cystic fibrosis (CF) epithelia reflects regulatory rather than conductive abnormalities of an apical membrane Cl^- channel, we investigated the role of different regulatory pathways in the activation of Cl^- secretion in freshly excised normal and CF nasal epithelia mounted in Ussing chambers. A β agonist (isoproterenol [ISO]), a Ca^{2+} ionophore (A23187), and a phorbol ester (PMA) were all effective Cl^- secretagogues in normal human nasal epithelia. Agonist addition studies indicated that ISO and PMA but not A23187 may share a common regulatory pathway. In contrast, only A23187 induced Cl^- secretion in CF epithelia. Bradykinin raised cytosolic Ca^{2+} and induced Cl^- secretion in both normal and CF tissues, indicating that receptor gated Ca^{2+} dependent Cl^- secretory mechanisms were preserved in CF. The defective Cl^- secretory response in CF epithelia to ISO and PMA did not reflect abnormalities in cAMP-dependent (A) and phospholipid Ca^{2+}-dependent (C) kinase activities. We conclude that (*a*) a Ca^{2+}-sensitive mechanism for regulating Cl^- secretion is maintained in CF airway epithelia, and (*b*) a regulatory pathway shared by two distinct protein kinases is defective in CF, indicating that the CF genetic lesion is not tightly coupled to a single (e.g., cAMP dependent) regulatory mechanism.

Introduction

The defective Cl^- transport exhibited by cystic fibrosis (CF)[1] airway epithelia reflects a defect(s) in regulatory processes rather than the conduction properties of apical membrane Cl^- channels (1, 2). Most studies agree that normal but not CF cells respond to β agonists or cAMP-dependent kinase with activation of Cl^- secretion or channels (1–6). However, because of the difficulty in obtaining human tissues, a characterization of other important regulatory pathways that control human airway Cl^- secretion has not been reported. Widdicombe raised the possibility that Ca^{2+} ionophores function as Cl^- secretagogues in cultured CF airway epithelia (7). However, patch clamp studies yielded conflicting results with respect to whether cultured airway epithelia exhibit a Ca^{2+}-dependent mechanism for activation of Cl^- channels (1, 2).

Therefore, the present study was undertaken with three specific aims. First, employing pharmacologic agonists of different classes, we sought to characterize the important pathways, and their interactions, that control Cl^- secretion in freshly excised normal and CF airway epithelia. Secondly, we tested whether Cl^- secretion can be induced in CF epithelia by both receptor-gated and ionophore-induced increases in cytosolic Ca^{2+}. Thirdly, because of the absent Cl^- secretory response to both isoproterenol and PMA in CF, we compared the form and activities of the intracellular enzymes that mediate the actions of these agonists (cAMP-dependent protein kinase [8] and protein kinase C [9]) in CF and normal cells.

Methods

Subjects

Nasal tissues were excised from 41 normal subjects (32±4 yr old); 22 males and 19 females were included. Tissues from all subjects were obtained from the operating room, typically after general anesthesia. The nasal polyps were excised from 16 cystic fibrosis patients (aged 14±5 yr; seven females, nine males). All CF patients had typical diagnostic clinical criteria for the disease and elevated sweat electrolytes.

Chemicals and solutions

Chemicals were obtained from the following sources. Hepes, bradykinin (BK) isoproterenol, PGE_1, isobutyl methyl xanthine (IBMX), and ampholines were obtained from Sigma Chemical Co., St. Louis, MO. LTC_4 and 15S-hydroperoxyeicosa-5Z, 8Z, 11Z, 13E-tetraneonic acid (15-HPETE) were obtained from Biomol, Plymouth Meeting, PA. PMA and A23187 were obtained from Calbiochem-Behring Corp., San Diego, CA or Sigma Chemical Co. The acetoxymethylester of Fura-2 (Fura-2/AM) was purchased from Molecular Probes, Eugene, OR. Bumetanide was a gift of Leo Pharmaceuticals; amiloride was a gift of Merck, Sharpe & Dohme, West Point, PA.

For bioelectric studies, a standard mammalian Krebs-Ringer bicarbonate buffer (KRB) was employed (3). For studies of intracellular Ca^{2+} levels, the NaCl Ringer contained the following (in millimolar): 150 NaCl, 2.5 K_2HPO_4, 1.0 $CaCl_2$, 1.0 $MgCl_2$, 5.0 glucose, and 10 Hepes, adjusted to pH 7.40 (37°C) with *N*-methyl-D-glucamine base.

In vitro bioelectric studies

Freshly excised specimens were mounted in Ussing chambers (0.25 cm^2 [3, 10]), short-circuited, and exposed to amiloride (10^{-4} M, luminal bath). The effects of log increasing additions of agonists (added bilaterally every 3 min) on short circuit current (I_{sc}) were tested. The following agonists were tested in the ranges listed: A23187, 10^{-9} to 3×10^{-6} M; PGE_1, 10^{-11} to 10^{-6} M; isoproterenol 10^{-9} to 10^{-5} M; phorbol myristate acetate (PMA), 10^{-11} to 10^{-6} M; leukotriene C_4 (LTC_4), 10^{-11} to 10^{-7} M; and 15-HPETE, 10^{-8} to 3×10^{-5} M. Because tachyphylaxis was observed in preliminary studies with BK, as previously observed in canine trachea (11), a single maximal concentration of BK (10^{-5} M) was added to the mucosal surface. In some cases after amiloride addition, a blocker of Cl^- secretion (bumetanide,

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1. *Abbreviations used in this paper:* BK, bradykinin; CF, cystic fibrosis; D, diacylglycerol; HPETE, 15S-hydroperoxyeicosa-5Z, 8Z, 11Z, 13E-tetraneonic acid; IBMX, isobutyl methyl xanthine; I_{sc}, short circuit current; PKC, protein kinase C; PS, phosphatidylserine.

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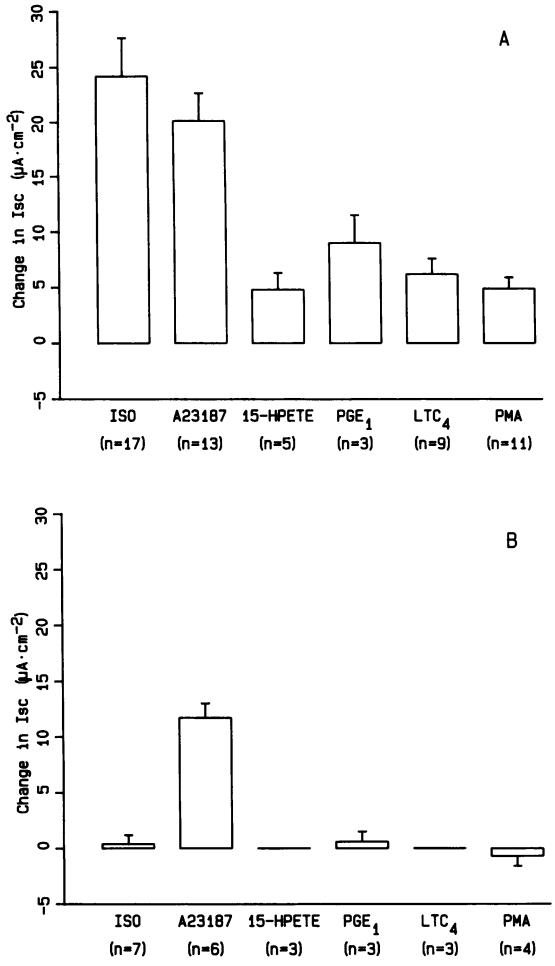

Figure 1. Changes in I_{sc} in amiloride-pretreated (*A*) normal and (*B*) CF nasal epithelia induced by addition of maximal effective concentrations of Cl^- secretagogues of different pharmacologic classes. The mean I_{sc} of normal and CF tissues was 63±4 and 143±13 $\mu A \cdot cm^{-2}$, respectively before amiloride exposure and 26±3 and 5±3 $\mu A \cdot cm^{-2}$ after amiloride, respectively. The half-maximal effective concentrations (EC_{50}) of agonists in normal tissues were as follows (M): isoproterenol (ISO), 5×10^{-8}; A23187, 5×10^{-7}; PMA, 10^{-9}; LTC_4, 10^{-9}; PGE_1, 10^{-7}; 15-HPETE, 10^{-6}. The maximal effective concentrations in normals were as follows (M): ISO, 10^{-6}; A23187, 10^{-6}; 15-HPETE, 10^{-5}; PGE_1, 10^{-7}; LTC_4, 10^{-8}; PMA, 10^{-7}. The EC_{50} for A23187 in CF tissues was 6×10^{-7} M; the maximal effective concentration was 10^{-6} M. *n* denotes number of different patients. Mean±SEM displayed.

10^{-4} M [12]) was added to the submucosal surface 30 min before subsequent addition of A23187, BK, or PMA. Finally, cumulative agonist addition experiments, employing maximal concentration(s) of agonists, were performed in CF and normal tissues.

Cell culture

Cells from freshly excised nasal specimens were grown in an F-12 hormone-supplemented medium as previously described (13) and used 5–7 d after plating. The substrata employed in the cultures included, as required, 35-mm plastic dishes, collagen matrices, and vitrogen-coated coverslips.

Loading cells with Fura 2 and cell chamber

Fura-2/AM was added to nasal cells grown on coverslips at a final concentration of 2 μM for 30 min at 37°C. After loading, cells were washed with NaCl Ringer and mounted in a chamber over the objective (UV-F ×100 glyc., NA 1.30, Nikon, Inc., Garden City, NY) of the microscope (see below). Both chamber and objective were maintained at 37°C by a heat lamp (model 335, Opti-Quip, Inc., Highland Mills, NY). The NaCl Ringer was prewarmed to 37°C and exchanged frequently (2–3 min; exchange time < 3 s) to remove any Fura-2 that leaked from the cell.

Microspectrofluorimeter and measurements of $[Ca]_i$

Measurements of $[Ca]_i$ in single nasal cells were obtained at 37°C using a fluorimeter system (Flurolog 2, model F2C, Spex Industries, Inc., Edison, NJ) attached to an inverted microscope (Nikon, Inc.). The fluorescence from nasal cells was acquired alternately at 340 and 380 nm (emission ≥ 450 nm). A photometer was used to pass the fluorescence arising from each of the two excitation wavelengths from the central region (spot diameter 5–10 μm) of a single nasal cell.

At the end of an experiment, background signals originating from

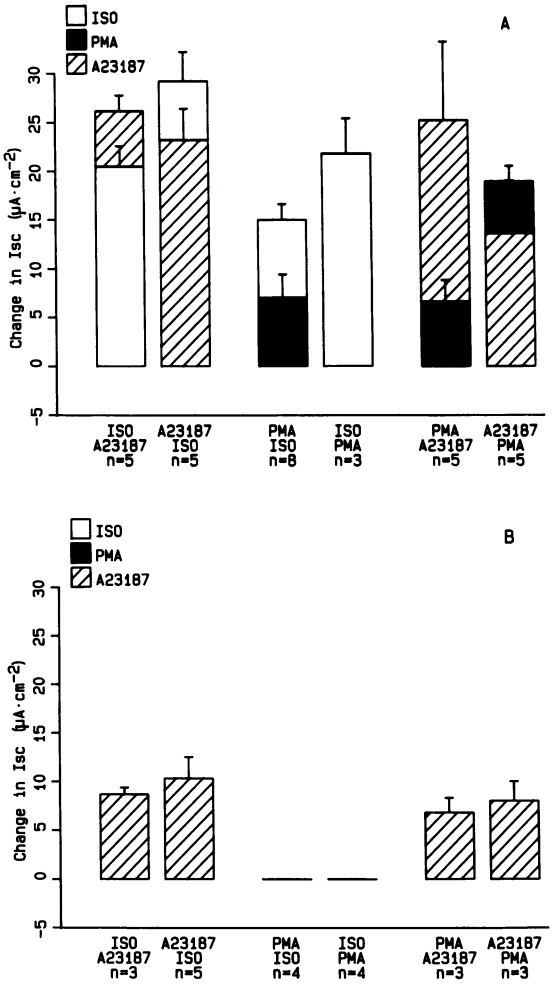

Figure 2. Changes in I_{sc} of amiloride-pretreated (*A*) normal or (*B*) CF tissues in response to cumulative agonist additions. The first agonist added is listed as the first agonist under the abscissa and the response is depicted as the change in current (±SEM) from 0. After the maximal increase in I_{sc} to the first agonist was obtained, the second agonist was added in a cumulative fashion. The second agonist added is listed underneath the first on the figure, and the change in current in response to the second agonist is depicted as the mean increment (±SEM of the increment) to the maximal response of the first agonist. The component contributed by each agonist to the total change in I_{sc} is depicted by the shading contained in the bars.

cells and incomplete deesterfied Fura-2 were measured by exposing cells to NaCl Ringer solution containing 10^{-5} M ionomycin and 10^{-3} M $MnCl_2$ (14). Background values obtained in this way were in good agreement with values measured in unloaded cells. The background signals were subtracted from the corresponding signals measured in Fura 2-loaded cells before taking the ratio (340/380). The corrected ratio was converted to $[Ca]_i$ using an external calibration standard and the formula derived by Grynkiewiez et al. (15) for dual wavelength measurements: $[Ca]_i = K(R_x - R_0)/(R_s - R_x)$, where R_0 and R_s are the ratios at 0 Ca and saturating Ca, respectively. R_x is the experimental ratio. K represents K_d (F_0/F_s), where K_d (2.25×10^{-7} M at 37°C) is the effective dissociation constant for Fura-2, and F_0 and F_s are the fluorescence intensities at 380 nm (or 385 nm) minus and plus Ca, respectively.

cAMP RIA

Cells were cultured on collagen matrices and grown to confluency. Preliminary time course studies indicated that cAMP levels peaked after a 10-min incubation with the agonists tested. A basal sample (time 0) was obtained after which a 10-min incubation in F12 medium containing the following agent(s) was carried out: isoproterenol (10^{-5} M), forskolin (10^{-5} M), PMA (10^{-7} M), A23187 (10^{-6} M), IBMX (10^{-5} M), PMA (10^{-7} M) + IBMX (10^{-5} M), isoproterenol (10^{-5} M) + A23187 (10^{-6} M), or vehicle (ethanol). The extraction and measurement of cAMP was performed as described by Steiner et al. (16) as previously described for this tissue (3).

Characterization of cAMP-dependent kinase

Isolation of cytosol. Cells were scraped from the plastic tissue culture dishes, homogenized in a glass pestle in a homogenization solution (50 mM Hepes, pH 7.2; 0.25 M sucrose; 0.15 M NaCl; 10 mM $MgCl_2$; 1 mM EDTA; 1 mM DTT; 20 mM benzamidine), and, after a low-speed spin (300 *g*) to remove nuclei and large debris, cell membranes were pelleted in an ultracentrifuge (100,000 *g*) and the supernatant (cytosol) was harvested.

cAMP-dependent kinase assay. cAMP-dependent protein kinase activities contained in the cytosolic fractions of normal and cystic fibrosis cells were assayed by the technique of Gill and Walton (17). In brief, aliquots of cytosol normalized for protein (40 μg per assay) were incubated (assay volume of 50 μl) with magnesium (10 mM), buffer (30 mM KH_2PO_4, 2 mM DTT), histone (Sigma Chemical Co. type VII, 1 mg/ml), and 10^{-5} M cAMP or vehicle (water). The reaction was initiated with the addition of 10 μM [^{32}P]ATP (3 μCi per tube) and continued for 10 min at 30°C after which 40 μl of the reaction mixture was pipetted onto filter paper (No. 3 M, Whatman Inc., Clifton, NJ) and washed in 10% TCA (17).

Characterization of isoforms of cAMP-dependent kinase. Cytosolic R_I and R_{II} isozymes were separated and identified by their salt elution profile (18) on a fast protein liquid chromotography/DEAE column (LKB Products, Inc., Gaithersburg, MD) eluted using a 20 mM Tris-HCl, 1 mM EDTA buffer (pH 7.4) with a linear 0–500 mM NaCl gradient. Column fractions were assayed for kinase activity±10 μM cAMP as described above.

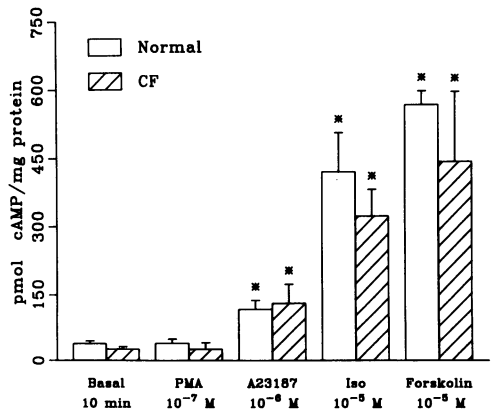

Figure 3. Intracellular cAMP concentrations of cultured normal and CF nasal epithelial cells after 10-min exposure to putative Cl^- secretory agonists in concentrations listed below the agonist. Numbers of 10-min basal, PMA, A23187, ISO, forskolin for normal were 20, 12, 18, 10, and 5; for CF, 11, 4, 8, 8, and 4, respectively. The 10-min basals were not significantly different from 0-min basals for NL (0-min basal = 31.75±5.77, n = 20; 10-min basal = 38.21±6.19, n = 20) or for CF (0-min basal = 26.63±5.72, n = 11; 10-min basal = 25.66±5.46, n = 11). Asterisks indicate that the amounts of cAMP generated were significantly different from the 10-min basals.

Characterization of protein kinase C (PKC)

Measurement of whole-cell PKC activity. PKC activity of extracted whole-cell homogenates of cystic fibrosis and normal cultured cells were compared by the technique of Halsey et al. (19). Cells were homogenized in the buffer described above, the membrane-associated PKC was extracted by incubation for 30 min at 0°C with 0.3% Triton X-100, and a 100,000-*g* supernatant was prepared. Aliquots of the supernatant (40 μg of protein) were incubated with Mg^{2+} (10 mM), Tris (20 mM, pH 7.2), 20 μM [γ-^{32}p] ATP (5 μCi per assay), and a fragment of a lysine-rich histone (Sigma Chemical Co. type III-S) purified by HPLC after cleavage with *N*-bromosuccinimide (19). ^{32}P incorporation for 10 min (30°C) without (basal) or with addition of Ca^{2+} (500 μM) or Ca^{2+} plus 500 μM phosphatidylserine (PS) and 80 μg of diacylglycerol (D) was terminated when 40 μl of the mixture was pipetted on filter paper (P-81, Whatman, Inc.) and washed in 10% TCA (19).

Activation of PKC in intact cells. Cultured normal and CF epithelial cells were incubated in phosphate-free minimal essential medium with 0.5% fetal bovine serum containing 250 μCi of $^{32}P_i$ for 4 h before each experiment. PMA (10^{-7} M) was added to the cultures for 10 min, cultures were rinsed with phosphobuffered saline (pH 7.4) and the incubation was stopped by adding electrophoresis buffer containing 9.5 M urea, 2% NP40, 2% ampholines (pH 3–10), and 5% β-mercaptoethanol. 25-μl aliquots of sample were subjected to nonequilibrium pH gradient electrophoresis followed by 10% PAGE in the second dimension (20). Gels were dried and subjected to autoradiography on AR film (Eastman Kodak Co., Rochester, NY).

Distribution of PKC activity. Cytosol and membrane fractions were prepared as above. The particulate fractions were solubilized with 0.3% Triton X-100 and recentrifuged at 100,000 *g* for 30 min. The activities of cytosol and solubilized particulates were measured using the standard PKC assay. In addition, to compare endogenous substrates in CF and normal cells, cytosol and solubilized membrane fractions were prepared as described above and incubated with [γ-^{32}P]ATP and with or without the addition of PS, D, and Ca^{2+}. Aliquots were electrophoresed on one-dimensional 10% SDS-PAGE gels, stained with Coomassie blue, destained, dried, and subjected to autoradiography.

Results

The nasal epithelial Cl^- secretory response to potential Cl^- secretagogues (1, 2, 21–25) was measured as the change in I_{sc} in tissues pretreated with amiloride to abolish the component of active Na^+ absorption to the I_{sc} (3, 7). In normal tissues (Fig. 1 *A*), isoproterenol and A23187 were equieffective. PMA, an activator of PKC (9), routinely induced Cl^- secretion but was less effective, as reported in canine trachea (24, 25). The arachidonic acid metabolites, LTC_4, PGE_1, and 15-HPETE, were less effective than A23187. Previous studies have shown that the increased I_{sc} of amiloride pretreated normal tissues in response to such agonists, e.g., isoproterenol or PGE_1, is paralleled by an increase in the isotopically measured secretory flux

Table I. Change in Cell Ca^{2+} Levels and I_{sc} Induced by BK (10^{-5} M) in Amiloride (10^{-4} M)-pretreated Normal and CF Tissues

	Normal (n = 4)			CF (n = 4)		
	Before	During	Δ	Before	During	Δ
Cell Ca^{2+}, nM	115±10	506±32	391±26*	118±12	515±51	397±43*
ΔI_{sc}, $\mu A \cdot cm^{-2}$	13.2±2.5	25.2±2.6	12.0±.5*	2.4±2.0	19.2±6.8	16.8±6.3*

Data expressed as mean±SE. * Different from zero ($P < .05$).

of Cl^-, and that this Cl^- secretory current is partially inhibited by bumetanide (3). In the present study, bumetanide pretreatment (10^{-4} M) inhibited 65% of the response to A23187 in normal tissues (ΔI_{sc} of bumetanide pretreated tissues in response to A23187 = 7.2±3 $\mu A \cdot cm^{-2}$, n = 4), indicating this change in I_{sc} also reflects Cl^- secretion. Bumetanide pretreatment inhibited a similar fraction of the PMA induced I_{sc} (70±8%; n = 3).

In contrast to normal tissues, only A23187 raised I_{sc} in amiloride pretreated CF tissues (Fig. 1 *B*). The A23187-induced response in CF differed from normals in that the maximal increase was smaller (~ 60% of the ΔI_{sc} in normals) and the effect was shorter in duration. Cl^- secretion could be induced in CF tissues bathed on the mucosal surface with a Ringer solution with 500 nM Ca^{2+} and exposed to mucosal A23187. This observation indicates that unphysiological levels of cytosolic Ca^{2+} may not be required to induce Cl^- secretion (also see Table I). The increase in I_{sc} induced by A23187 in CF tissues was partially inhibited by bumetanide pretreatment (62% inhibition; ΔI_{sc} = 4.6±0.1 $\mu A \cdot cm^{-2}$, n = 4), indicating that at least a major fraction of the stimulated I_{sc} reflects the induction of chloride secretion.

Studies of drug interactions with the Cl^- secretory path were performed in normal nasal epithelia with cumulative additions of maximal effective agonist concentrations (Fig. 2 *A*). Isoproterenol, added during the maximal sustained (5 min) effect of A23187, routinely further increased the Cl^- current; A23187 also routinely added to the maximal sustained effect induced by isoproterenol. Similarly, A23187 added to the Cl^- current induced by maximal effective concentrations of PMA, and PMA added to the maximal response induced by A23187. In contrast, isoproterenol added to the maximal effect induced by PMA but PMA did not add to the maximal I_{sc} induced by isoproterenol. Consequently, whereas fully additive effects were not observed between A23187 and isoproterenol or PMA, the highly reproducible increment in I_{sc} after A23187 addition to isoproterenol or PMA prestimulated tissues indicates that components of the Ca^{2+}-dependent pathway(s) are separate from the isoproterenol- and/or PMA-dependent path.

In CF, no combination of PMA or isoproterenol with A23187 induced a larger response than A23187 alone (Fig. 2 *B*). The combination of PMA and isoproterenol was ineffective.

The interactions between these agonists were explored at the level of cAMP accumulation. As shown in Fig. 3, isoproterenol and forskolin were potent stimulators of cAMP accu-

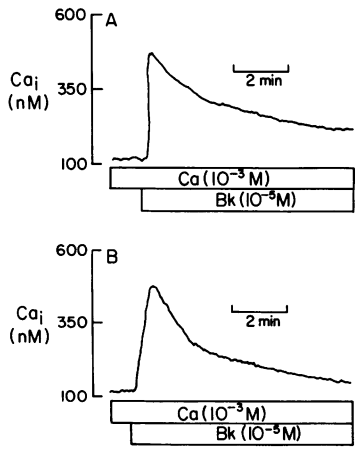

Figure 4. Effect of bradykinin (*Bk*, 10^{-5} M) on $[Ca]_i$ in (*A*) normal or (*B*) CF human nasal cells grown on a vitrogen-coated coverslip. Each trace represents one of four similar experiments.

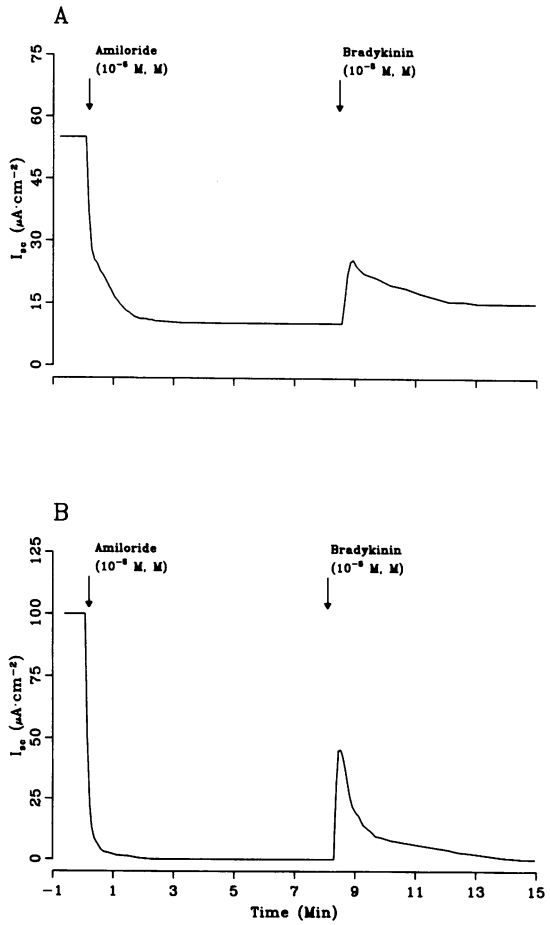

Figure 5. I_{sc} tracings for (*A*) normal and (*B*) CF nasal epithelia. Amiloride (10^{-5} M, mucosal surface [*M*]) and bradykinin (10^{-5} M, mucosal surface [*M*]) were added cumulatively at times noted.

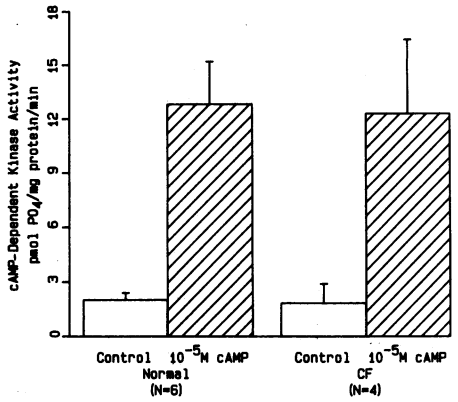

Figure 6. cAMP-dependent protein kinase activity of cytosol from normal and cystic fibrosis cultured nasal epithelial cells. Enzyme activity is expressed as picomole of PO_4 transferred to substrate per milligram of protein per minute. *N* reflects number of patient samples. The mean±SEM is shown.

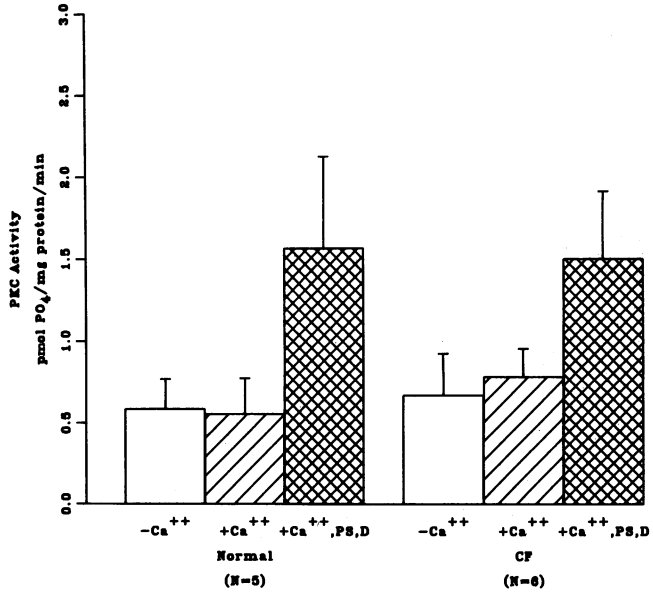

Figure 8. PKC activity of homogenates extracted with 0.3% Triton X-100 from normal and cystic fibrosis cultured nasal epithelial cells. Enzyme activity in the presence or absence of PS, D, and Ca^{2+} is expressed as picomole of PO_4 transferred to substrate per milligram of protein per minute. *N* reflects number of patient samples. Mean±SEM is shown.

mulation in normal cells. A23187 induced smaller but significant changes in cell cAMP concentrations. PMA did not detectably alter cell cAMP levels, as previously observed in canine trachea (24, 25). No systematic difference in responses to these agonists was observed between normal and CF cells.

Additional studies revealed that addition of IBMX, a phosphodiesterase inhibitor, increased basal cAMP concentrations but did not reveal a PMA-induced increment in cAMP for normal (IBMX, 137.46±22.32 pmol/mg protein; IBMX plus PMA, 101.93±21.02 pmol/mg protein) or CF (IBMX, 53.01±19.75 pmol/mg protein; IBMX plus PMA, 40.4±23.97 pmol/mg protein) cells. Further, no additivity in cell cAMP concentrations was detected comparing incubation with A23187 and isoproterenol to isoproterenol alone in CF (isoproterenol, 421.22±51.87 pmol/mg protein; isoproterenol plus A23187, 426.61±92.01 pmol/mg protein) or normal (isoproterenol, 418.73±85.87 pmol/mg protein; isoproterenol plus A23187, 536.34±49.11 pmol/mg protein) cells.

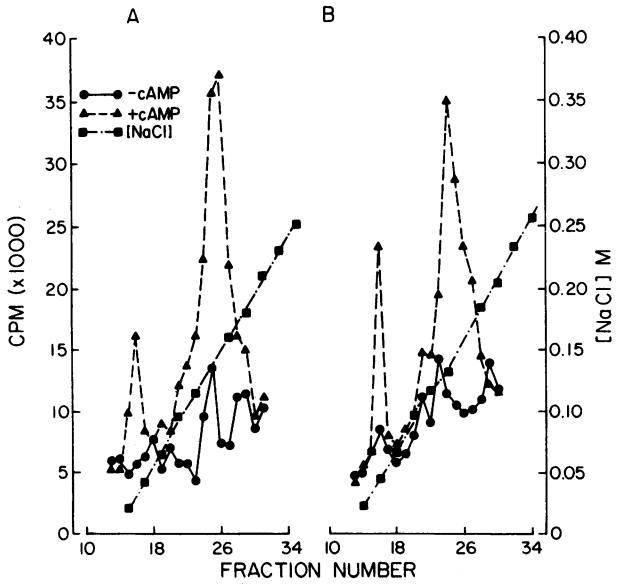

Figure 7. DEAE chromatographed isoforms of cytosolic cAMP-dependent kinase in (*A*) normal and (*B*) CF cells. The activity in the presence (▲) and absence of 10 μM cAMP (●) is shown.

These findings may have implications with regard to pathways that control the rate of Cl^- secretion in human airway epithelia. First, because PMA does not raise cAMP concentrations, the effect of PMA is clearly not mediated by cAMP. In addition, the additive effects of isoproterenol and PMA are not due to any additive effect on cAMP accumulation. Secondly, because little additivity in cellular cAMP concentrations was detected with A23187 and isoproterenol, the additivity observed for these two agonists with respect to rates of Cl^- secretion observed also is not likely to be a consequence of the level of cellular cAMP.

To test whether the activation of Cl^- secretion induced by the calcium ionophore A23187 is mimicked by receptor-gated increases in cytosolic Ca^{2+}, intracellular Ca^{2+} levels and I_{sc} of amiloride-pretreated tissues were measured before and after exposure to BK ([10^{-5} M], Table I). No differences in basal Ca^{2+} levels between CF and normals were noted, as previously reported (26). BK-induced rapid rises (Fig. 4) in intracellular Ca^{2+} levels that were not different in CF and normal (Table I). The response of the I_{sc} to BK administration is shown in Fig. 5 and summarized in Table I. In amiloride-pretreated tissues, BK induces a rapid increase in I_{sc} with a variable plateau phase. Pretreatment with bumetanide inhibited the maximal BK-induced change in I_{sc} by 65% to 74% in normal and CF tissues, respectively.

The defect in CF Cl^- secretion in response to isoproterenol and PMA was further explored by measuring the maximal stimulated activities of protein kinase activities of cells cultured from CF and normal subjects. Fig. 6 shows that the cAMP-dependent kinase activity of cytosolic fractions was not different in CF and normal cells. When DEAE chromatography was used to analyze the holoenzyme isotypes in CF and

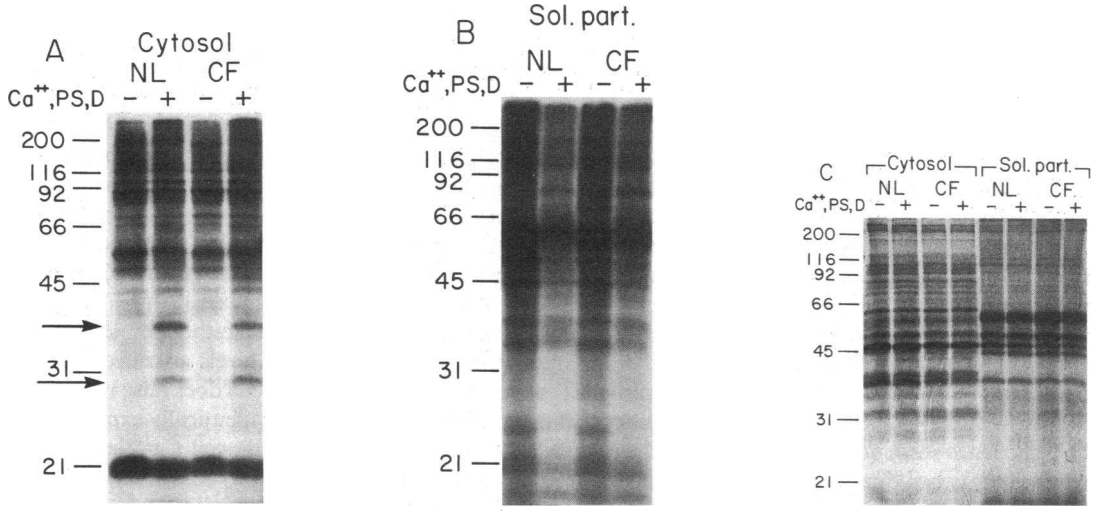

Figure 9. Phosphorylation of endogenous substrates from subcellular fractions of normal and CF-cultured respiratory epithelial cells. Autoradiography was performed after gel electrophoresis of fractions phosphorylated in the presence or absence of PS, D, and Ca^{2+}. (*A*) Autoradiograph of phosphorylated cytosolic proteins; (*B*) autoradiograph of phosphorylated solubilized particulate fraction; (*C*) the Coomassie blue staining pattern of the gel shown in *A* and *B*. The arrow points to prominent PKC-dependent substrates.

normal, the type II (R_{II} containing) isoform of the cAMP-dependent kinase was the dominant isoform in both CF and normal cells (Fig. 7).

We next tested whether total PKC activity differed in CF and normal cells. Total cell PKC activity, measured at maximally stimulating concentrations of PS and D (18) after quantitative extraction of homogenates with 0.3% Triton X-100, was not different in CF and normal cells (Fig. 8). Further, fractionation of PKC activity into cytosolic and detergent extractable particulate fractions showed that > 80% of the basal activity resided in the cytosolic compartment in both CF and normal cells.

Endogenous substrates in the cytosol and extracted membrane fractions were studied in the absence and presence of PS, D, and Ca^{2+}. The radioautograph of the phosphorylated cytosolic and solubilized membrane fractions from CF and normal cells is shown in Fig. 9. Two substrates, ~ 43 and ~ 30 kD, were phosphorylated in both CF and normal cells to the same extent upon the addition of activators of PKC. The solubilized particulate fractions were devoid of detectable PKC substrates.

To obtain a measure of PKC activity in intact normal and CF cells, cells were labeled for 4 h in phosphate-free media with $^{32}P_i$. Endogenous substrate phosphorylation was then assessed after 10 min in control and PMA-treated cells. Two substrates were detected in the 35–40- and 17–20-kD region of the gel. The pI of the substrates was ~ 6.2 and 9.0, respectively. Equivalent phosphorylation of each substrate was seen in both CF and normal (Fig. 10) cells.

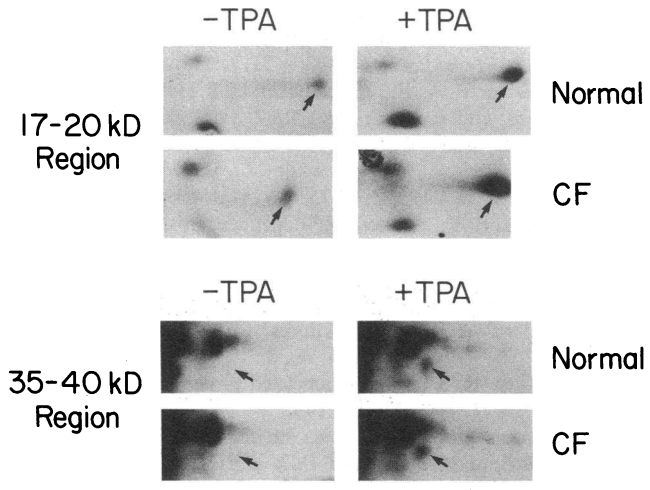

Figure 10. Nonequilibrium pH gradient and PAGE of $^{32}P_i$-labeled intact CF and normal cultured cells treated for 10 min with or without PMA (10^{-7} M). Arrows demonstrate two substrates that were phosphorylated in a PMA-dependent manner in both (*A*) CF and (*B*) normal cells. The ~ 34–40-kD substrate has a pI of ~ 6.2 and a 17–20-kD substrate has a pI of 9.0.

Discussion

In normal human airway epithelia, agonists that raise cytosolic Ca^{2+} or that activate cAMP-dependent protein kinase (isoproterenol) or PKC (PMA) induce Cl^- secretion. In contrast, only a Ca^{2+}-dependent Cl^- secretory mechanism appears functional in freshly excised CF nasal epithelia (7, 27). Double-barreled Cl^- microelectrode experiments have pinpointed the locus of action of Ca^{2+} ionophores in both normal and CF airway epithelia to the apical membrane Cl^- conductance (28). Unfortunately, neither the data cited above nor those presented in this paper elucidate the mode(s) of the A23187 activation of apical membrane Cl^- channels in human respiratory epithelia. However, the addition both of the first product (15-HPETE) produced via the major arachidonic acid metabolic pathway in human epithelial cells (15-lipoxygenase [29]) and other eicosanoids (PGE_1, LTC_4) failed to induce chloride secretion in CF tissues. This finding indicates that, unlike the canine trachea (23), the A23187-induced activation of Cl^- secretion in CF tissues probably is not mediated by effects on AA metabolism.

A key finding in this study is that Cl^- secretion (Fig. 5, Table I) can be induced in CF tissues by BK receptor gated

increases in cytosolic Ca^{2+} (Fig. 4). These findings indicate that (*a*) the coupling mechanisms, presumably inositol trisphosphate mediated, for receptor activation and increases in cytosolic Ca^{2+} activities are intact in CF cells and (*b*) that receptor-gated increases in Ca^{2+} levels are equieffective in initiating Cl^- secretion in CF and normal cells. This finding consequently provides an important physiologic control for the specificity of action of calcium ionophore responses. Like the actions of A23187, the mechanism of activation of Cl^- secretion by BK is not clear. However, the failure of exogenous additions of PMA or eicosanoids to initiate Cl^- secretion in CF tissues makes it likely that BK-induced increases in cell Ca^{2+} levels are working through a mechanism that does not involve activation of PKC or phospholipase A_2, respectively.

As previously shown in fresh tissue (4, 30) and cultured cells (2, 26), the failure of CF tissues to respond to Cl^- secretagogues of the beta adrenergic class is not due to a failure to produce cAMP. The data in Fig. 6 confirm the report of Berthelson and Widdicombe (31) that cytosolic A kinase in CF and normal cells exhibits the same maximal activity in the presence of exogenous cAMP. In addition, the data in Fig. 7 indicate that the R_{II} isoform of the A kinase dominates in both CF and normal airway epithelia. The observation that the R_{II} isoform is expressed in airway epithelia suggest that this form, which is often associated with the plasma membrane, is present in respiratory epithelial cells. Preliminary patch clamp data indicate that cAMP plus ATP can activate Cl^- channels in off cell patches of airway epithelium, consistent with the notion that some kinase is membrane associated (32).

A second key finding in this study is that the PKC system appears to have a role in the activation of Cl^- secretion in normal human tissues but is ineffective in inducing Cl^- secretion in CF tissues. Whereas PKC isoforms were not compared in CF and normal airway epithelia, several types of experiments indicate that total PKC activity is similar in CF and normals. Assays of total cell PKC activity do not detect a difference in maximal activity between CF and normal tissues (Fig. 8). Our experiments also indicate that the distribution of basal PKC activity in cytosol (> 80% of activity) and membrane fractions are equivalent, as is the ability to phosphorylate endogenous substrates in cell-free assays (Fig. 9). Lastly, the capacity of PMA to activate PKC in intact cells also is similar in CF and normal cells (Fig. 10). Thus, it appears that whereas normal tissues develop Cl^- secretion in response to the activation of two independent kinases (A kinase, C kinase), CF tissues, despite apparently normal kinase activities, do not.

The finding that activation of C kinase is ineffective in initiating Cl^- secretion in CF epithelia indicates that the activation defect in CF is not limited to the A kinase path. This finding increases the complexity of the CF regulatory defect, but suggests that proteins whose state of phosphorylation is governed by both A and C kinase can be considered important targets when searching for differences in CF and normal phosphoprotein profiles.

Our data suggest a complex mechanism of regulation for Cl^- secretion in human airway epithelia with at least two distinct pathways. First, agents that activate A and C protein kinases appear to work via a common path that converges upon a regulatory protein(s) whose activity is altered by phosphorylation. The lack of efficacy of both isoproterenol and PMA in CF, and the failure of the two agents to act additively in normals, lend support to this concept. Secondly, evidence for a distinct calcium-dependent path is derived from the additivity of A23187 and isoproterenol or PMA in normals, and the retention of A23187 efficacy in CF tissues.

The retention of the Ca^{2+} but not cAMP-dependent path for secretion in airways appears to mimic the pattern observed in the CF sweat gland wherein the cholinergic (Ca^{2+}-mediated) but not β-adrenergic (cAMP-mediated) path is effective in initiating sweat formation, presumably through a Cl^- secretory mechanism (30). The recent report that Ca^{2+} ionophores and carbachol are ineffective in the CF small intestine (33) indicates retention of Ca^{2+}-dependent mechanisms for Cl^- secretion may not be a uniform feature of CF epithelium. However, the finding that increases in cell Ca^{2+} levels are effective initiating Cl^- secretion in CF airway epithelia does raise the possibility that this mechanism may be therapeutically exploited in the future. Specifically, agents that raise cell Ca^{2+} levels, via direct or receptor-mediated actions, may effectively initiate Cl^- and water fluxes toward the airway lumen.

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