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Cell Calcium Levels of Normal and Cystic Fibrosis Nasal Epithelium

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ABSTRACT. To determine whether epithelial ion transport abnormalities in cystic fibrosis (CF) might reflect abnormal regulation of intracellular Ca^{2+} levels, cytosolic free calcium (Ca_i^{2+}) was measured using fura-2 or quin2 in suspensions of normal or CF nasal epithelial cells derived from primary cell culture. The basal Ca_i^{2+} level measured with fura-2 in CF nasal epithelia was 155 ± 9 nM (n = 5), a value not significantly different from normal nasal epithelia (143 ± 16 nM, n = 5). Total cell calcium was measured by atomic absorption spectroscopy and no differences were observed between CF (6.3 ± 0.5 nmol/mg protein; n = 3) and normal (6.2 ± 1.2 nmol/mg protein; n = 3) nasal epithelial cells. Placing Na^+ loaded cells in a low (10 mM) extracellular Na^+ solution resulted in a rapid increase in Ca_i^{2+} consistent with Ca^{2+} uptake via a plasmalemmal Na^+-Ca^{2+} exchanger. The level of Ca_i^{2+} achieved by this low Na^+ maneuver was not significantly different in CF cells compared to normal cells. Neither isoproterenol (10^{-5} M) nor forskolin (10^{-6} M) had any effect on Ca_i^{2+} in normal or CF nasal epithelial cells. Thus, it appears that differences in cell Ca_i^{2+}, as measured by fluorescent chelators in suspensions of cultured cells, do not account for the abnormalities in basal or isoproterenol stimulated ion transport in CF tissues. (*Pediatr Res* 24: 79–84, 1988)

Abbreviations

Ca_i^{2+}, cytolsolic free calcium
CF, cystic fibrosis
AM, acetoxymethyl ester
TCA, trichloroacetic acid
PMN, polymorphonuclear cells

The pathophysiology of CF appears to reflect abnormal epithelial ion transport functions in specific target epithelia (1, 2). Recent evidence has focused on the notion that the abnormalities in epithelial ion transport in the lung reflect dysfunctions in regulatory processes rather than abnormalities in the permeability properties of channel proteins. Evidence for this notion has accrued from two lines of investigation. First, studies of freshly excised nasal epithelia have shown that there is 1) an imbalance between the basal rate of Na^+ absorption and the capacity to secrete Cl^- in CF as compared to normal tissues (3); and 2) the response to β-adrenergic agonists qualitatively differs in the two preparations, *i.e.*, isoproterenol induces Cl^- secretion in normal tissues whereas Na^+ absorption is accelerated in CF tissues (4). Second, evidence from patch clamp studies of cultured tracheal epithelial cells indicate that the conductive properties of Cl^- channels from CF patients are not different from normal patients when the patches are excised from the cell, implying a defect of cellular control of channel activation (5, 6).

Because regulation of many cellular functions, including both Na^+ and Cl^- transport, may be linked to changes in cell calcium (7, 8), total cell calcium and Ca_i^{2+} have been measured in a number of nonepithelial cell types from normal and CF patients. The results of studies of cultured fibroblasts and circulating blood elements are contradictory and have not been correlated with specific ion transport defects (9–15). Ca_i^{2+} or total cell calcium have not been measured in target epithelia from patients with this disease.

Herein we have measured Ca_i^{2+} with fluorescent chelators in suspensions of normal and cystic fibrosis nasal epithelial cells grown in culture. Basal Ca_i^{2+} levels were measured and the presence of a Na^+-Ca^{3+} exchange process, and responses to selected pharmacologic agonists were tested. Cultured nasal cells were chosen for study because of their availability and because the Cl^- permeability defect that characterizes CF has been reported to be retained in this preparation (16).

MATERIALS AND METHODS

Tissues and subjects. All tissues were obtained from patients undergoing clinically indicated surgery, through an arrangement approved by the Human Rights Committee for the School of Medicine at the University of North Carolina at Chapel Hill. CF subjects were diagnosed by standard clinical and sweat electrolyte criteria and required excision of nasal polyps to relieve obstruction. There were nine males and five females, age 16.3 ± 2.4 yr (range 8 to 38 yr). Normal nasal tissue was inferior turbinate that was removed during reconstructive procedures. The patients were 12 males and four females, age 34.0 ± 3.9 yr (range 7 to 69 yr). Some CF and non-CF patients had received medications chronically, *e.g.* antibiotics and inhaled steroids, but these were usually discontinued 7 to 14 days before surgery.

Cell isolation and culture. The culture conditions were described in detail by Wu *et al.* (17). Freshly excised polyp or nasal turbinate mucosa was rinsed and incubated in sterile Joklik's modified Eagle medium with 0.1% protease (Sigma Chemical Co., St. Louis, MO, type 14), penicillin (60 U/ml), streptomycin (60 μg/ml), and gentamicin (50 μg/ml). The tissue and medium were maintained at 4° C for 36 to 48 h with periodic gentle agitation. For tissues obtained from distant CF centers, this period included transit via commercial carriers. Fetal bovine serum (10%) was added to neutralize the protease, and the resulting suspension of epithelial cells was passed through nylon mesh to remove clumps and debris. The cells (ciliated, mucous,

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and basal) were centrifuged (500 × *g*; 5 min) and resuspended in protease-free medium twice. Washed cells were plated on plastic tissue culture dishes (Falcon Labware, Oxnard, CA) at a density of 2 to 3 × 10^4 cells/cm^2 in serum-free, growth factor supplemented Ham's F12 medium (17). The cells were incubated at 37° C in a humidified 5% CO_2:95% air atmosphere. Cells not attached to the culture dish (attachment efficiency was 20–30%) were removed after 24 h by replacement of the medium with fresh supplemented F12. The cells were grown for another 14–21 days. The medium was changed three times per week. Four to 11 days before use, at about 70% confluency, the growth medium was changed to a 1:1 mixture of supplemented F12 medium and Dulbecco's modified Eagle medium conditioned by exposure to NIH/3T3 fibroblasts (17). Conditioned medium contained 1% fetal bovine serum and appeared to further increase cell density at confluence.

Fluorescence measurement of Ca_i. Ca_i^{2+} was measured with the fluorescent dyes quin2 or fura-2, as described by Tsien and coworkers (18–20). The nasal epithelial cells from five 60-mm culture dishes (3 × 10^5 cells/dish) were removed by a brief (6-min) exposure to 0.1% trypsin in a Ca^{2+}-free solution. The cells were then centrifuged and resuspended in a modified Hanks' HEPES solution containing in mM: NaCl, 120; K_2HPO_4, 1.2; $MgSO_4$, 1.2; KCl, 5; glucose, 10; sodium HEPES, 10; $CaCl_2$, 1.25. The cells were allowed to reequilibrate in this solution for 30 min after which they were loaded with the AM of the calcium indicator by incubating them at 10^6 cells/ml for 20 min at 37° C with either quin2-AM (50 μM; Calbiochem-Behring, La Jolla, CA) or fura-2-AM (1 μM; Molecular Probes, Eugene, OR). After loading, the cells were washed twice with modified Hanks'-HEPES and resuspended in the same solution. Fluorescent measurements of quin2 and fura-2 were made on an Aminco spectroflurometer (model SPF-500, Aminco, Silver Spring, MD) with excitation at 340 nm and emission at 510 nm.

Quin2 measurements. The quin2 loaded cells (~3 × 10^5 cells/3 ml) were placed in a stirred water-jacketed cuvette (37° C). Ca_i was measured using the method described by Tsien *et al.* (19), modified slightly to allow correction for extracellular dye (21, 22). Two methods have been described for correcting for extracellular dye (22, 23); a correction using Mn^{2+} or EGTA. As described below, our experiments with quin2-loaded cells were designed to allow a comparison of both methods of correcting for extracellular dye.

Mn correction. After obtaining a baseline fluorescence (F1), 5 μM $MnCl_2$ were added to reduce the fluorescence of extracellular quin2 in 1.25 mM $CaCl_2$ by 0.5 (F2). Excess EGTA (2 mM) plus tris hydroxymethylaminomethane (pH 8.1) were added to obtain F3. The 2 mM EGTA binds the 5 μM Mn^{2+} allowing an independent measure of extracellular dye, as well as measurement of F_{max} and F_{min} without interference from Mn. Digitonin (70 μg/ml) was then added to obtain the fluorescence of quin2 in the absence of Ca^{2+} (F4). Saturating levels of $CaCl_2$ (10 mM) were then added to obtain F5. Ca_i was then calculated using the equation described by Tsien *et al.* (19).

$$Ca_i = K_D * (F-F_{min})/(F_{max}-F)$$

In this equation K_D, the quin2 dissociation constant is 115 nM (19). F is the initial level of fluorescence, F_{max} is the fluorescence with saturating Ca^{2+}, and F_{min} is the fluorescence in the presence of excess EGTA. To correct the variables F, F_{max} and F_{min} for the presence of extracellular quin2, we have subtracted from F, F_{max}, and F_{min} the fluorescence due to extracellular dye: F = F1 - [(F1-F2)/0.5]; F_{max} = F5 - [(F1-F2)/0.5]; and F_{min} = F4 - (0.3 [(F1-F2)/0.05]). F_{min} must be corrected because there is a calcium-independent component of quin2 fluorescence (30%), and therefore extracellular dye will also contribute to F_{min}. Mn^{2+} quenches all quin2 fluorescence, both calcium dependent and calcium independent.

Finally, to determine if there were significant levels of uncleaved dye in our cells, we made use of the observation that uncleaved dye has a higher K_D for Ca binding than completely hydrolyzed dye (24, 25). To test whether the intracellularly generated quin2 has the same K_D as the free acid, we did the following experiment. A Ca-EGTA buffer was prepared and the free-Ca^{2+} value, determined with commercially produced free acid of quin2 (Molecular Probes), was found to be 288 nM. Cells that had been loaded with quin2 by exposure to the acetoxymethyl ester were then lysed in this solution. The free-Ca level was then determined using the fluorescence of the intracellularly generated quin2 and was found to be 299 nM. These data suggest that the K_D for quin2 generated intracellularly is similar to that of the free acid. Furthermore, our method of calibration automatically circumvents the contribution of uncleaved, Ca^- insensitive dye, to the measurement of Ca^{2+}.

EGTA correction. Inasmuch as EGTA has a high affinity for Mn and EGTA was added after Mn, we can also correct for extracellular dye by using the difference between the fluorescence before and after EGTA (F1–F3). Thus, we can compare these two methods for correction in each experiment. F1 through F5 are defined as in the Mn correction, and Ca_i^{2+} is calculated from equation 1 with F_{max} and F corrected for extracellular dye as follows: F_{max} = F5 - (F1–F3), F = F1 - (F1–F3). F_{min} (F_{min} = F4) does not need to be corrected because unlike Mn, EGTA does not quench Ca-independent fluorescence.

We found that estimates of extracellular dye and calculated Ca_i levels corrected with Mn^{2+} and EGTA agreed within 10%. Further, there were no systematic differences in the two methods of correction. Extracellular dye was corrected for in each experiment.

Dye loading was calculated from the difference between F_{max} and F_{min} compared to an addition of a known concentration of quin2. Intracellular quin2 concentrations were ~6.4 nmol/10^6 cells.

Fura-2 measurements. These measurements were similar to those for quin2-loaded cells with the only difference being that we used only the EGTA addition to correct for extracellular dye. A K_D of 224 nM was used for fura-2 (20). Intracellular fura-2 concentrations were ~0.6 nmol/10^6 cells.

Measurement of total cell calcium. Total cell calcium was measured as described in detail elsewhere (21). Briefly, the cells on the culture dish were washed three times with an ice-cold calcium-free buffer. After this the calcium was extracted with 1 N HNO_3. Aliquots of the HNO_3 were taken, diluted with a calcium blank solution, and the samples were read with standards on an atomic absorption spectrometer. The remaining HNO_3 was removed from the dish and 0.6 N NaOH was added to dissolve the cell protein, following which protein was measured by the procedure of Lowry *et al.* (26).

Protocols. Initial experiments were performed to estimate the rate of dye leakage over intervals that spanned those of experimental protocols. The rate of dye leakage was found to be negligible. Consequently, the fluorescence before addition of the hormone (or other agent) served as the baseline for each experiment's manipulation. In addition, a control measurement of Ca_i was made on each aliquot of cells.

Cells were preincubated in a stirred, thermostated (37° C) cuvette for 10 min. For most experiments the appropriate drug or vehicle was then added and the fluorescence was monitored for an additional 10 min. This sequence was followed by calibration of Ca_i^{2+} (see Fig. 1). Experiments designed to test for the presence of a Na^+-Ca^{2+} exchanger had a slightly different protocol. For this series of experiments, a stock cell suspension was preincubated in a shaker bath at 37° C at 15× the normal concentration. At t = 0, 200 μl of the cell suspension were diluted into 3 ml Hanks'-HEPES buffer in the cuvette (1:15 dilution), the basal fluorescence was noted, and 10^{-4} M ouabain was then added to both the cells in the cuvette and the stock cell suspension. After a 10-min incubation, the Ca^{2+} level of the cells in the cuvette was measured by calibration with EGTA and Tris base, digitonin, and excess Ca^{2+}. Also after 10 min exposure to ouabain (10^{-4} M), 200 μl of the stock cell suspension were diluted into 3

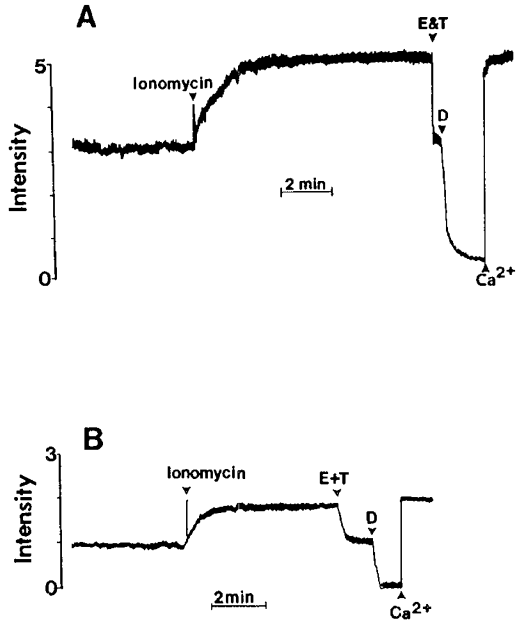

Fig. 1. Effect of ionomycin (10^{-5} M) on Ca_i^{2+} in fura-2-loaded nasal epithelial cells. *A* depicts the fluorescence intensity and changes in intensity (excitation 340 nm; emission 510 nm) from a cell suspension from a normal subject. *B* is the fluorescence intensity and changes in intensity as a function of added reagents in a cell suspension from a CF patient. *E&T*, 2 mM EGTA plus Tris base (pH = 8.1); *D*, 70 μg/ml of digitonin; Ca^{2+}, 10 mM $CaCl_2$.

ml of Na^+-free Hanks'-HEPES buffer (choline chloride substituted; final concentration Na_o^+ = 10 mm), and Ca_i^{2+} was measured.

Measurements of cAMP. Cells were removed from dishes by trypsinization as described above. The cells were washed, resuspended in Hanks'-HEPES solution, and allowed to equilibrate for 60 min at 37° C in a 100% O_2 atmosphere. Aliquots of cells (3×10^5) were exposed to vehicle (basal values) or to isoproterenol (10^{-5} M) for 2 and 10 min after which the cells were pelleted ($600 \times g$; 10 min) and the cAMP content of a TCA (10%) extract measured as previously described (4). The TCA-precipitated pellet was solubilized in 0.5 M NaOH solution and protein measured as described previously by the Bradford technique with bovine serum albumin as a standard (4). Each time point for each tissue was measured in triplicate, the results averaged, and expressed as pmol/mg protein. Recovery of added cAMP exceeded 90%.

Statistics. All values represent the mean ± SEM. Comparisons between values obtained with fura-2 and quin2 were made using *t* test for independent means. Comparisons of effects of drugs and low Na_o^+ on Ca_i^{2+} were made with *t* tests for paired samples.

RESULTS

Table 1 shows basal Ca_i^{2+} levels measured with quin2 and fura-2 in nasal epithelial cells from normal subjects and CF patients. There is no significant difference in basal Ca_i^{2+} levels between normal and CF cells measured with either quin2 ($p > 0.2$) or fura-2 ($p > 0.2$). However, the levels of Ca_i^{2+} measured with fura-2 were significantly higher than those measured with quin2. The total cell calcium content in cultured normal and CF nasal epithelial cells were not significantly different ($p > 0.2$; Table 1).

To investigate whether the measurement system was sensitive to changes in cell Ca_i^{2+}, fura-2-loaded cells were exposed to a Ca^{2+} ionophore. Figure 1 and Table 2 show that addition of 10 μM ionomycin, a nonfluorescent Ca^{2+} ionophore, induced increases in Ca_i^{2+} in both CF and normal nasal epithelial cells. In both CF and normals, ionomycin elevated Ca_i^{2+} to a level that

Table 1. *Basal Ca^{2+} levels and contents of cultured normal and CF nasal epithelia**

		Normal	CF
Ca_i^{2+} level (nM)	Quin2	81 ± 16 ($n = 5$)	106 ± 12 ($n = 3$)
	Fura-2	143 ± 16† ($n = 5$)	155 ± 9† ($n = 5$)
Total cell Ca^{2+} (nmol/mg protein)		6.2 ± 1.2 ($n = 3$)	6.3 ± 0.5 ($n = 6$)

* Values are mean ± SEM; n = number of separate patient specimens tested.

† Fura-2 value different than quin2 value ($p < 0.05$).

saturated fura-2. Because fura-2 is saturated with Ca^{2+} at approximately 2000 nM activity, we have listed the concentration in Table 2 for ionomycin exposed cells as equal to or more than 2000 nM.

We also tested whether basal cell Ca_i^{2+} levels in fura-2 loaded cells were in part regulated by Na^+-Ca^{2+} exchange. We reasoned that if a Na^+-Ca^{2+} exchanger existed in these cells, we should see a significant elevation in Ca_i^{2+} upon placing sodium-loaded cells (exposed to 10^{-4} M ouabain for 10 min in a 140 Na_o^+ solution) into low-Na^+ (10 mM) solution. As shown in Table 2, this treatment caused a significant elevation in Ca_i^{2+} of both normal and CF cells. The level of Ca_i^{2+} achieved in response to exposure to low Na^+ solutions was not significantly different in CF cells as compared to normal cells ($p > 0.1$). Ten min of exposure to ouabain alone caused no change in Ca_i^{2+} in normal or CF nasal epithelia (Table 2).

We next tested the response of CF and normal cells to β-agonist exposure. In three experiments with quin2-loaded cells from normals, the level of Ca_i^{2+} 10 min after isoproterenol addition (106 ± 12 nM, $n = 3$) was not different from the level of Ca_i before addition (105 ± 6 nM, $n = 3$). Similarly, in CF cells isoproterenol did not cause a significant increase in Ca_i^{2+} (97 ± 16 nM, $n = 3$) compared to Ca_i^{2+} levels before addition (102 ± 8 nM, $n = 3$).

To assure that we were not missing a Ca_i^{2+} response upon β-agonist stimulation due to buffering of Ca_i^{2+} by the relatively large intracellular levels of quin2, we repeated these studies using fura-2-loaded cells. Fura-2, recently introduced by Tsien and coworkers (20), has a 30-fold increased fluorescence intensity as compared with quin2 and can therefore be used at much lower loading levels. Typical results obtained using fura-2 are shown in Figure 2 and summarized in Table II. As with the results with quin2-loaded cells, isoproterenol had no effect on Ca_i^{2+} when added to either fura-2-loaded normal or CF epithelial cells. Furthermore, forskolin, a direct activator of adenylate cyclase, had no effect on CF or normal epithelial cell Ca_i^{2+}. Comparable responses were observed in cells grown in the presence or absence of cholera toxin (data not shown).

Figure 3 demonstrates an increase in cell cAMP content in response to isoproterenol, measured in cells treated identically to those used in the Ca measurements. These data suggest that the lack of effect of isoproterenol on Ca_i is not due to the inability of isolated cells to respond to the agonist.

DISCUSSION

The central role of Ca^{2+} in regulation of epithelial transport processes has led many investigators to postulate a defect in cell Ca^{2+} homeostasis as being a central defect in CF. Studies of both secretory and absorptive epithelia have indicated that substantial species and organ variability exists but have generally led to the notion that raised cell Ca_i^{2+} levels activate, directly or indirectly, cell Cl^- permeabilities (27), but decrease cell Na^+ permeability (7). Because CF respiratory epithelia exhibit a low basal Cl^- permeability but raised basal Na^+ permeability, a possible regulatory defect that would qualitatively account for these findings would be a low basal Ca_i^{2+} level (1, 3, 4). Consequently, we

Table 2. *Cell Ca^{2+} levels (Ca_i^{2+}, nM) of normal and CF nasal epithelia measured with fura-2 before and during exposure to drugs and ion substitution**

	Normal			CF		
	n	Before	During	*n*	Before	During
Ionomycin (10^{-5} M)	3	140 ± 20	≥2000†	3	143 ± 21	≥2000†
Ouabain (10^{-4} M)	3	122 ± 6	112 ± 11	3	92 ± 13	92 ± 13
Low Na‡		112 ± 11	259 ± 22†		92 ± 13	238 ± 44†
Isoproterenol (10^{-5} M)	4	142 ± 19	144 ± 21	3	135 ± 10	132 ± 11
Forskolin (10^{-6} M)	4	138 ± 22	134 ± 23	3	119 ± 14	130 ± 15

* Values are mean ± SEM, *n* = number of separate patient samples tested.
† Different than before value ($p < 0.05$).
‡ The before condition is during ouabain.

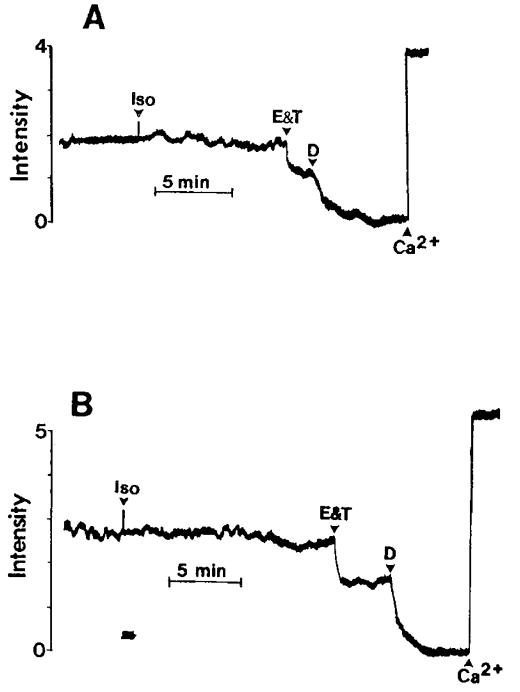

Fig. 2. Effect of isoproterenol on Ca_i^{2+} in fura-2-loaded nasal epithelial cells. *A* depicts the changes in fluorescence intensity (excitation 340 nm; emission 510 nm) induced by sequential addition of reagents from a cell suspension from a normal subject. *B* depicts the changes in fluorescence in a cell suspension from a CF patient. After a preincubation period, isoproterenol (10^{-5} M) was added to the cell suspension. *E&T*, *D*, and *Ca^{2+}* are defined in the legend to Figure 1. As seen in Table 2, the errors in these measurements are ~5–15%, thus an increase in Ca_i of less than ~50% would be undetectable. Additions are made through a hole drilled into the lid to the fluorometer. Thus the time resolution after addition of hormones is the mixing time which is less than 5 s.

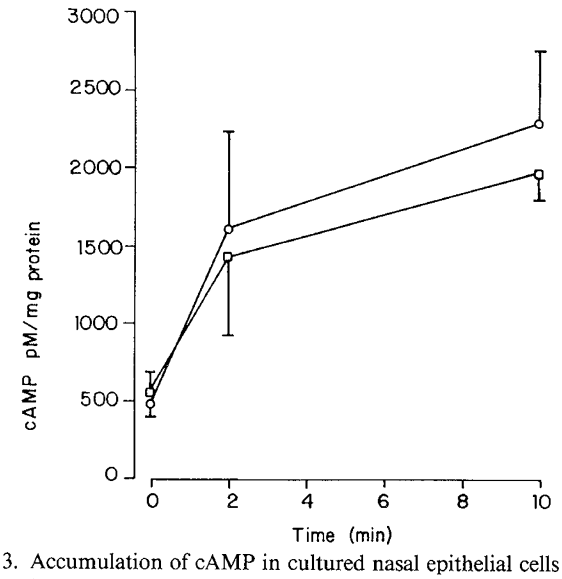

Fig. 3. Accumulation of cAMP in cultured nasal epithelial cells as a function of time after exposure to isoproterenol (10^{-5} M). *Circles* reflect data from cells of four CF subjects; *squares* reflect data from cells of five normal subjects; *points* reflect mean ± SEM.

initially sought to measure basal Ca_i^{2+} and assess at least one transport process that contributes to the regulation of cell Ca_i^{2+}, *i.e.* Na^+-Ca^{2+} exchange.

We used the fluorescent calcium indicators quin2 and fura-2 to monitor changes in Ca_i^{2+}. These fluorescent indicators, introduced by Tsien and coworkers (18–20), are loaded into cells as the acetoxymethyl ester of quin2 or fura-2. Once inside the cell intracellular esterases cleave the acetoxymethyl ester to produce the free acid of the calcium chelator and by-products acetate and formaldehyde. Although this technique has become widely used for Ca_i^{2+} measurements, potential problems with the technique exist. First, there are reports of adverse effects of the formaldehyde by-products (23). However, toxic effects appear rare. Second, quin2 has a low fluorescence intensity and this property, coupled with cell autofluorescence, necessitates intracellular quin2 loadings in the 1.0 mM range (23). This high level of loading is capable of buffering calcium transients (19, 23). We therefore repeated certain studies on fura-2-loaded cells because fura-2 has a higher fluorescent intensity and consequently can be used to measure Ca^{2+} when present at much lower intracellular levels. Finally, cell Ca^{2+} measurements are an average of ionized Ca^{2+} in cell compartments into which the dyes permeate. Preliminary optical microscopy indicates that fura-2 is not selectively concentrated in intracellular organelles in nasal epithelial cells.

As seen in Table 1, basal Ca_i^{2+} levels measured with fura-2 were slightly higher than those measured with quin2. This discrepancy, which may reflect the decreased buffering with fura-2, has also been observed by others (22, 28). However, basal Ca_i^{2+} levels measured with both indicators are well within the range of values reported by others using a variety of techniques in epithelial cells (28–30) as well as other tissues (19, 21, 22, 31). Impor-

tantly, the Ca_i^{2+} values for CF compared to normal cells do not differ by either method.

Na^+-Ca^{2+} exchange has been described in many cell types (32), including epithelial cells (33), and it appears to be involved in regulating basal Ca_i^{2+} levels. We tested for the presence of a Na^+-Ca^{2+} exchange process in fura-2-loaded nasal epithelial cells by imposing ion gradients that would be expected to favor Na^+_{in} for Ca^{2+}_{out} exchange. Lowering to 10 mM the extracellular Na^+ bathing Na^+ loaded cells resulted in a large increase in Ca_i^{2+}, consistent with an exchange of extracellular Ca^{2+} for intracellular Na^+ (Table 2). The increase in Ca_i^{2+} seen upon lowering extracellular Na^+ in Na^+-loaded cells was similar in normal compared to CF nasal epithelial cells. This finding suggests that the response of the Na^+-Ca^{2+} exchanger is similar in CF and normal epithelia.

We also sought to investigate the possible relationship between changes in cell Ca^{2+} activity and the observations that freshly excised normal tissues respond to isoproterenol or forskolin with Cl^- secretion whereas Na^+ absorption but not Cl^- secretion is accelerated in CF tissues (4). As recently reviewed by Rasmussen (34), the interactions between agonists that increase cAMP in cells and Ca^{2+} metabolism are complex. However, interactions between agonists that might be expected to raise cell cAMP levels (epinephrine) and Ca_i^{2+} levels have been reported in a variety of epithelia, including canine respiratory epithelia (30). Based on such studies, we reasoned that isoproterenol might act to increase cell Ca_i^{2+} in normal tissues, in concert with the induction of Cl^- secretion, but might decrease cell Ca_i^{2+} in CF tissues, in parallel with the increase in Na^+ absorption.

No changes in cell Ca_i^{2+} levels after exposure to isoproterenol or forskolin were detected in either CF or normal cells. The failure to detect a response is probably not related to a failure of cultured cells to respond to the addition of agonists for several reasons. First, cells treated in an identical protocol to that used for the Ca_i^{2+} measurements increased cell cAMP content in response to isoproterenol (Fig. 3). Second, the absence of change in Ca_i^{2+} after agonist exposure was also observed in cells cultured in the presence or absence of a culture media additive, cholera toxin, that may be expected to alter basal cAMP levels. Third, the bioelectric correlates of isoproterenol effects on ion transport of cultured CF and normal cells mimic those of fresh tissue, indicating that the linkage between cAMP accumulation and the functional response is not lost in these cells (35). Whereas it is conceivable that cell disaggregation influenced our findings, it appears more likely that substantial changes in Ca_i^{2+} levels in response to cAMP-accumulation are not a major feature in human respiratory epithelia. Consequently, the differing responses in CF and normal cells in responses to β-agonists may not be mediated by Ca^{2+}-sensitive paths.

The role of Ca^{2+} in directly activating Cl^- channels in normal and CF epithelial Cl^- channels is unclear. Data from two reports of patch clamp studies of excised patches differ in their assessment of the role of bathing solution Ca^{2+} to activate Cl^- channels (5, 6). Frizzell *et al.*(5) have also reported that CF Cl^- channels monitored in the on-cell patch mode are activated by the addition of Ca^{2+} ionophores. The activation of CF Cl^- channels by ionophore A23187 could either reflect 1) the elevation of cell Ca^{2+} levels from an abnormally low basal level, to a normal basal level or a supra-basal level required for activation or 2) an elevation of Ca_i^{2+} from a "normal" basal level to a supra-basal level required for activation. Our data that cell Ca_i^{2+} levels are comparable in CF and normal cells and that the ionophore response is roughly comparable in both cell populations would favor the latter interpretation.

Substantial variability is apparent in reports of total cell calcium values from a variety of CF *versus* normal cells. Our finding of comparable total cell calcium in CF and normals is in agreement with the findings in PMN (14) and lymphocytes (11) but is in disagreement with other reports describing raised total cell calcium levels in CF fibroblasts (9, 10), PMN (13), and lymphocytes (12). Somewhat more agreement has emerged from studies that have measured cell Ca_i^{2+} levels. Cell Ca_i^{2+} levels have been reported to be comparable in CF and normal RBC (15), PMN (14), and lymphocytes (11, 12). Our findings extend the observation from circulating blood elements that cell Ca_i^{2+} levels do not distinguish CF from normal cells to a target epithelium, *i.e.* the respiratory epithelium.

In summary, Ca_i^{2+} levels are similar in CF and normal respiratory epithelium. Ca_i^{2+} appears to be regulated in part by an Na^+-Ca^{2+} exchange process that is expressed in cells from both CF and normal subjects. Changes in cell Ca_i^{2+} in response to agonists that modulate ion permeabilities do not appear to induce responses in cell Ca_i^{2+} that distinguish CF from normal. Consequently, paths that are sensitive to very small changes in cell Ca_i^{2+}, or more likely regulatory paths or channels that may operate independently of Ca_i^{2+}, appear important to the pathophysiology of CF.

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