

Cyclic Adenosine Monophosphate-dependent Kinase in Cystic Fibrosis Tracheal Epithelium

Roger Barthelson and Jonathan Widdicombe

Cardiovascular Research Institute, University of California San Francisco, San Francisco, California 94143

Abstract

Cl^- impermeability in cystic fibrosis (CF) tracheal epithelium derives from a deficiency in the β -adrenergic regulation of apical membrane Cl^- channels. To test the possibility that cAMP-dependent kinase is the cause of this deficiency, we assayed this kinase in soluble fractions from cultured airway epithelial cells, including CF human tracheal epithelial cells. Varying levels of cAMP were used in these assays to derive both a V_{\max} and apparent dissociation constant (K_d) for the enzymes in soluble extracts. The cAMP-dependent protein kinase from CF human tracheal epithelial cells has essentially the same V_{\max} and apparent K_d as non-CF human, bovine, and dog tracheal epithelial cells. Thus, the total activity of the cAMP-dependent kinases and their overall responsiveness to cAMP are unchanged in CF.

Introduction

Cystic fibrosis (CF)¹ is an inherited disorder characterized by deficient Cl^- permeability in epithelia, including those of the sweat gland duct (1) and the airways (2, 3). Cl^- impermeability in CF secretory epithelia probably reflects a deficiency in the β -adrenergic regulation of apical membrane Cl^- channels. In the coil of the normal human sweat gland, fluid secretion is stimulated by methacholine and isoproterenol, but in individuals with CF, only methacholine stimulates (4). In airway epithelium, β -adrenergic agents are potent stimulators of Cl^- secretion, and cAMP serves as second messenger in this response (5). Recent Ussing chamber studies show that Cl^- secretion across human tracheal epithelial cells can be stimulated by isoproterenol, and by A23187 in the presence of Ca^{2+} . With CF human tracheal epithelial cells, A23187 can elicit a response, but not isoproterenol (6). Similarly, patch clamp studies of human tracheal epithelial cells demonstrate the presence of functional apical membrane Cl^- channels in CF cells, but these channels fail to open in response to β -adrenergic stimulation (7, 8).

The lack of response to β -adrenergic agonists by CF cells does not involve a problem with the activation of adenylate cyclase; intracellular levels of cAMP rise by similar amounts in CF and non-CF tracheal epithelial cells treated with isoproterenol (6, 8). Elevated cAMP levels are probably linked to increased Cl^- secretion by the cAMP-dependent protein kinase (9). Regulation of ion transport by activation of the cAMP-dependent kinase has been described in a number of systems (10, 11).

The following experiments test for a possible deficiency in the cAMP-dependent kinase in cultured CF tracheal epithelial cells. Levels of the kinase activity were compared for cell extracts from cattle, dog, and humans, both with and without CF. Cultures of bovine and canine cells behave identically to cultured human cells in Ussing chamber studies of ion transport (12). Dog tracheal cells respond in a manner qualitatively identical to the original epithelium (13).

Methods

γ -[^{32}P]-ATP was obtained from NEN DuPont Co., Wilmington, DE. All other biochemicals were from Sigma Chemical Co., St. Louis, MO.

Tracheal epithelial cells were grown on 60-mm dishes coated with human placental collagen as described previously (12, 13). The cells were washed in PBS, scraped from the dishes with a rubber policeman, and centrifuged at 1,000 *g*. The pellet was resuspended in homogenization buffer and sonicated on ice for 1 min with a sonic dismembrator (Quigley Co., Inc., Rochester, NY), using the needle tip. Homogenization buffer consisted of 20 mM Tris/HCl, pH 7.5, 0.5 mM EGTA, 1.0 mM dithiothreitol, and three protease inhibitors in 0.1% NP-40, diluted into the buffer to give 2 mM benzamidine, 1 $\mu\text{g}/\text{ml}$ leupeptin, 0.1 $\mu\text{g}/\text{ml}$ pepstatin, and 0.01% NP-40. Cell sonicates were centrifuged at 1,000 *g* to remove unbroken cells and then separated into supernatant and membrane fractions by centrifugation at 47,000 *g* for 1.5 h. Cell extracts were stored at -100°C .

Transfer of [^{32}P]- PO_4 from γ -[^{32}P]-ATP to the acceptor histone was assayed essentially as described by Burnham and Williams (14). The following mixture was incubated at 30°C : 20 mM Pipes buffer, pH 7.0, 5 mM MgCl_2 , 0.1 mM EGTA, 30 $\mu\text{g}/\text{ml}$ supernatant protein or 60 $\mu\text{g}/\text{ml}$ membrane protein, 0.5 mg/ml histone, and 25 μM γ -[^{32}P]-ATP, $\sim 15,000$ cpm/ μl . Timed 100- μl samples were removed and added to 4 ml of 5% TCA, 1.5% sodium pyrophosphate, and 1.0% sodium phosphate (monobasic). These precipitated samples were filtered on HATF 25 mm/0.45 μm filters (Millipore/Continental Water Systems, Bedford, MA). The filters were washed eight times with 2 ml of the acid mixture, and their radioactivity was measured with an aqueous scintillation mixture in a scintillation counter. Control experiments showed that eight rinses were sufficient to reduce radioactivity from the reaction mixture to a level not more than twice the background level of 30 cpm. In the absence of cAMP, $\sim 1,000$ cpm were transferred to the sample per minute. For the experiments with varying concentrations of cAMP, duplicate samples were taken at 6 min to

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1. Abbreviations used in this paper: CF, cystic fibrosis; K_{ap} , apparent affinity constant.

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determine the rate of phosphate incorporation into histone. Isobutylmethylxanthine (1 mM) was added to the reaction mixture of some preliminary experiments, but it had no significant effect on phosphorylation and was omitted from all histone kinase assays.

For phosphorylation of endogenous proteins, the same incubation conditions as just described were used, except protein concentration was 0.2 mg/ml, EGTA was 0.3 mM, isobutylmethylxanthine was 1 mM, γ - ^{32}P -ATP was 2.5 μM , 70 $\mu\text{Ci/ml}$. Samples (100 μl) were incubated for 2 min at 30°C. The reaction was quenched by the addition of 25 μl of a solution of 100 mM EDTA, 100 mM NaF, and 5 mM ATP. Samples were chilled on ice, and 100 μl each was passed through a G-50 Sephadex column (15) to remove radioactive ATP. A sample (64 μl) of the void fraction was prepared and run on SDS-polyacrylamide gel electrophoresis using 10% acrylamide as described (16).

Results and Discussion

Activity of the cAMP-dependent kinase was measured as the cAMP-stimulated incorporation of ^{32}P into the exogenous phosphate acceptor histone. Fig. 1 shows the results of the cAMP-dependent kinase assay for the high-speed supernatant fraction from dog tracheal epithelial cells. Incorporation of ^{32}P into histone was dependent on the presence of both histone and cytosolic protein and was linear with time for at least 8 min even in the presence of supramaximal concentrations of cAMP (Fig. 1).

Most of the cAMP-dependent kinase activity was found in the soluble fraction. For example, the pellet portion for human cells had a maximum of 15 $\text{pmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$ activity, which was only a 10% increase over baseline phosphate incorporation for these experiments, and only 4% of the mean activity in the supernatants. This relatively low association of the kinase with the membrane fraction does not necessarily reflect the physiological distribution of the enzyme, because the association of the kinase with membranes can be affected by ionic strength (17) and possibly by metal chelators.

Fig. 2 shows examples cAMP-dependent phosphorylation of endogenous proteins separated by SDS-PAGE. As expected from the data on histone phosphorylation, no cAMP-dependent kinase activity towards endogenous acceptors was present in the membrane fraction (lane C), whereas cAMP caused a very large increase in phosphorylation of supernatant proteins

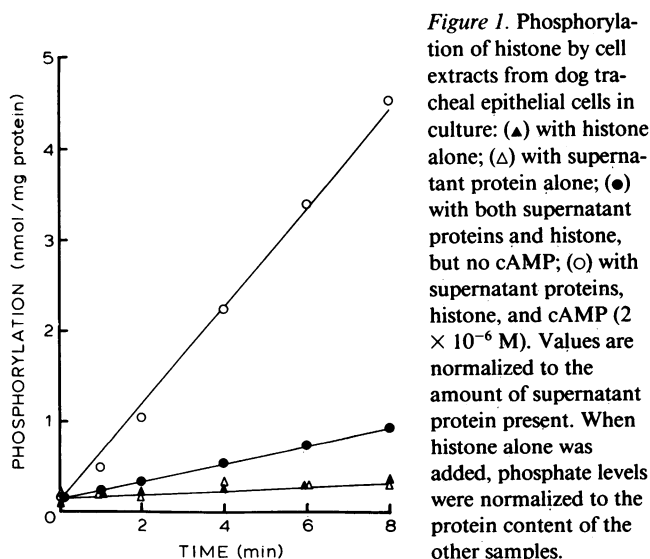


Figure 1. Phosphorylation of histone by cell extracts from dog tracheal epithelial cells in culture: (▲) with histone alone; (Δ) with supernatant protein alone; (●) with both supernatant proteins and histone, but no cAMP; (○) with supernatant proteins, histone, and cAMP (2×10^{-6} M). Values are normalized to the amount of supernatant protein present. When histone alone was added, phosphate levels were normalized to the protein content of the other samples.

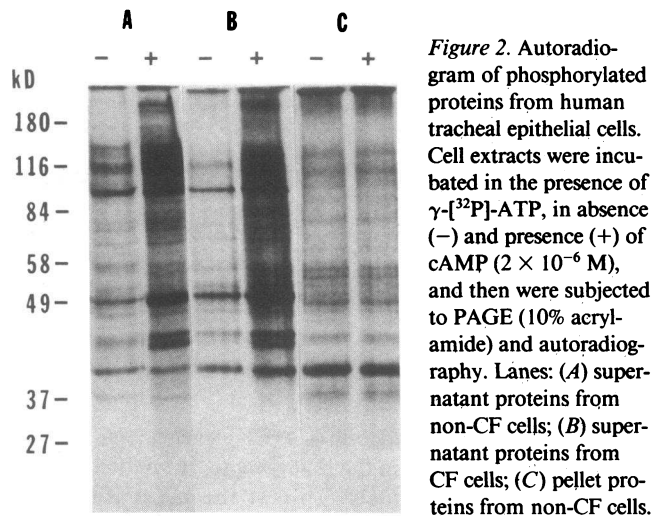


Figure 2. Autoradiogram of phosphorylated proteins from human tracheal epithelial cells. Cell extracts were incubated in the presence of γ - ^{32}P -ATP, in absence (-) and presence (+) of cAMP (2×10^{-6} M), and then were subjected to PAGE (10% acrylamide) and autoradiography. Lanes: (A) supernatant proteins from non-CF cells; (B) supernatant proteins from CF cells; (C) pellet proteins from non-CF cells.

(lanes A and B). There was essentially no difference in kinase activities in the soluble fractions of non-CF (lane A), and CF (lane B) cell extracts.

Supernatant proteins and histone were incubated with varying concentrations of cAMP to determine the sensitivity of the kinase activity to cAMP. The cAMP-dependent kinases in many tissues consist of at least two different holoenzymes, each constructed of interchangeable catalytic subunits and differing regulatory subunits (18, 19). Because it is the regulatory subunit that binds cAMP, these experiments yield an apparent affinity constant (K_{ap}) for the binding of cAMP to the collective holoenzymes in the supernatant fraction.

Soluble fractions from all the tracheal epithelial cells show a typical saturation binding curve for cAMP activation of kinase activity (Fig. 3). Eadie-Hofstee analysis of these data gives a K_{ap} ranging from 1.3×10^8 to 2.3×10^8 for tracheal cells of the three species tested (Table I). These values are near to those

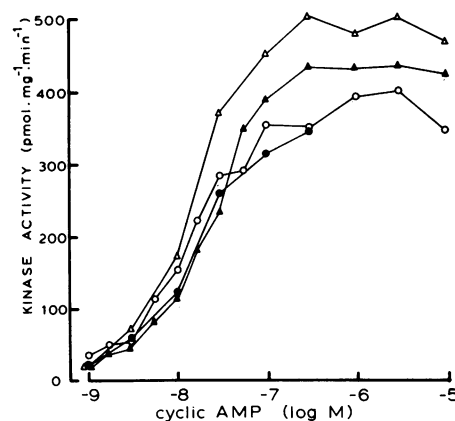


Figure 3. cAMP-dependent kinase activity at different concentrations of cAMP in soluble fractions from tracheal epithelial cells in culture. The difference between the rates of phosphate incorporation for the experimental (+cAMP) and control incubations was calculated for (Δ) bovine, (▲) canine, and both (○) non-CF and (●) CF human tracheal epithelial cells. Each curve represents the mean data for four experiments from two batches of cells for bovine cells, three from two for dog, seven from three for non-CF, and three from two for CF cells.

described for the pancreas (14) and for skeletal muscle (20). In muscle, the exact K_d for cAMP depends on the concentration of ATP (+Mg²⁺) and of the phosphate acceptor. The K_{ap} of $\sim 1.5 \times 10^{-8}$ M observed here for 25 μ M ATP falls above a K_d of 6×10^{-9} M given by Beavo (20) for cAMP binding in the absence of ATP and is considerably lower than the K_d of 6×10^{-7} M for kinase activity in muscle extracts in the presence of 125 μ M ATP. Table I shows that the K_{ap} for tracheal epithelial cells from all three species are very similar.

Similarly, Table I shows that the V_{max} for cAMP-dependent kinase activity (normalized to supernatant protein) is also very similar for tracheal epithelial cells from the three species tested. The maximal activity seen in these tracheal epithelial cells (350–500 pmol \cdot mg⁻¹ \cdot min⁻¹) is higher than that reported for mouse pancreatic acinar cells, (100 pmol \cdot mg⁻¹ \cdot min⁻¹) (14), and lower than that found for rabbit skeletal muscle (690 pmol \cdot mg⁻¹ \cdot min⁻¹) (21).

Epithelial cells cultured from tracheas of patients with CF show normal protein kinase activity in response to cAMP. Fig. 3 shows that the dose-response curve for cAMP-dependent kinase activity in the CF cells follows very closely the activity in non-CF human cells. The mean K_{ap} and V_{max} (normalized to supernatant protein) from these experiments are also not significantly different from those for non-CF human cells (Table I).

These data indicate that the predominant cAMP-dependent kinase activity is essentially the same in non-CF and CF tissue. However, these experiments do not distinguish between the different regulatory subunits or catalytic subunits (22) which may be present in trachea, nor do they consider the possible segregation of a subpopulation of holoenzyme into a subcellular compartment that may be critical to the regulation of Cl⁻ secretion. For example, in cardiac myocytes (23), the protein kinase that regulates glycogen hydrolysis is in the gly-

cogen granules. In tracheal epithelium, isoproterenol acts from the serosal side of the tissue, thus activation of apical membrane Cl⁻ channels must involve cytosolic messengers, and these may include the protein kinase itself. The specific holoenzyme that regulates Cl⁻ secretion may, nonetheless, be associated with the plasma membrane or otherwise represent a small fraction of the total kinase activity.

Whatever the composition of the holoenzymes in the trachea, the total catalytic activity is not affected in patients with CF. The overall sensitivity of cAMP-dependent protein kinase to cAMP is also unaffected in CF. These results suggest that if CF reflects a defect in the cAMP-dependent protein kinase, the isozyme involved represents a small fraction of the total. Alternatively, the deficient β -adrenergic response in these cells may stem from a defect further down the regulatory chain that leads to the apical Cl⁻ channel. Very little is known about this pathway, which may simply consist of the kinase acting directly on the channel or may involve one or more intermediary steps.

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Table I. Summary of V_{max} and K_{ap} Values for all Epithelial Cells Tested

Source of epithelial cells	V_{max}	K_{ap}	n
	pmol \cdot mg ⁻¹ \cdot min ⁻¹	$\times 10^6$ M	
Bovine trachea, combined	516 \pm 18	1.6 \pm 0.2	4
Canine trachea, combined	449 \pm 10	2.3 \pm 0.2	3
Human (non-CF)			
Trachea 1	474 \pm 35	1.3 \pm 0.3	3
Trachea 2	355 \pm 25	1.1 \pm 0.3	3
Trachea 3	193 \pm 13	1.3 \pm 0.3	1
Combined	378 \pm 11	1.3 \pm 0.1	7
Human (CF)			
Trachea 1	471 \pm 19	1.3 \pm 0.3	1
Trachea 2	244 \pm 9	1.6 \pm 0.2	2
Combined	359 \pm 19	1.4 \pm 0.3	3

Values are means \pm SE from computer-fitted least squares nonlinear regression analysis. n , number of experiments. Experiments were performed on two separate batches of both bovine and canine cells. For individual tracheas, V_{max} and K_{ap} were obtained from the curves fitted to the means of the kinase activities from all the individual experiments on that tracheal preparation. "Combined" values were derived from the fit to the mean kinase activities of all the experiments, as illustrated in Fig. 3. No attempt was made to weight the results for differences in the numbers of experiments performed per trachea.

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