Scanning Microscopy

Volume 7 | Number 4 Article 12

12-29-1993

X-Ray Microanalysis of cAMP-Induced Ion Transport in NCL-SG3 Sweat Gland Cells

Ann-Christin Mörk
University of Uppsala

Godfried M. Roomans
University of Uppsala

Scanning Microscopy, Vol. 7, No. 4, 1993 (Pages 1233-1240)
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

0891-7035/93$5.00+.00

X-RAY MICROANALYSIS OF cAMP-INDUCED ION TRANSPORT IN NCL-SG3 SWEAT GLAND CELLS

Ann-Christin Mörk* and Godfried M. Roomans

Medical Ultrastructure Research Unit, Department of Human Anatomy,
University of Uppsala, Box 571, S-75123 Uppsala, Sweden

(Received for publication December 1, 1993, and in revised form December 29, 1993)

Abstract

cyclic AMP (cAMP)-induced ion transport in a human sweat gland cell line, NCL-SG3, was investigated by X-ray microanalysis. Stimulation with cAMP caused a decrease in cellular Cl and K content in cells grown on impermeable as well as permeable supports. In cells grown on plastic coverslips or Transwell inserts stimulation with cAMP caused a significant decrease of the Na concentration, but no significant effect on the intracellular Na was seen on cells grown on grids. cAMP had no significant effect on the intracellular Ca concentration.

Key Words: Sweat gland, ion transport, cell cultures, cyclic AMP, substrate, X-ray microanalysis

* Address for correspondence:
Ann-Christin Mörk
Medical Ultrastructure Research Unit,
Dept. of Human Anatomy,
University of Uppsala, Box 571,
S-75123 Uppsala, Sweden
Phone No.: +46(18)174292
Fax. No.: +46(18)551120

Introduction

The sweat gland consists of two functionally different parts, the secretory coil where a sodium-chloride rich primary sweat is produced, and a duct where part of the sodium and chloride is reabsorbed. Chloride reabsorption in the duct appears to be mediated by cyclic AMP (cAMP), although some investigators have claimed that duct cells in addition are sensitive to cholinergic agonists via a Ca^{2+}-mediated pathway (Pedersen, 1987, 1991). The interest in studying ion transport in sweat glands mainly derives from the fact that in patients with cystic fibrosis (CF) the cAMP-mediated chloride absorption in the duct is defective, which results in an abnormally high concentration of chloride (and sodium) in the sweat.

NaCl uptake from the duct involves passive diffusion of Na^+ across the apical membrane into the duct cell through an amiloride-sensitive pathway and an active extrusion of Na^+ from the cell across the basolateral membrane requiring an ouabain-sensitive Na^+-K^+-ATPase (Bijman and Quinton, 1984). The normal sweat duct appears to be highly conductive to Cl^- so that its movement seems to be governed by the electrochemical gradients established by active Na^+ transport. Cl^- ions move passively across both the apical and the basolateral membrane down electrochemical gradients (Quinton, 1990).

Lee and Dessi (1989) established a sweat gland cell line (NCL-SG3) by infection of primary cultures with Simian virus 40 (SV40). It is not known whether the cell line was derived from the secretory or the reabsorptive components of the sweat gland or both. It could be shown that electrogenic ion transport (chloride secretion) can be stimulated by the β-adrenergic agonist isoproterenol and by lysylbradykinin.

Previously we have shown that electron probe X-ray microanalysis (XRMA) is a useful method to measure ion fluxes after activation (von Euler and Roomans, 1992; von Euler *et al.*, 1993). Since X-ray microanalysis allows measurements on single cells, inhomogeneities in cell cultures can be relatively easily detected. In the present study we therefore investigated whether the sweat gland cells were able to secrete chloride after

stimulation with cAMP. Since the preferred substrate for culturing cells for X-ray microanalysis, Formvar-coated titanium grids, is rather unusual, we tested whether the response of the cells to cAMP was dependent on the culture substrate.

Materials and Methods

Cell culture

A human eccrine sweat gland cell line NCL-SG3 (Lee and Dessi, 1989) was used in the study. NCL-SG3 cells were cultured in William´s E medium (Gibco) supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), insulin (10 μg/ml), transferrin (10 μg/ml), hydrocortisone (5 ng/ml), epidermal growth factor (10 ng/ml), sodium selenite (10 ng/ml) and 20 mM HEPES.

Experimental procedures

Cells grown on plastic were trypsinized with 0.05% trypsin (Sigma) in 0.02% EDTA according to standard methods. The cells were seeded out on titanium grids (75 mesh or 100 mesh, Agar Scientific), plastic coverslips (Thermanox tissue culture coverslips), glass coverslips (Agar Scientific) or Transwell inserts (Costar). The grids had been coated with a Formvar film and a thin carbon layer and sterilized under ultraviolet-light before the cells were seeded out. The cells were allowed to attach and spread for 2 days at 37 °C in a humidified atmosphere of 5% CO_2/95% air in a culturing chamber.

In stimulation experiments, the cells were, after rinsing with Krebs-Ringer buffer pH 7.4 to remove the culture medium, stimulated with 5 mM 8-bromo-cAMP in Krebs-Ringer buffer for 5 minutes at room temperature. The incubation was stopped by a quick (2-3 seconds) rinse in 0.15 M ammonium acetate (von Euler *et al.*, 1993). Ammonium acetate removed the salt-rich Ringer's buffer. After the rinsing, the grids, coverslips and Transwell inserts were quickly frozen in liquid propane cooled by liquid nitrogen (-180 °C) and then freeze-dried overnight in vacuum at -80 °C. The freeze dried specimens were covered with a conductive carbon layer before analysis.

Electron microscopy

For transmission electron microscopy (TEM), cells were fixed with glutaraldehyde (2.5%) in 0.1 M cacodylate buffer for 30 minutes, and washed in 0.1 M cacodylate buffer before postfixation in osmium tetroxide (1%) for 30 minutes. The cells were dehydrated in an ethanol series, infiltrated in absolute ethanol containing increasing concentrations of Polybed 812. The resin was polymerized overnight at 60 °C. Sections were cut, contrasted with uranyl acetate and lead citrate, and examined in a Hitachi H-7100 electron microscope.

For scanning electron microscopy (SEM), cells were fixed with glutaraldehyde (2.5%) in 0.1 M phosphate buffer for 60 minutes, and washed in 0.1 M phosphate buffer before postfixation in osmium tetroxide (1%) for 2 hour and then washed in phosphate buffer. The cells were dehydrated in an acetone series and then dried by the critical point method. Gold was sputtered onto the cells before they were examined in a Philips scanning electron microscope 525 using 20 kV as acceleration voltage.

X-ray microanalysis

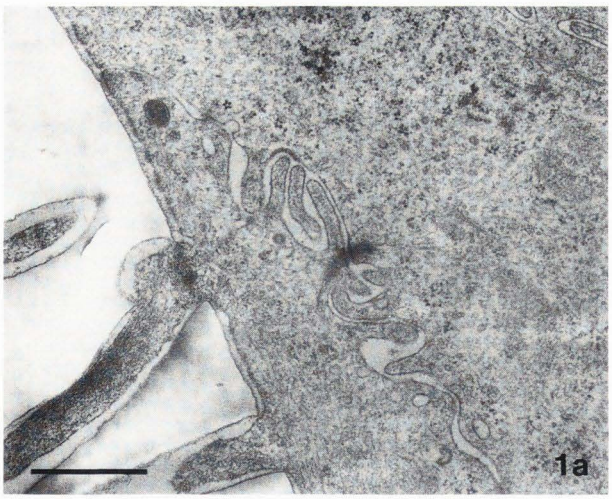
Cells grown on grids were analyzed at 100 kV in the transmission mode of a Philips 400 transmission electron microscope (TEM) with a field emission gun using a LINK QX200 energy-dispersive X-ray microanalysis system. Cells grown on coverslips or Transwell inserts were analyzed at 20 kV in the scanning mode of a Philips 525 scanning electron microscope using a LINK AN10000 energy-dispersive X-ray microanalysis system. The cells grown on grids were considered to be semi-thick specimens. Quantitative analysis was carried out by determining the ratio of characteristic counts to the background under the peak (P/B), and by comparing P/B ratios obtained from standards consisting of a gelatin/glycerol matrix containing mineral salts in known concentrations (Roomans, 1988). However, on the cells cultured on the solid substrates no fully quantitative data could be obtained, since the substrate contributes significantly to the background. Therefore, the analytical results are presented as elemental ratios, with phosphorus (P) as the internal standard. Spectra were acquired for 100 seconds. Only one spectrum was acquired from each cell. Student´s t-test was used to evaluate statistical significance.

Results

Morphology

Cells on Transwell inserts grew with their basolateral membrane apposed to the substratum and with a deposition of a thin basal lamina. These cells formed small cytoplasmic projections growing into the pores of the membrane (Fig. 1a). Cells grown on impermeable supports (plastic coverslips) seemed to deposit a thicker basal lamina (Fig. 2a). All cells had microvilli on their apical surface, irregular nuclei and intercellular connections (desmosomes) typical of epithelia *in situ* (Fig. 1b and 2b). The cytoplasm contained mitochondria, endoplasmic reticulum and ribosomes. Cultures on both supports were monolayered.

Scanning electron microscopy of critical point dried cells with microvilli (Fig. 3a) and freeze-dried cells on glass coverslips (Fig. 3b) showed that the cells grow in a monolayer and are flattened with a rather prominent nucleus. The freeze-dried cells show obvious freezing damage but since whole cells are analyzed this does not compromise the analysis. Sweat gland cells cultured on Formvar-coated titanium grids could easily be identified in the TEM (Fig. 4).



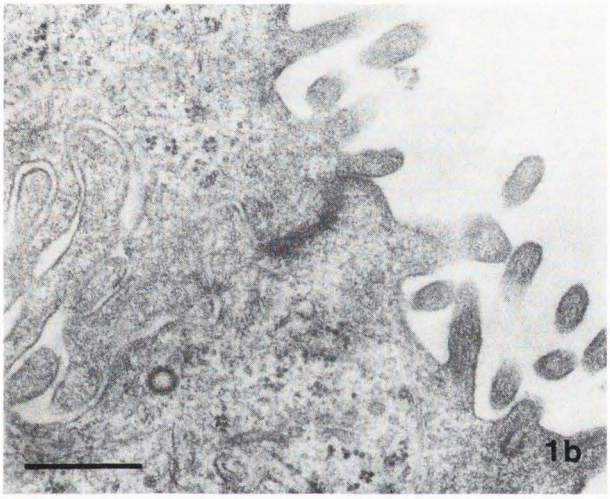
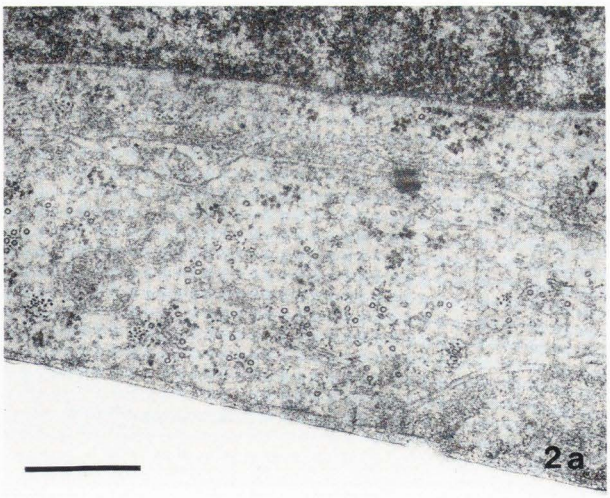


Figure 1a. NCL-SG3 cell grown on Transwell insert with the basolateral membrane apposed to the substratum and small cytoplasmic projections growing into the pores of the membrane. A desmosome can be seen between two cells. The cells seems to deposit a thin basal lamina. Bar indicates 4 μm.

Figure 1b. Cells grown on Transwell inserts with microvilli (M) on the apical surface. The figure also shows what is probably a junctional complex (J) at the apical side of the cell. Bar indicates 2 μm.

X-ray microanalysis of cells grown on permeable and impermeable supports

Stimulation with cAMP caused a significant decrease of the cellular Cl and K concentration in cells grown on impermeable as well as on permeable supports. Cells cultured on glass coverslips also showed a decrease of the cellular Cl content. To be able to compare the results obtained from cells grown on different supports, we calculated the Cl/P and K/P ratios (Fig. 5 and 6). We could not calculate K/P for the cells grown on glass coverslips because analysis of the glass itself by



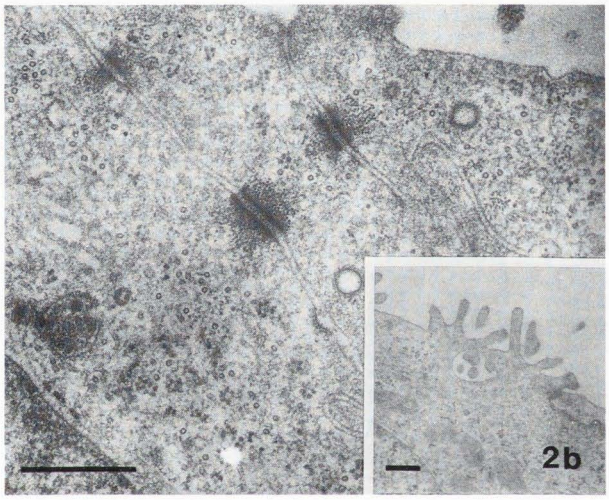


Figure 2a. Cells grown on impermeable support (plastic) seem to deposit a thicker basal lamina. A nucleus and a desmosome can be discerned. Bar indicates 2 μm.

Figure 2b. Clearly defined desmosomes in cells grown on plastic coverslips. Inset: Microvilli on the apical surface. Bars indicate 2 μm.

SEM XRMA gave background spectra with elemental peaks for Na, Al, Si, K, Ca, Ti and Zn. Background spectra from Transwell inserts and plastic coverslips showed no observable elemental peak (with the exception of Si, which is a normal contaminant). Titanium grids gave background spectra with Ti, Si and Cu (from the holder). A correction for these extraneous peaks was made in the quantitative analysis. In cells grown on plastic coverslips or Transwell inserts stimulation with cAMP caused a significant decrease of the Na concentration, but no significant effect on Na was seen in cells grown on grids. cAMP had no detectable effect on the intracellular Ca concentration. The data in figs. 5 and 6 are from cells in late passages of the NCL-SG3 cell line (passage number 64-70 for cells grown on grids, passage

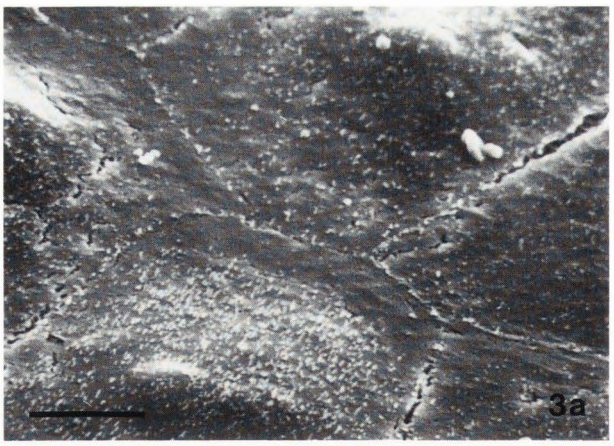


Figure 3a. Cells grown on a glass coverslip (critical-point dried) with microvilli and prominent nuclei. Bar indicates 10 μm.

Figure 3b. Individual freeze-dried cells that were grown on a glass coverslip can easily be identified, although they show obvious freezing damage. Bar indicates 50 μm.

number 72-74 for cells grown on other substrates).

Initially (passage number 58-63) the cultured sweat gland cells on grids appeared to be a homogeneous population as seen from the distribution of the K concentrations (Fig. 7a). The distribution of the K concentration in cells with a higher passage number (passage 64-70) seemed to be inhomogeneous and two populations of control cells could be distinguished (Fig. 7b). Cells grown on Transwell inserts, plastic coverslips and glass coverslips are all from a higher passage number (passage 72-74). They all appeared to be a homogeneous population with regard to the distribution of K concentrations.

Discussion

It is of interest to know whether the NCL-SG3 cells have the ion transport characteristics of duct cells

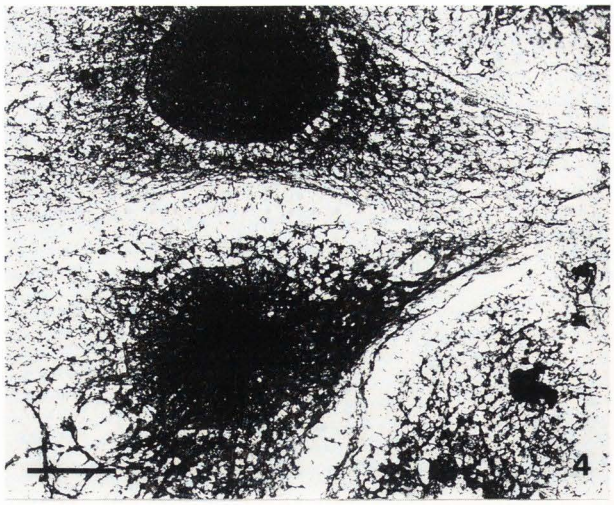


Figure 4. Carbon-coated freeze-dried cells grown on Formvar-coated titanium grids viewed in the transmission mode. Bar indicates 50 μm.

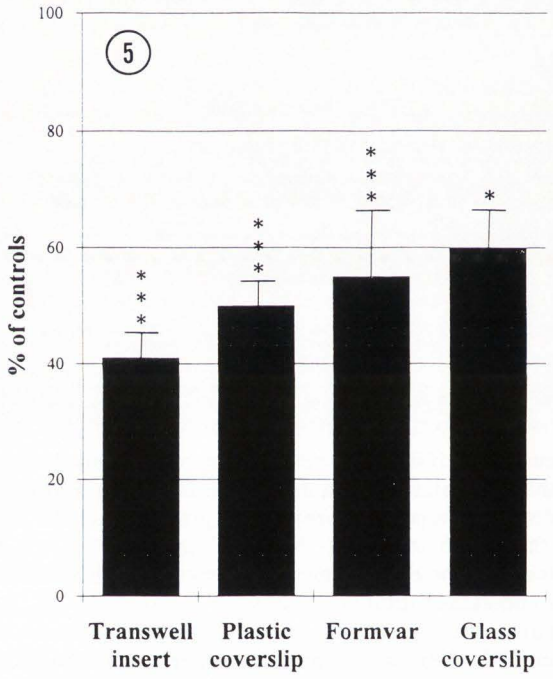
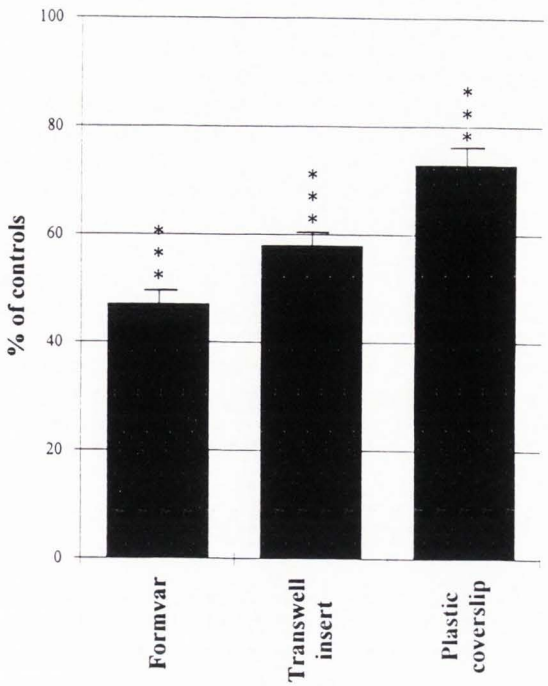


Figure 5. Effects of cAMP (5 mM, 5 min) treatment of sweat gland cells grown on different supports. Chloride concentrations are related to phosphorus and given as % of controls. Each column represents the mean $\pm$ standard error (SE). Significant differences between unstimulated and cAMP-stimulated cells are indicated: *** $p < 0.001$, * $p < 0.05$.

or those of secretory coil cells.

In the coil cells, NaCl secretion requires the movement of Cl^- ions from the basolateral to the apical side of the cell. This creates a potential difference across the epithelium driving Na^+ ions into the lumen via a paracellular route. Chloride enters the secretory cells via

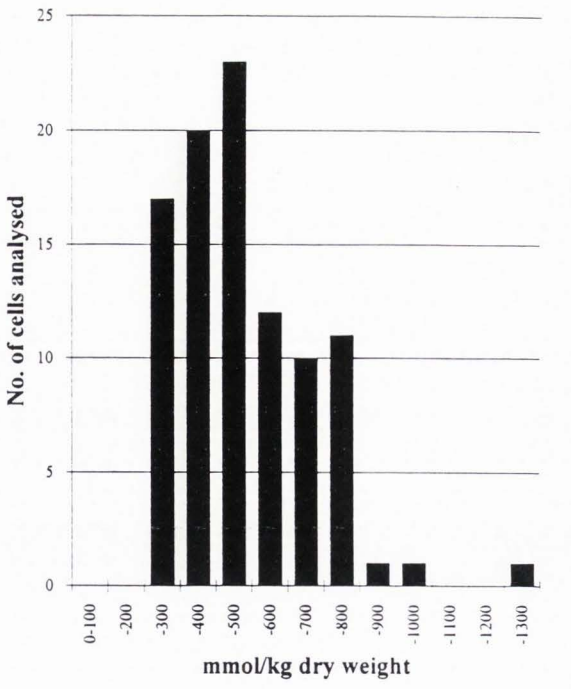


6

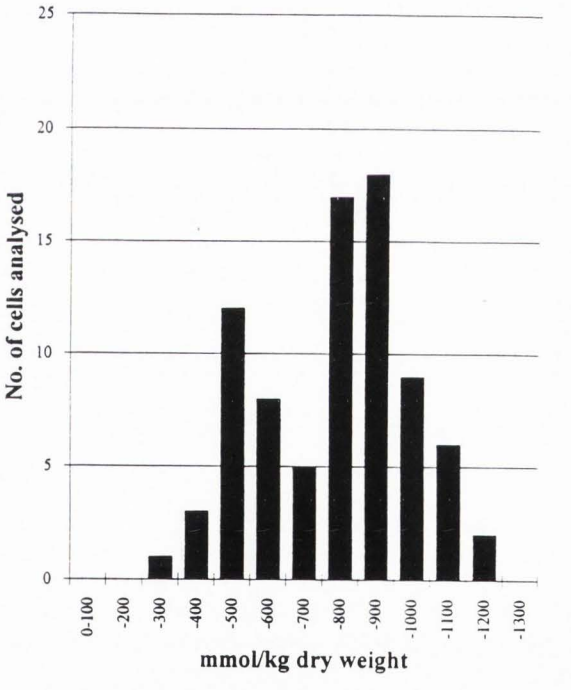
Figure 6. Effects of cAMP (5 mM, 5 min) treatment of sweat gland cells grown on different supports. Potassium concentrations are related to phosphorus and given as % of controls. Each column represents the mean ± SE. Significant differences between unstimulated and cAMP-stimulated cells are indicated: *** $p < 0.001$.

the Na^+-$2Cl^-$-K^+ co-transport. The secretion of chloride requires an activation of cellular K^+ efflux at the basolateral membrane. The K^+ efflux hyperpolarizes the cell to create a favorable electrical gradient for Cl^- exit across the apical surface. That basolateral K^+ efflux is linked to apical Cl^- secretion is substantiated by findings that K^+ channel blockers, such as barium, inhibit Cl^- secretion (Krouse *et al.*, 1989). The increase in cellular potassium permeability is mediated by a rise in intracellular free calcium (Bovell *et al.*, 1989) and this in turn can be brought about by cholinergic stimulation.

In duct cells, Na^+ is removed from the cell by Na^+/K^+-ATPase in the basolateral membrane. Following this, the concentration of cytoplasmic Na^+ is low in spite of a passive movement of Na^+ into the cell across the apical membrane. This process of Na^+ reabsorption leads to a favorable electrochemical gradient for the passive movement of Cl^- to accompany Na^+ into the cell. Cl^- ions also move passively across the basolateral membrane down electrochemical gradients (Quinton, 1990). This process is activated by cAMP. In cells from patients with cystic fibrosis (CF), this passive movement of Cl^- is impaired and resistant to β-adrenergic stimulation (Bijman and Frömter, 1986; Pedersen, 1990). The



7a



7b

Figure 7a. Frequency histogram of K in NCL-SG3 cells (passage 58-63). The X-axis shows intervals for K concentrations (in mmol/kg dry weight), the Y-axis the number of cells within a particular interval.

Figure 7b. Frequency histogram of K in NCL-SG3 cells (passage 64-70).

basic cellular defect in CF is due to a mutation in the gene coding for a membrane protein, CFTR (cystic fibrosis transmembrane conductance regulator) (Riordan *et al.*, 1989). The CFTR protein is identical with the cAMP-activated apical Cl^- channel, and in CF patients the mutated form of the gene product cannot be activated by cAMP.

Most XRMA studies on sweat glands have been concerned with the secretory cells. In monkey secretory cells, cholinergic stimulation caused a decrease in K and Cl, and an increase in Na (Saga and Sato, 1989; Takemura *et al.*, 1991). In rat secretory cells, cholinergic stimulation also was seen to cause a decrease in K and an increase in Na, but no change in Cl concentration was noted (McWilliams *et al.*, 1988). In equine sweat glands, thermally-induced sweating caused a decrease in K and a rise in Na and Cl (Wilson *et al.*, 1988a). Only in one study have the secretory cells been compared to duct cells. Wilson *et al.* (1988b) studied the effects of thermally-induced activity *in vivo* both in normal sweat glands and in glands from CF patients. In normal glands, a decrease in K and an increase in Na was observed in the secretory part of the gland (the fundus) and an increase in Na and Cl was found in the duct. None of these changes were observed in the CF sweat glands. In addition it was found that in normal glands Ca stores are mobilized during fluid secretion in the secretory cells, but not in the duct cells (Wilson *et al.*, 1989).

In the present study, stimulation with cAMP caused a decrease in cellular Cl and K content in NCL-SG3 cells grown on impermeable as well as permeable support. The changes in Na were not consistent, but the data on cells grown on grids are for technical reasons most reliable, and in these cells, no significant changes in Na were observed. Since the Na K-line is at the low-energy end of the spectrum and Na X-rays are absorbed by the beryllium window, the increased peak-to-background ratio attainable at high accelerating voltage with cells grown on thin supports provides better statistics. Our data on the NCL-SG3 cells do not completely fit in with the published data, since we do not find the increase in Na found by other groups both in the duct and in the secretory coil cells. The decrease in cellular K is found in both duct and secretory coil cells in the majority of studies on glands *in situ* and is also observed in our study. The effect of stimulation on the cellular Cl content differs widely between published studies. We have, however, in other Cl^--secreting epithelial cell types observed a decrease of the cellular Cl content after stimulation with β-adrenergic agonists or with cAMP both in cultured cells (von Euler and Roomans, 1992; von Euler *et al.*, 1993; Sagström *et al.*, 1992) and in cells stimulated *in situ* (Spencer and Roomans, 1989).

The cAMP-induced loss of Cl from the sweat gland also confirms earlier electrophysiological studies of these cells (Lee and Dessi, 1989), and the apparent presence of a cAMP-regulated chloride channel would give the NCL-SG3 cells duct characteristics. Whether the chloride channel in these cells is of the CFTR type cannot be concluded from our study. On the other hand, Whiteford *et al.* (1993) found that chloride permeability in NCL-SG3 cells can be increased by ATP via a Ca^{2+}-dependent mechanism. This could suggest that the NCL-SG3 cells might have both Ca^{2+}-dependent and cAMP-dependent chloride channels, and thus combine properties of duct and secretory coil cells. This contradicts results by Lee and Dessi (1989) who did not find a Ca^{2+} activation of chloride transport in these cell lines.

One problem with the cell line appears to be that there might have been a differentiation of the original cell population into two subpopulations along with increasing passage number. It is possible that the expression of ion transport characteristics is not completely stable but varies with the passage number. The characteristics and regulation of chloride transport in NCL-SG3 cells is now the subject of more detailed studies.

Acknowledgements

The authors are indebted to Dr C.M. Lee, University of Newcastle upon Tyne, UK for a gift of the NCL-SG3 cell line. The technical assistance of Mr Leif Ljung and Mrs Marianne Ljungkvist is gratefully acknowledged. The study was supported by grants from the Swedish Medical Research Council (project 7125) and the Swedish Association against Heart and Lung Diseases.

References

Bijman J, Frömter E. (1986). Direct demonstration of high transepithelial chloride-conductance in normal human sweat duct which is absent in cystic fibrosis. Pflügers Arch **407**: S123-S127.

Bijman J, Quinton PM (1984). Influence of abnormal Cl^- impermeability on sweating in cystic fibrosis. Am J Physiol. **247**: C3-C9.

Bovell DL, Elder HY, Jenkinson DMcE, Wilson SM. (1989).The control of potassium ($^{86}Rb^+$) efflux in the isolated human sweat gland. Quart J Exp Physiol **74**: 267-276.

Krouse ME, Hagiwara G, Chen J, Lewiston NJ, Wine JJ. (1989). Ion channels in normal human and cystic fibrosis sweat gland cells. Am J Physiol **257**: C129-C140.

Lee CM, Dessi J. (1989). NCL-SG3: a human eccrine sweat gland cell line that retains the capacity for transepithelial ion transport. J Cell Sci **92**, 241-249.

McWilliams SA, Montgomery I, Elder HY, McEwan Jenkinson D, Wilson SM. (1988). Effects of stimulation on the ultrastructure and Na, K, Cl composition of the fundus of the rat plantar sweat gland. Tissue & Cell **20**: 109-118.

Pedersen PS. (1987). Cellular mechanism of cholinergic action in epithelial cell cultures derived from the human reabsorptive sweat duct. In: Genetics and Epithelial Cell Dysfunction in Cystic Fibrosis (eds

Riordan JR, Buchwald M) Alan R Liss, New York, 151-165.

Pedersen PS. (1990). Chloride permeability regulation via a cyclic AMP pathway in cultured human sweat duct cells. J Physiol **421**: 379-397.

Pedersen PS. (1991). Separate agonist-specific oscillatory mechanisms in cultured human sweat ducts. J Physiol **433**: 549-559.

Quinton PM. (1990). Cystic fibrosis: a disease in electrolyte transport. FASEB J **4**: 2709-2717

Riordan JR, Rommens JM, Kerem B, Alon N, Rozmahel R, Grzelzak Z, Zelenski Z, Lok J, Plavsik N, Chou, JL, Drumm ML, Ianuzzi MC, Collins FC, Tsiu LC. (1989). Identification of the cystic fibrosis gene: cloning and characterization of complemetary DNA. Science **245**: 1066-1073.

Roomans GM. (1988). Quantitative X-ray microanalysis of biological specimens. J Electr Microsc Techn **9**: 19-44.

Saga K, Sato K. (1989). Electron probe X-ray microanalysis of cellular ions in the eccrine secretory coil cells during methacholine stimulation. J Membrane Biol **107**: 13-24.

Sagström S, Roomans GM, Wroblewski R, Keulemans JLM, Hoogeveen AT, Bijman J. (1992). X-ray microanalysis of cultured respiratory epithelial cells from patients with cystic fibrosis. Acta Physiol Scand **146**: 213-220.

Spencer AJ, Roomans GM. (1989). X-ray microanalysis of hamster tracheal epithelium. Scanning Microsc **3**: 505-510.

Takemura T, Sato F, Saga K, Suzuki Y, Sato K. (1991). Intracellular ion concentrations and cell volume during cholinergic stimulation of eccrine secretory coil cells. J Membrane Biol **119**: 211-219.

Whiteford ML, Bovell DL, Pediani JD, Smith GL, Wilson SM. (1993). ATP regulation of chloride permeability in an established cell line (NCL-SG3) from sweat gland epithelium. Proc XXXII Congress Int Union Physiol Sci, IUPS Program Committee, Glasgow, 114-115.

Wilson SM, Elder HY, McEwan Jenkinson D, McWilliams SA. (1988a). The effects of thermally induced activity *in vivo* upon the levels of sodium, chlorine and potassium in the epithelia of the equine sweat gland. J Exp Biol **136**: 489-494.

Wilson SM, Elder HY, Sutton AM, McEwan Jenkinson D, Cockburn F, Montgomery I, McWilliams SA, Bovell DL. (1988b). The effects of thermally-induced activity *in vivo* upon the ultrastructure and Na, K and Cl composition of the epithelial cells of sweat glands from patients with cystic fibrosis. Tissue & Cell **20**: 691-700.

Wilson SM, Elder HY, McEwan Jenkinson D, Sutton AM, Cockburn F. (1989). The measurement of intracellular calcium by energy dispersive X-ray microanalysis in normal and cystic fibrotic human sweat glands. Inst Phys Conf Ser **98**: 735-738.

von Euler A, Roomans GM. (1992). Ion transport in colon cancer cell cultures studied by X-ray microanalysis. Cell Biol Int Rep **16**: 293-306.

von Euler A, Pålsgård E, Vult von Steyern C, Roomans GM. (1993). X-ray microanalysis of epithelial and secretory cells in culture. Scanning Microsc **7**: 191-202.

Discussion with Reviewers

J.E. Beesley: The authors find that after cAMP stimulation there is a significant decrease in Na in those cells (of highest passage number) cultured on plastic coverslips and Transwells but no change of Na in those cells (of lower passage number) cultured on grids and consider the reason to be because data on cells grown on grids are technically most reliable. Would the authors please comment on the initial and final levels of elements in cells grown on each of these substrates and discuss further whether the differences in Na changes observed in cells from the three substrates are of technical and physiological origin, thereby explaining the observed differences?

Authors: The Na concentrations in the sweat gland cells were as follows (data in mmol/kg dry weight, mean ± standard error):

substrate	unstimulated	cAMP-stimulated
grid	106 ± 6	87 ± 11
Transwell	45 ± 4	32 ± 5
plastic	58 ± 8	32 ± 5

(data based on 80 measurements for the unstimulated cells on grids and 20 measurements in each of the other groups).

It should be noted that the absolute data for cells grown on Transwell or plastic coverslip substrates are in error because the analytical volume includes the substrate. In a more extensive series of measurements (Mörk *et al.*, in preparation) we could not find a significant effect of cAMP-stimulation on the Na content of cells grown on grids. The difference in passage number between cells grown on grids and those grown on other substrates is not very much, and does not seem a likely explanation for the observed differences. We prefer the explanation that the difference in results between the substrates is of technical, rather than physiological nature.

A.J. Spencer: Since the cells were grown to confluency, showed a high degree of cell to cell attachment and were polarised, i.e., basal surfaces towards the support, did you ever observe accumulation of (reabsorbed) fluid following stimulation, between the cells and the support, particularly with the impearmeable plastic coverslips?

Authors: We did not do any morphological studies of cells *after stimulation*, so we cannot answer the question at present. However, we are grateful for the suggestion and will attempt to carry out such a study.

A.J. Spencer: Did passages 64-70 (the heterogeneous population) show a different response to cAMP than passages 58-63?
Authors: Qualitatively no; quantitatively yes: the earlier passages lost about 66% of their Cl after stimulation, the later passages only 52%.

H.Y. Elder: For SEM, why did you use the traditional route of chemical fixation followed by acetone dehydration, critical point drying (CPD) and gold coating? Would it not have been a better comparison with the specimens for microanalysis (less shrinkage than CPD) also to gold-coat some of the cryofixed and freeze-dried cells for SEM?
Authors: You are in principle correct. Our home-made freeze-drier was during this part of the study not performing well enough for morphological studies.

H.Y. Elder: You found ice crystal artefact by TEM in the plunge frozen, freeze-dried samples. Did this crystal damage occur during freezing or drying do you think? These flat cultures should lend themselves to slam freezing; have you tried this method on any samples?
Authors: The specimens are so thin that freezing by immersion should be good enough, but we have not investigated the origin of the ice crystal remnants. Since we analyze at the cell level, and the ice crystal damage is small in relation to the analyzed volume, the problem has no practical importance.

H.Y. Elder: Did you consider sectioning the cells as a way of avoiding some of the extraneous background problems and quantification difficulties you report? Zierold (1989) has described an elegantly simple arrangement for obtaining sections of cells grown as monolayers on flexible supports (Petriperm foil). Sections would also avoid potential problems of electrolyte washout during the ammonium acetate rinse and would allow use of an external standard solution.
Authors: The present paper is part of a larger study where the effect of various pharmacological agents on the cultured sweat gland cells is investigated. The large number of analyses necessary can be carried out much faster on whole-mount specimens as described here and we have found Formvar-covered titanium grids a good substrate for a variety of cell types (von Euler *et al.*, 1993). Nevertheless, the method used by Zierold (1989) is very useful, in particular if analysis at the subcellular level gives valuable information. Of course, sections have to be positioned on grids for analysis, so the problems of extraneous background would persist.

Additional Reference

Zierold K (1989) Cryotechniques for biological microanalysis. In: Microbeam Analysis 1989 (PE Russell, ed), San Francisco Press, San Francisco, pp. 109-111.