Vol. 180, No. 1, 1991
October 15, 1991

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 342-348

HIGH INTRACELLULAR PH IN CFPAC: A PANCREAS CELL LINE FROM A PATIENT WITH CYSTIC FIBROSIS IS LOWERED BY RETROVIRUS-MEDIATED CFTR GENE TRANSFER

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Received August 26, 1991

Expression of CFTR from a retroviral vector in CFPAC, a pancreatic adenocarcinoma cell line derived from a patient with Cystic Fibrosis, causes a decrease in the average intracellular pH (pH_i) in these transduced clones (PLJ-CFTR), as compared to CFPAC or CFPAC transduced with control virus (PLJ clones). Whereas the average pH_i calculated based on results obtained in two PLJ-CFTR clones, PLJ-CFTR-20 (n=2) and PLJ-CFTR-6 (n=5), was 7.46±0.07, the average pH_i calculated from results obtained in CFPAC (n=13), PLJ-6 (n=11) and PLJ-10 (n=3) was pH 7.83±0.11. This finding suggests that CFTR may be involved, directly or indirectly, in the regulation of pH_i in the pancreas.

Cystic Fibrosis (CF) is regarded as the most common severe autosomal recessive disorder in the Caucasian population, with a disease frequency of 1 in 2000 live births and a calculated frequency of about 5% (1). The predominant clinical manifestations of the disease result from improper function of epithelial tissues (1-3) and are related to two major anomalies, abnormal electrolyte composition of epithelial secretions and abnormal properties of the mucous secretions resulting in obstruction and frequently infection within mucus secreting organs including the lung, intestine, pancreas, biliary tract, salivary glands and genitourinary tract (3,4).

Recent progress in our understanding of the genetic and functional basis of CF indicates that CF is caused by mutations in the gene for the cystic fibrosis transmembrane conductance regulator (CFTR) (5-10). In epithelia affected by CF, Cl^- transport is abnormal (11-14). Expression of normal CFTR has been shown to correct the Cl^- channel defect in airway epithelial cells (5) and pancreas epithelial duct cells (6). Although recent evidence demonstrates that CFTR acts as a Cl^- channel, these studies do not exclude the possibility that CFTR could have an additional function (5-7).

We have recently found that sulfate transport via SO_4^{2-}/Cl^- anion exchange is altered in a pancreas epithelial cell clone from a CF patient (CFPAC) and that the defect is corrected in CFPAC clones exposed to a CFTR-expressing virus (PLJ-CFTR clones), but

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not in CFPAC clones exposed to control virus (PLJ clones) (30). The altered anion exchange activity in this pancreas epithelial cell system was not totally unexpected, since impaired HCO_3^- secretion via HCO_3^-/Cl^- anion exchange in the CF pancreas has been previously reported (16,17) and the duodenal fluid is rarely alkaline in CF patients with abnormal pancreas activity (18). The main objective of the present study was to determine whether a pancreas epithelial clone from one patient with CF displays altered intracellular pH and whether such a defect is corrected by retrovirus-mediated CFTR gene transfer.

METHODS

PANC-1, a permanent epithelioid cell line initiated from a pancreatic carcinoma of ductal origin (19), was obtained from the American Type Culture Collection (ATCC CRL 1469) and maintained at 37°C in a humidified environment of 5% CO_2 in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum and 100 μg/ml Gentamicin. Cell monolayers were grown almost to confluence in 35 mm dishes (Corning) in 3 ml growth medium. An epithelioid cell line (CFPAC) has been established from a CF patient with pancreatic adenocarcinoma at the Gregory Fleming James CF Research Center. Stable cell clones were obtained after subsequently exposing CFPAC to CFTR-expressing virus (PLJ-CFTR cell lines) or control virus (PLJ cell lines) (6). All cell lines were grown under identical conditions, as described above. The weak acid [^{14}C]benzoic acid was used to determine intracellular pH (pH_i), as previously described by L'Allemain et al. (22) and by us (20,21). Transport was measured in confluent cultures, as previously described by us (20,21,23). Protein concentrations were determined by the method of Lowry et al. (24). All transport experiments and assays were performed with 2-4 samples on 3-13 cell preparations. Results are given as means$\pm$SD (σ_{n-1}) and the number of experiments *n* is given with them. The kinetic constants apparent K_m and k_{cat} obtained in kinetic studies were calculated using Enzfitter, a data analysis program for the IBM PC (25). For a kinetics plot, the k_{cat} value represents the limiting rate as the substrate concentration tends to infinity (25).

RESULTS

Measurement of the distribution of a labeled weak acid in the intracellular and extracellular space is a simple method for estimating the intracellular pH that has been widely used by others (22) and by us (20,21). Typical time course of [^{14}C]Benzoic acid uptake in several clones is given in Fig. 1. In most clones, the accumulation reached steady state within 20 sec and remained constant for at least 120 sec. For the rest of the pH_i measurements presented, a convenient incubation time of 90 sec was chosen to estimate pH_i in the various clones. The average pH_i (n=13) measured in CFPAC, the clone isolated from the CF patient, was 7.87$\pm$0.13.

Amphotropic retroviruses have been used to transduce a functional CFTR cDNA into CFPAC (6). CFPAC cells were exposed to control virus (PLJ) or CFTR-expressing virus (PLJ-CFTR). Isolated viral-transduced PLJ-CFTR clones were shown to display normal Cl^- channel activity (6). We have used the same clones to test the possibility that pH_i may be lowered in the clones transduced with CFTR. The steady state level of

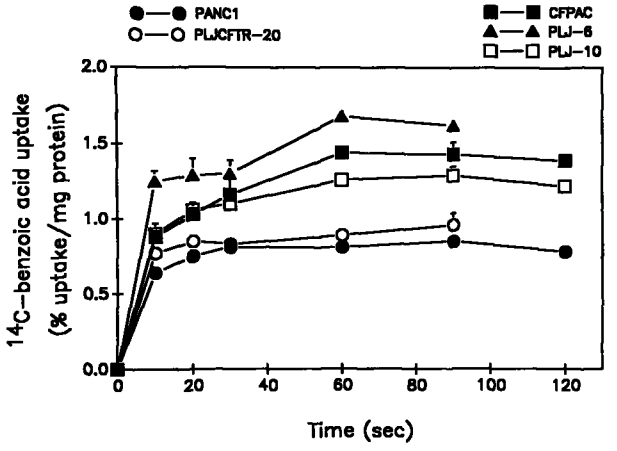

Fig. 1. Time course of [^{14}C]Benzoic acid uptake in cell clones from the pancreas. Cells were equilibrated for 1 hr at 37°C, in a 5% CO_2 environment, in 1 ml of medium containing 150 mM NaCl, 5 mM KH_2PO_4, 1 mM Ca gluconate, 1 mM Mg gluconate, 5 mM D-glucose, 1 mM Na_2SO_4, 10 mM Tris Hepes, pH 7.5. Uptake of [^{14}C]Benzoic acid was then followed for various times in the same medium, in an environment containing 0.03% CO_2. Value at each time point is the mean+standard deviation of results obtained in triplicate samples and represents cpm of [^{14}C]Benzoic acid taken up as a percentage of the cpm added to the cell monolayer, normalized by the amount of protein determined in the monolayer.

[^{14}C]Benzoic uptake was significantly higher in CFPAC, as compared to PLJ-CFTR-20 (Fig. 1). PLJ-6 and PLJ-10 displayed steady state [^{14}C]Benzoic acid uptake values comparable to CFPAC and significantly higher than PLJ-CFTR-20. The steady state levels in PANC1, a non-CF cell line, were comparable to those in PLJ-CFTR-20 and were significantly lower than the levels observed in CFPAC, PLJ-6 and PLJ-10 (Fig. 1).

Based on similar steady state [^{14}C]Benzoic acid uptake values, pH_i was estimated in several pancreas epithelial clones (Fig. 2). Cells were first equilibrated for 1 hr at 37°C, in a 5% CO_2 environment. [^{14}C]Benzoic acid was then added and incubation proceeded for 90 sec at 37°C, in an environment containing 0.03% CO_2. Under these conditions, pH_i in CFPAC, PLJ-6 and PLJ-10 was higher than that estimated in PLJ-CFTR-6 and PLJ-CFTR-20 (Fig. 2). To assess the significance of the differences observed, the combined pH_i measured in CFPAC (n=13), PLJ-6 (n=11) and PLJ-10 (n=3) were averaged and the result obtained was 7.83 ± 0.11 as compared to the average of combined results obtained in PLJ-CFTR-6 (n=5) and PLJ-CFTR-20 (n=2), which was 7.46 ± 0.07. The average pH_i value obtained in PANC1 clones was 7.28 ± 0.3 (n=5).

pH_i was estimated in selected clones in the presence of added HCO_3^- (Fig. 3). For these experiments, cells were first equilibrated for 1 hr at 37°C, in a 5% CO_2 environment, in a medium containing 15 mM $NaHCO_3$. [^{14}C]Benzoic acid uptake was then measured for 90 sec in the same medium, but in an environment containing 0.03% CO_2. Under these conditions, pH_i in CFPAC and PLJ-6 was also found to be higher than that in PLJ-CFTR-20 (Fig. 3).

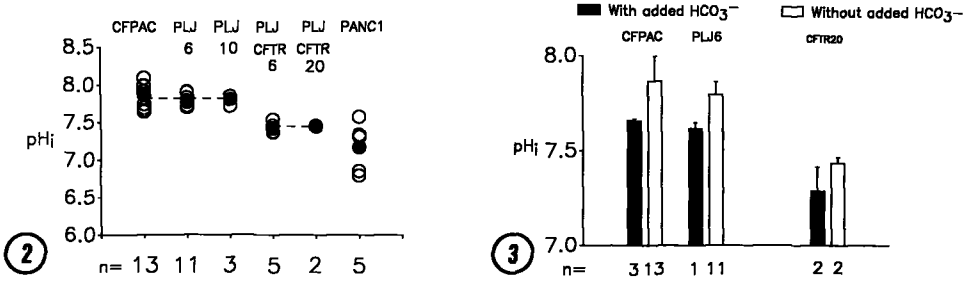

Fig. 2. pH_i is lower in cells expressing CFTR than in cells that do not express it. Cells were equilibrated for 1 hr at 37°C, in a 5% CO_2 environment, in 1 ml of medium containing 140 mM NaCl, 5 mM KH_2PO_4, 1 mM Ca gluconate, 1 mM Mg gluconate, 5 mM D-glucose, 0.3 mM Na_2SO_4, 10 mM Tris Hepes, pH 7.5. Uptake of [^{14}C]Benzoic acid was then followed for 90 sec in the same medium, in an environment containing 0.03% CO_2. pH_i (*open circles*) was estimated based on 4 replicate samples of [^{14}C]Benzoic acid uptake values obtained in each experiment. *n* is the number of experiments carried out with each clone type. The closed circle is the average pH_i calculated for *n* experiments. The upper dashed line represents the average pH_i calculated from the combined results obtained in CFPAC, PLJ-6 and PLJ-10. The lower dashed line represents the average pH_i calculated from the combined results obtained in PLJ-CFTR-6 and PLJ-CFTR-20.

Fig. 3. pH_i is lower in cells expressing CFTR than in cells that do not express it. For experiments in the presence of HCO_3^- (*filled bars*), cells were equilibrated for 1 hr at 37°C, in a 5% CO_2 environment, in 1 ml of medium containing 125 mM NaCl, 15 mM $NaHCO_3$, 5 mM KH_2PO_4, 1 mM Ca gluconate, 1 mM Mg gluconate, 5 mM D-glucose, 0.3 mM Na_2SO_4, 10 mM Tris Hepes, pH 7.5. For experiments without HCO_3^- (*open bars*), the medium was identical, but without $NaHCO_3$ and with 140 mM NaCl. After the 1 hr incubation, uptake of [^{14}C]Benzoic acid was followed for 90 sec in the same medium, respectively, in an environment containing 0.03% CO_2. pH_i was estimated based on 4 replicate samples of [^{14}C]Benzoic acid uptake values obtained in each experiment. Results are mean$\pm$standard deviation of all pH_i results obtained in *n* experiments.

Detailed sulfate transport studies carried out in these clones will be published elsewhere (30). Kinetic constants of sulfate transport measured in the presence or absence of HCO_3^- are given in Fig. 4. The apparent K_m values were quite variable in the various clones and may represent differences among the clones which are unrelated to CFTR. In contrast, k_{cat} values were consistently higher in PLJ-CFTR clones as compared to CFPAC or PLJ clones, suggesting that expression of CFTR affects, directly or indirectly, the activity of the SO_4^{2-}/Cl^- anion exchanger in these cells. k_{cat} was lower when HCO_3^- was added than when HCO_3^- was omitted from the medium (Fig. 4). This finding is consistent with the fact that pH_i was lower in the presence of HCO_3^-, as compared to that in its absence (Fig. 3), since our previous studies have shown that sulfate uptake is inhibited by lowering the intracellular pH (20).

DISCUSSION

These findings indicate that expression of CFTR from a retroviral vector, which confers normal Cl^- channel activity (6), is also affecting intracellular pH (pH_i) (Figs. 1-3).

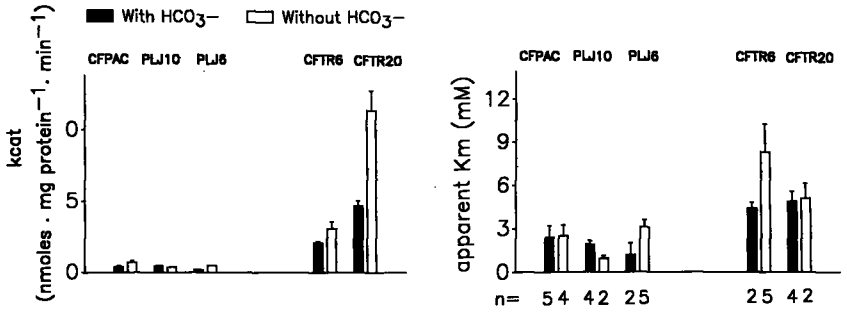

Fig. 4. The capacity of a sulfate transporter is low in cells that do not express CFTR. For experiments in the presence of HCO_3^-, cells were equilibrated for 1 hr in a medium containing 140 mM NaCl, 10 mM $NaHCO_3$, 5 mM KH_2PO_4, 1 mM Ca gluconate, 1 mM Mg gluconate, 5 mM D-glucose, 10 mM Tris Hepes, pH 7.5 and varying concentrations of sulfate (25 μM < $[SO_4^{2-}]$ < 7.5 mM). For experiments without HCO_3^-, cells were equilibrated in an identical medium, but without $NaHCO_3$ and with 150 mM NaCl. Both equilibration steps were in an environment containing 5% CO_2. Sulfate uptake was then measured for 1 min in an identical medium, respectively, but with $^{35}SO_4^{2-}$, in the presence or absence of 0.1 mM of the anion exchange inhibitor DIDS (0.03% CO_2). Kinetic constants for the DIDS-sensitive component of sulfate uptake were calculated using *Enzfitter*, a data analysis program for the IBM PC (25). Each bar is the mean+standard deviation of values obtained in *n* separate experiments. *n* for each cell type is given at the bottom of the graph.

Under conditions in which cells were first equilibrated in the presence of 5% CO_2/15 mM $NaHCO_3$ and [^{14}C]benzoic uptake, on the basis of which pH_i was estimated, was then measured in an identical medium but low CO_2 levels (0.03%) (Fig. 3), the net effect of lowering CO_2 is expected to be a rapid rise in pH_i (27). pH_i should recover provided that mechanisms of intracellular acidification, such as Na^+-independent HCO_3^-/Cl^- exchange are active. The fact that clones that do not express CFTR display higher pH_i than those that express it suggests that an anion exchange-mediated mechanism of acidification or its regulation may be altered in the former. On one hand, this possibility is supported by the change in the capacity of sulfate transport via SO_4^{2-}/Cl^- anion exchange in clones that express CFTR as compared with those that do not express it (Fig. 4). SO_4^{2-} has been shown to be a substrate for the band 3 HCO_3^-/Cl^- anion exchanger in erythrocytes (31). On the other hand, pH_i is higher in the clones that do not express CFTR and, under these conditions, sulfate uptake would be expected to be stimulated rather than inhibited (20,30). However, both findings would be explained by lower levels of expression or activity of an anion exchange protein responsible for both sulfate transport and intracellular acidification in cells which lack CFTR.

Some of the alternative possible explanations for the effect of CFTR on pH_i are (1) altered metabolic processes in the CFTR-deficient clones lead to alkalinization of the cell interior; (2) impaired regulation of a Na^+/H^+ antiport causes its activity to overshoot, leading to intracellular alkalinization; or (3) impaired coupling of the activities of HCO_3^-/Cl^- and Na^+/H^+ leads to an overshoot of pH_i towards more alkaline values. The second

possibility may be supported by findings in CF airway epithelia, in which increased amiloride-sensitive Na^+ transport has been demonstrated (28). The third possibility is supported by findings in lymphocytes in which stimulation of HCO_3^-/Cl^- anion exchange was eliminated when the alkalinization caused by Na^+/H^+ exchange was precluded, indicating that the two ion transport processes are tightly coupled (15).

In spite of recent discoveries (6,8-11,14), an elucidation of the mucus secretion abnormalities in CF has not yet been forthcoming and it is not yet known whether the mucous glycoprotein and the ion transport abnormalities are primary or secondary, or whether they are regulated or interfaced by an as yet unidentified common modulator. The pH sensitivity of virtually all enzymatic reactions implies that animal cells must maintain their cytoplasmic pH within a narrow range to provide a favorable environment for various intracellular activities (26). Altered pH_i in epithelial cells from the CF pancreas may be a possible link between electrolyte transport abnormalities and the mucus anomaly in the CF pancreas.

ACKNOWLEDGMENTS

We wish to express our thanks to Dr. Raymond Frizzell, Co-Director, Gregory Fleming James CF Research Center, Birmingham Al, for making the pancreas cell clones available to us. The excellent technical assistance of Rebecca Reed, Zhengfang Liang, Xi Ling Xe and Linda Hereford is gratefully acknowledged. This study was supported by a grant from the Cystic Fibrosis Foundation and a Career Investigator Award from the American Lung Association of Alabama/ American Lung Association.

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