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IN VITRO SYSTEMS AND CULTURED CELLS AS SPECIMENS FOR X-RAY MICROANALYSIS

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Abstract

In vitro systems and cultured cells are recognized as useful systems in many areas of biomedical research, including X-ray microanalysis. To be reliable, in *an vitro* system should have an elemental composition close to that of the tissue *in situ*, react in the same way to stimuli, and retain the *in situ* regulation of ion transport. In the present paper, four of the most commonly used *in vitro* systems will be reviewed: incubated tissue slices (liver and pancreas), isolated glands (submandibular gland acini, sweat glands), primary cell cultures (sweat glands, endometrium), and cell lines (the colon cancer cell line T84, immortalized sweat gland cells). Incubation of tissue slices of liver in Krebs-Ringers buffer caused a significant increase in Na and Cl and a decrease in K. Initially, these changes were also observed in the pancreas, but here the values gradually returned to normal. Isolated submandibular gland acini, and isolated sweat gland ducts and coils react in a similar way to stimulation as their *in situ* counterparts. In primary cultures of coil cells, however, part of the cell population acquires different ion transport characteristics. Technically simplest is the use of cell lines originating from cancer cells (e.g., the T84 cell line) and immortalized cell lines. X-ray microanalysis not only confirms data on ion transport obtained with other techniques, but adds the possibility to investigate the presence of subpopulations within a culture.

Key Words: X-ray microanalysis, specimen preparation, *in vitro* systems, cell culture, diffusible ions, cryotechniques, chloride transport, epithelia.

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Introduction

The usefulness of *in vitro* systems and cultured cells has been recognized in many areas of biomedical research, and it is not surprising that this type of specimens is increasingly being used in X-ray microanalysis. In general, *in vitro* systems and cultured cells allow a wider range of experimental conditions to be applied to the system, in comparison to *in situ* experiments. It has also been argued (Roomans, 1991; Hongpaisan *et al.*, 1994; Hongpaisan and Roomans, 1995) that especially in X-ray microanalysis of tissue in human pathology, the use of *in vitro* systems and cell cultures would have advantages: since it is difficult to optimize the freezing conditions in a clinical setting, *in vitro* systems would make it possible to separate taking the biopsy from the freezing process, and to perform both in an optimal way.

In the present paper, we will review four of the most commonly used *in vitro* systems: incubated tissue slices, isolated glands, primary cell cultures, and cell lines. To be useful in X-ray microanalysis, the *in vitro* systems should ideally have an elemental composition similar to the *in situ* systems from which they are derived, and they should react in a similar way to physiological stimuli.

Incubated tissue slices are routinely used in many physiological and biochemical studies (e.g., brain slices, see Ballyk and Goh, 1992 and McIlwain, 1987) but have been rarely used in X-ray microanalytical studies. The tissue slices are incubated in an aerated physiological buffer (Krebs-Ringers buffer, artificial cerebrospinal fluid) at physiological temperature, and left for a period of minutes to hours under these conditions. It could be shown (Hongpaisan *et al.*, 1994; Hongpaisan and Roomans, 1995) that even a brief exposure to a physiological buffer introduced significant changes in the elemental composition of the cells. In some cases (pancreas, submandibular gland) the situation was stabilized by prolonged incubation, in other cases (brain tissue) the situation deteriorated during incubation with further increase of Na and Cl and decrease of K. Isolated glands are used in physiological studies, but

have so far not been used for X-ray microanalysis.

Primary cell cultures have been used in a number of studies (Wroblewski and Roomans, 1984): one of the first such studies used cultured vascular smooth muscle cells (James-Kracke *et al.*, 1980), but also epithelial cells, such as respiratory epithelium (Sagström *et al.*, 1992) and sweat gland epithelium (Mörk *et al.*, 1995), and endocrine cells such as thyroid follicular cells and endocrine pancreatic cells (Wróblewski and Wroblewski, 1994) have been used. Often it has been noted that the primary cultures have higher concentrations of Na and Cl, and lower concentrations of K than the corresponding *in situ* system (Wróblewski and Wroblewski, 1994) and the situation may deteriorate with increasing passage number (Sagström *et al.*, 1992).

Cell lines (fibroblasts, cancer cells) have been used on numerous occasions (reviewed, e.g., in Von Euler *et al.*, 1993, Hongpaisan *et al.*, 1994 and Warley, 1994). If the analysis is not carried out on sections (Zierold and Schäfer, 1988) but rather on entire cells, a washing step has to be introduced to remove the culture medium and the possibility that the washing procedure may result in changes in elemental concentration has been a cause of some concern (Wroblewski and Roomans, 1984; Abraham *et al.*, 1985; Borgmann *et al.*, 1994). For X-ray microanalysis, cells may be cultured on a variety of substrates (reviewed, e.g., by Warley, 1994) that may be classified into two groups: ultrathin or thick. In the former case, the specimens can be quantitatively analyzed as thin sections, in the latter case, there may be a problem due to the presence of the substrate, which may contribute to the spectrum. There is some advantage in culturing cells on an ultrathin substrate (Von Euler *et al.*, 1993) because a better signal can be obtained in the (scanning) transmission electron microscope with a higher accelerating voltage, and because of the more straightforward quantitative procedure, but not all cells can be cultured in this way and good results may also be obtained with cells grown on a thick substrate and analyzed in the scanning electron microscope, particularly if the accelerating voltage is adjusted to give optimal results (Borgmann *et al.*, 1994).

The experiments described in this paper were carried out on epithelial cells. Since epithelia form the body's boundary and therefore regulate and control the passage of substances (including ions and water) into and out of the organism, regulation of epithelial ion transport is an important biological problem. In this study, in particular the aspect of chloride transport in epithelial cells is considered. Most epithelial cells have, or are assumed to have, an apical chloride channel responsible for chloride efflux. In the duct of the sweat gland, the apical cell membrane has an inwardly-directed chloride channel whereas the basolateral membrane has an outwardly directed chloride channel. Together these channels are responsible for reabsorption of Cl^- from the primary sweat. Some chloride channels are regulated by Ca^{2+} ions, and are activated by substances that increase the intracellular Ca^{2+} level. Other chloride channels are activated by cAMP. In the genetic disease cystic fibrosis, the cAMP-activated channels are defective, whereas the Ca^{2+} activated channels appear to function in a normal way. In some epithelial cells, both types of Cl^- channel may occur, whereas in other epithelial cell types, either cAMP- or Ca^{2+}- activated Cl^- channels are present. Whether this regulation of chloride secretion is retained in different *in vitro* systems is one aspect to be considered in the present paper.

Materials and Methods

Incubated slices of liver and pancreas

For incubation experiments on liver and pancreas, Sprague-Dawley rats (8-9 weeks old, 190-220 g) were used. The animals were anesthetized with pentobarbital (45 mg/kg body weight). In some animals, the tissue was frozen *in situ* with liquid-nitrogen cooled brass clamps. In other animals the tissue was dissected to 1-1.5 mm thick slices and incubated in a Krebs-Ringers buffer (KRB), containing 140 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 5 mM HEPES (2-hydroxyethylpiperazine-2'-ethanesulfonic acid), and 1 mM $MgCl_2$ supplemented with 5 mM D-glucose at pH 7.4. In some experiments, NaCl was exchanged for sodium gluconate or potassium gluconate. The tissue was superfused with the incubation fluid at a rate of 2 ml/min for 2 h at 36 ± 1°C and oxygenated with 95% O_2 and 5% CO_2; samples were taken at half-hour intervals and frozen in liquid propane cooled by liquid nitrogen.

For X-ray microanalysis either 16-μm thick (Wróblewski *et al.*, 1978, 1987; McMillan and Roomans, 1990) or 4 μm-thick (Wroblewski *et al.*, 1983) cryosections were cut on a conventional cryostat, mounted on a carbon support (the thinner sections were mounted over a Formvar (Merck, Darmstadt, Germany) film covered hole in the support), freeze-dried, and coated with a thin carbon layer to prevent charging in the electron microscope. Sections were cut a few cell layers away from the dissected edge of the tissue block.

The 16 μm-thick cryosections were analyzed in a Philips 525 scanning electron microscope in the secondary electron mode at 20 kV with a LINK AN10000 (Oxford Instruments, Oxford, UK) energy-dispersive spectrometer system. The 4 μm-thick sections were analyzed in a JEOL 1200EX TEMSCAN in the scanning transmission mode at 100 kV with a Tracor 5500 energy-dispersive spectrometer system. All analyses were carried out with a stationary beam (probe size 100

nm). Quantitative analysis was carried out based on the ratio of characteristic counts to background intensity in the same energy region (P/B-ratio) (Roomans, 1988; Von Euler *et al.*, 1992). P/B-ratios obtained on the samples were compared with those obtained on standards consisting of a gelatin/glycerol matrix containing mineral salts in known concentrations (Roomans, 1988). Data from 4 μm-thick sections were not significantly different from those obtained on 16 μm-thick sections and the results of these two specimen types were pooled.

Isolated submandibular gland acini

Sprague-Dawley rats (male, 200 g, about 5 weeks old) were used for these experiments. Animals had access to food and water *ad libitum* until the moment of the experiment. The animals were anesthetized with pentobarbital sodium (30 mg/kg body weight). The submandibular glands were dissected free, removed and placed in a small volume of Dulbecco's Modified Eagle Medium (DMEM; Gibco, Paisley, UK), which had previously been aerated with 95%O_2/5%CO_2 for 30 minutes at 37°C; the pH of this medium was adjusted to 7.4 with 1M NaOH before use. The submandibular glands were then chopped into about 1 mm^3 pieces, and digested in DMEM containing 1000U/15ml collagenase (Worthington Biochemical Corporation, Freehold, New Jersey, USA) and 11mg/15ml hyaluronidase (Sigma, St. Louis, Missouri, USA). The pH of the digestion medium was adjusted to 7.4 at 0, 5, 15, 30, 40 and 50 minutes. To promote dissociation of gland fragments, the digestion medium with the fragments was sucked through a glass pipette with a broken tip at 30 and 40 minutes, and through a 10 ml plastic pipette at 50 minutes. After digestion, the suspension was filtered through a nylon mesh and the fragments were washed 3 times with DMEM containing 2% albumin. The fragments were then allowed to sediment for 4-5 minutes, the supernatant was removed, and finally the fragments were resuspended in DMEM containing 2% albumin. All the steps of isolation were performed under 95%O_2/5%CO_2 at 37°C. To check the effect of the various steps of the isolation procedure, gland fragments were frozen by slam-freezing against a liquid-nitrogen cooled, gold-coated copper block (CF100, Life Cell, Houston, TX) after collagenase digestion for 15 and 50 minutes, as well as after 50 minute digestion followed by disaggregation. As controls, glands were dissected and frozen without any treatment. The frozen samples were transferred into liquid nitrogen in which they were kept until cryosectioning. Ultrathin cryosections (0.2 μm or less) were cut with an LKB (Uppsala, Sweden) Cryo Nova cryoultramicrotome at a specimen temperature of -140°C and a knife temperature of -135°C. The sections were collected dry onto copper grids and sandwiched between two grids. The grids had been covered with a Formvar film and then coated with a thin carbon layer before they were used to carry sections. The sections were freeze-dried at -80°C and 10^{-5} torr in an external freeze-drier. X-ray microanalysis was performed at 100 kV in the transmission (TEM) mode of a Philips 400 electron microscope (spot size about 0.2 μm) with a LINK QX200 energy-dispersive X-ray microanalysis system. Quantitative analysis was carried out based on the peak-to-continuum ratio after correction for extraneous background (Roomans, 1988) and by comparing the spectra from the cells with those from a standard. Only one spectrum was acquired from each cell. Spectra were acquired for 100 seconds.

Primary cultures of sweat glands

The coil or duct part, respectively, was cultured in 25 cm^2 tissue culture flasks (Costar, Cambridge, MA) containing 800 μl of the culture medium (1% fetal calf serum added to the pre-culture medium). When cellular outgrowth was seen, several ml of the culture medium was added. After 10-14 days the cells were incubated with 1 ml dispase (Boehringer Mannheim, Germany) for 30-45 minutes. Sheets of cells detached from the floor of the culture flask were allowed to recover in culture medium. For X-ray microanalysis the cells were seeded out on 75 mesh titanium grids (Agar Scientific, Stansted, UK). The grids had been covered with a Formvar (Merck) film coated with a thin carbon layer. The grids were sterilized under ultra-violet light before use. The cells were allowed to attach and spread for 3-7 days at 37 °C in a humidified atmosphere of 5% CO_2/95% air in a culturing chamber. In stimulation experiments, the cells were exposed to an agonist (as stated in the Results section). As controls, unstimulated cells, or cells exposed to buffer only were used; there was no significant difference between these types of controls. The incubation was stopped by a 4 second rinse in 0.15 M ammonium acetate to remove the salt-rich Krebs-Ringer solution, which would otherwise disturb the analysis. After the rinsing, the grids were blotted on filter paper and frozen in liquid propane cooled by liquid nitrogen and allowed to freeze dry at -80 °C in vacuum overnight. The freeze-dried grids were coated with a conductive carbon layer before analysis. X-ray microanalysis was performed at 100 kV in the TEM mode of a Philips 400 electron microscope as described above.

Primary cultures of endometrium

Female NMRI (Naval Medical Research Institute) mice (4-5 weeks old), obtained from B&K, Sollentuna, Sweden, were used for this part of the study. The animals were kept in separate cages until defecation habits became normal, which usually took 1 week.

Primary cultures of epithelial cells were isolated and cultured using a method modified from Glasser *et al.* (1988). Briefly, the uterine horns were rapidly removed, cut lengthwise, and placed (10 uterine horns from 5 mice) in 4 ml sterile Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS -Ca^{2+} -Mg^{2+}) (Gibco) at room temperature. They were transferred to a 35 mm Petri-dish with 2 ml sterile enzyme solution containing 0.5% trypsin and 2.5% pancreatin which were prepared in HBSS (-Ca^{2+} -Mg^{2+}) and incubated for 60 min at 4°C and for 60 min at room temperature. The enzyme solution containing part of uterine epithelial cells was collected, centrifuged, and the supernatant was discarded. The uterine horns were washed using 3 ml HBSS on a vortex mixer. The washing solution was collected and used to suspend the epithelial cell pellet. The washing was repeated once. The epithelial pellet was washed again 2 more times using HBSS by centrifugation at 1200 rpm. The pellet was finally resuspended in 5 ml normal culture medium by gentle aspiration. The cell plaques were allowed to settle by gravity (10-15 min). The supernatant, approximately 4.5 ml, was discarded, and the remaining cells were resuspended in the culture medium (50 μl medium per uterine horn). A drop of the cell suspension (40-50 μl) was seeded out on a titanium grid as described above. The cells were allowed to attach and spread in a humidified chamber overnight at 37°C in an atmosphere of 5% CO_2/95% air. Then 2.5 ml culture medium was added to each culture dish. The medium was changed every 2 days and the cells were cultured for 6 days. The cells were washed in Krebs-Ringer buffer (KRB), pH 7.4. Experiments were carried out by exposing cells to stimulants or inhibitors of ion transport dissolved in KRB, and the incubation was terminated by a quick rinse in KRB solution followed by a quick rinse in the distilled water in order to remove the salts from the KRB solution. The grids were then blotted dry on a filter paper. The whole rinsing procedure did not take more than 10 seconds. The grids with the cells were frozen in liquid nitrogen and freeze-dried at -80°C at 10^{-5} torr overnight. For X-ray microanalysis, the specimens were viewed in the STEM mode of a Hitachi 7100 electron microscope and analyzed at an accelerating voltage of 100 kV with an Oxford Instruments (Oxford, UK) ISIS X-ray microanalysis system. Quantitative analysis was carried out as described above.

Immortalized sweat gland cell lines.

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) and sodium selenite (10 ng/ml). Cells grown on plastic were trypsinized with 0.05% trypsin in 0.02% ethylenediaminetetraacetic acid (EDTA) according to standard methods. As soon as the cells disengaged from the culture dish they were flushed with culture medium containing 10% fetal calf serum (FCS), collected in a sterile centrifugation tube and pelleted at low speed. The cell pellet was gently agitated in fresh medium and for X-ray microanalysis, the cells were then reseeded on titanium grids as described above for the primary cultures. 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 (Mörk and Roomans 1993; Von Euler *et al.* 1993). Stimulation experiments were carried out as described above, the cell cultures were rinsed with 0.15 M ammonium acetate or with distilled water (there was no difference between these methods) and X-ray microanalysis was carried out in the TEM mode at 100 kV as described above.

In some experiments, cells were also cultured on other substrates (Mörk and Roomans, 1993), namely, glass or plastic coverslips, or Transwell inserts (Costar). These cells were after freezing and freeze-drying analyzed in a Philips 525 scanning electron microscope at 20 kV, using a LINK AN 10000 energy-dispersive spectrometer system. Semi-quantitative analysis was carried out (because the analytical volume presumably included the substrate) by normalizing the values for the elemental concentrations to phosphorus and/or sulfur.

Tumor cell lines

The colonic tumor cell line T84 was cultured as described previously (von Euler and Roomans, 1992), and for X-ray microanalysis, the T84 cells were grown on Formvar-covered titanium grids and analyzed as described above.

Statistics

Comparisons between two experimental groups were carried out by Student's t-test; for comparisons between more groups, analysis of variance (ANOVA) was used, followed by Student's t-test.

Results

Incubated slices of liver and pancreas

Rapid dissection of the tissue did not cause marked changes in the elemental composition of the hepatocytes or the pancreatic acinar cells, but even a brief incubation in KRB caused an increase in Na and Cl and a decrease in K (Fig. 1). For the pancreas, incubation for 2h in standard KRB resulted in a decrease of Na and an increase of K, so that after 2h incubation the elemental composition was more close to the *in situ* values. For

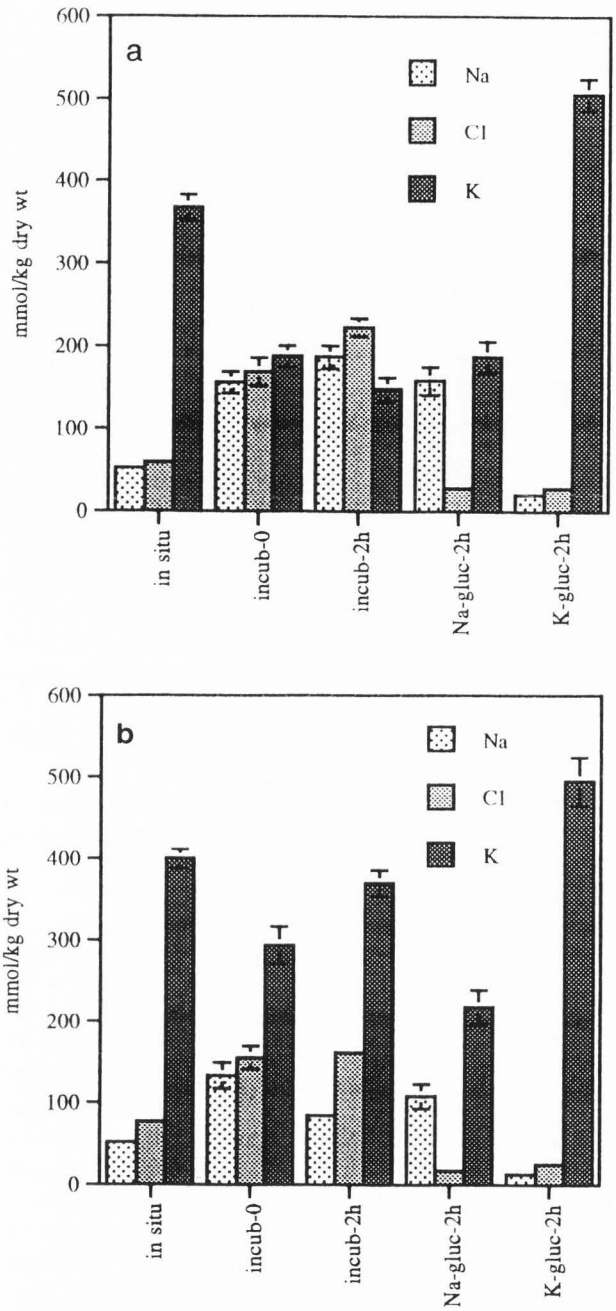

Figure 1: Effect of incubation (at 36°C) of (a) rat liver and (b) rat pancreas in standard KRB, compared to buffer in which the NaCl was replaced by Na gluconate or K gluconate, respectively. The following data are given: *in situ* concentrations, incubation for a few seconds in standard KRB (incubation-0), incubation for 2h in standard KRB (incubation-2h), incubation for 2h in Na-gluconate buffer (Na-gluconate) and incubation for 2h in K-gluconate buffer (K-gluconate). Data mainly from Hongpaisan and Roomans (1995).

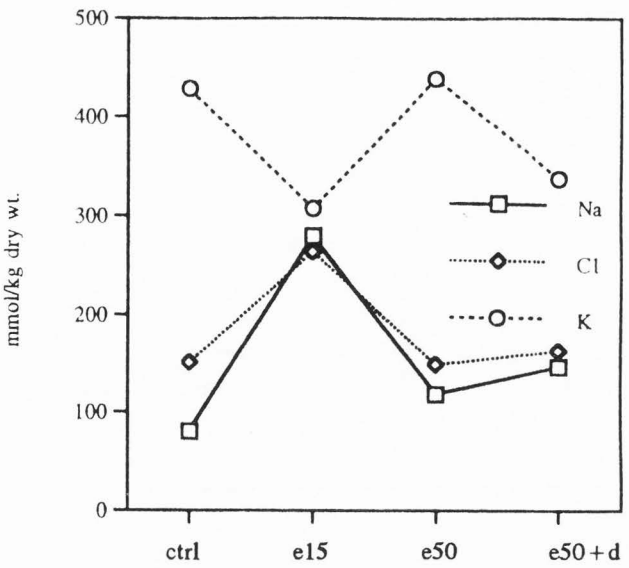

Figure 2: Concentrations of Na, Cl and K in the nucleus of submandibular gland acinar cells, determined on ultrathin cryosections, under the following conditions: *in situ* (ctrl), after 15 min collagenase treatment (e15), after 50 min collagenase treatment (e50), and after collagenase treatment and disaggregation (e50+d). Mean and standard error, data in mmol/kg dry weight.

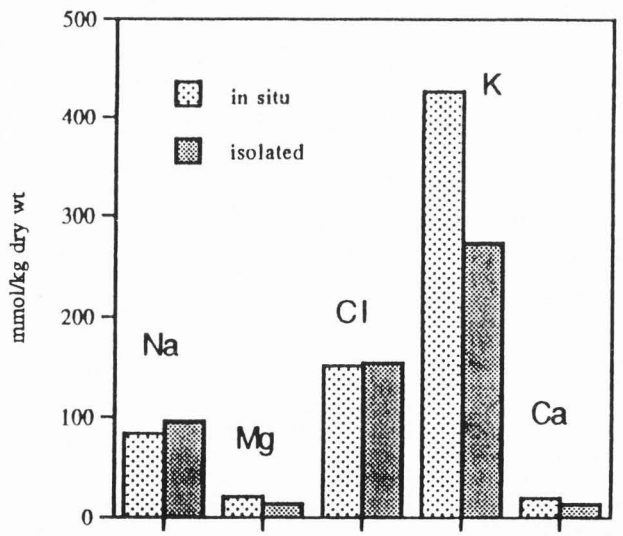

Figure 3: Nuclear elemental concentrations in a typical preparation of isolated submandibular gland acinar cells compared to data obtained from the gland *in situ*. Only for K, the isolated cells differ significantly from the *in situ* concentrations. Data in mmol/kg dry weight.

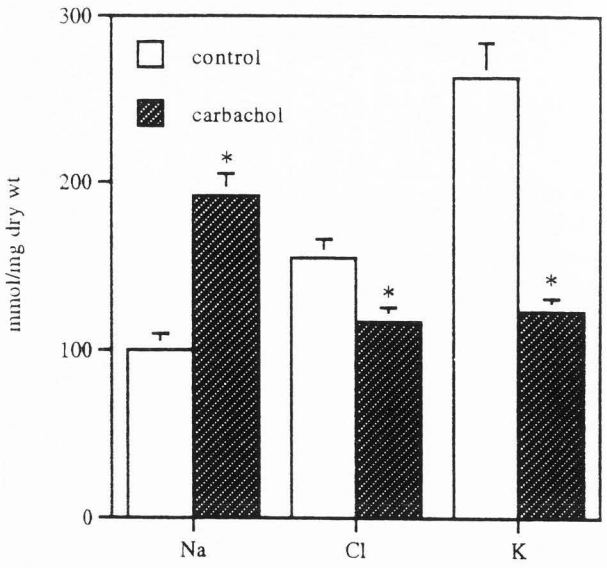

Figure 4. Effect of carbachol stimulation on the elemental concentrations in the nucleus of isolated submandibular gland acini. Data in mmol/kg dry weight, mean and standard error (number of measurements: 26 in each group), significance of differences indicated by an asterisk (*). Differences were considered significant for $p < 0.05$.

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liver, on the other hand, a further decrease of K and increase in Na and Cl was noted. Exchanging NaCl in the KRB for Na gluconate had only minor effects on the intracellular Na concentration. The exchange of chloride for gluconate resulted, regardless of the accompanying cation, in a decrease of the cellular Cl concentration. When NaCl was replaced by K gluconate, the intracellular K concentration increased, and intracellular Na decreased (Fig. 1). Marked oedema could be observed not only in tissues incubated in KRB, but also in the tissue incubated for 2 hours in Na gluconate or K gluconate (Hongpaisan and Roomans, 1995).

Isolated submandibular glands

While incubation with collagenase gave rise to a marked increase of the Na/K ratio of the acinar cells in comparison to the *in situ* situation, the value improved after prolonged incubation and even after the disaggregation step (Fig. 2). It was well possible to obtain an acinar preparation with elemental concentrations quite close to the in situ values (Fig. 3). Carbachol caused Cl^- and K^+ efflux and an increase in the intracellular Na^+ concentration (Fig. 4).

Isolated sweat glands

In one series of experiments, the effect of the isolation procedure on the elemental composition of sweat gland cells was investigated. Dissected sweat glands showed a Na/K ratio of about 2, which was much higher than literature data on *in situ* coils and ducts, which show Na/K ratios in the range of 0.2-0.3 (Wilson *et al.*, 1988). When the glands were incubated, the Na/K ratio increased additionally, to about 4. Addition of collagenase to the incubation medium had no significant effect on the Na/K ratio. After the glands had been incubated with collagenase, they could be separated into coil parts and duct parts. At this stage of the procedure, the sweat gland coils had a Na/K ratio of about 8, whereas the ductal part had an even higher Na/K ratio, about 16 (Fig. 5).

If at this stage, isolated ducts were stimulated with cAMP, a significant decrease of the intracellular Cl^- concentration was observed. Most of this decrease could be inhibited by the chloride channel blocker NPPB (5-nitro-2-(3-phenylpropylamino)-benzoicacid). Carbachol, on the other hand, did not cause a significant loss of Cl^- from duct cells. If, on the contrary, isolated coils were stimulated by cAMP, no significant effect on the intracellular Cl was noted. Carbachol, however, caused Cl^- efflux, which to a large part could be inhibited by NPPB (Fig. 6).

While coil cells could be routinely cultured, it proved to be very difficult to obtain primary cultures of the duct part.

Primary cultures of sweat gland cells

Stimulation with carbachol of cultured coil cells resulted in a significant decrease (about 60%) in the cellular content of Cl and a slight decrease in the cellular content of K and Na. This chloride efflux could be blocked by NPPB (Fig. 7). Also acetylcholine and the calcium ionophore A23187 stimulated chloride secretion (Mörk *et al.*, 1995). NPPB inhibited acetylcholine-induced chloride secretion. Stimulation of cultured coil cells with 5 mM cAMP caused a significant decrease in the cellular content of Cl and K. The decrease in Cl after stimulation with cAMP (about 30%) was, however, much smaller than after stimulation with carbachol (Fig. 7). In the control cells, the frequency distribution of the cellular Cl concentration displayed a bell-shape with a median concentration close to the mean concentration of Cl in the cultured cells. However, after stimulation with cAMP the frequency distribution of Cl showed two peaks, one with a maximum at the control value, and the other with a maximum at about 40-50% of the control value (Fig. 8). Hence, it appears, that only part of the cells secrete Cl^-. NPPB (but not another chloride channel blocker, diphenylamine-2-carboxylate (DPC)) inhibited cAMP-induced Cl^- secretion to a small extent.

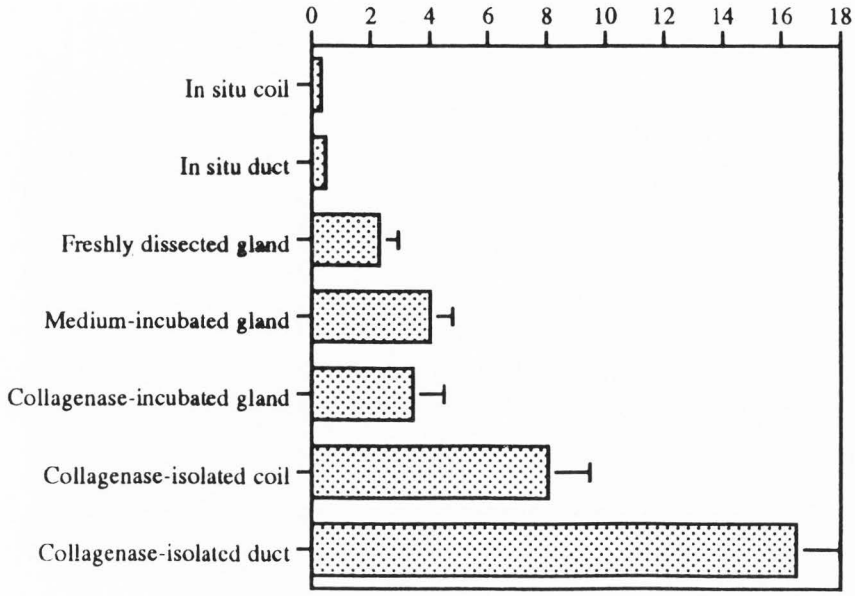

Figure 5. The Na/K ratio in the cytoplasm of cells in the human sweat gland. The data for *in situ* coil and duct were taken from Wilson *et al.* (1988). Data were obtained from freshly dissected glands (several hours after surgery), glands incubated in buffer, glands during collagenase treatment (no distinction made between coils and ducts in these cases), and isolated coils and ducts after collagenase treatment.

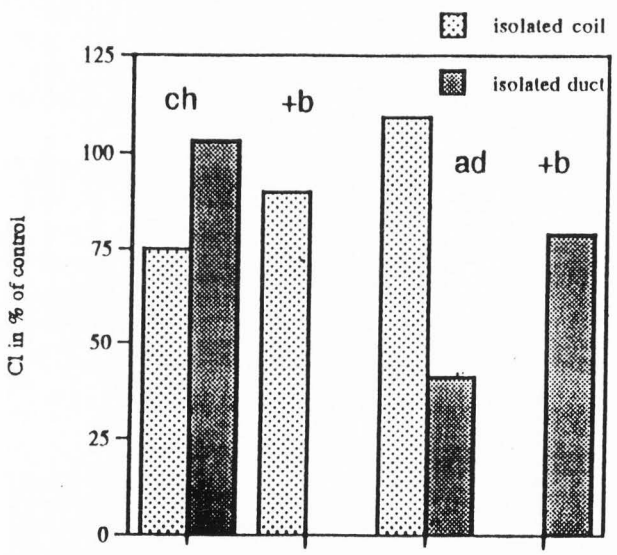

Figure 6. Intracellular Cl concentrations in sweat gland cells (data in % of unstimulated glands). Cholinergic stimulation (ch) causes a 25% loss of Cl from isolated coil cells, but no loss from isolated duct cells. In the isolated coil cells, the Cl loss can be reduced in the presence of the chloride channel blocker (+b) NPPB. β-Adrenergic stimulation (ad) causes no loss of Cl from isolated coil cells, but a substantial loss from the isolated duct cells. The Cl loss from the duct can be reduced by NPPB (+b).

Primary cultures of endometrium

The epithelial cells of the uterus had grown into continuous epithelium monolayer after 5 days in culture. Transmission electron microscopy showed that the cells had formed both apical microvilli and tight junctions (Fig. 9). Stimulation of the cells with cAMP caused a highly significant decrease of intracellular Cl and K and an increase in Na. Ouabain (10^{-4} M) caused an increase in Na and Cl and a decrease in intracellular K (Fig. 10).

Immortalized sweat gland cell lines

Stimulation of NCL-SG3 with cAMP caused a significant net Cl^- and K^+ secretion from the cells (Fig. 11). cAMP had no significant effect on the intracellular Na and Ca concentrations, although a tendency to increased Na levels could be observed. Cl^- secretion was blocked by the chloride channel blockers NPPB (Fig. 11), DPC, and 9-AC (Mörk *et al.*, 1996a). Administration of these chloride channel blockers (without cAMP) or of dimethylsulfoxide (DMSO) or ethanol in the concentration needed to dissolve the chloride channel blockers did not cause significant changes in ion content of the cells. The effect of cAMP was independent on the type of substrate used to culture the cells (Mörk and Roomans, 1993). Also carbachol caused a decrease of the intracellular Cl content, indicative of Cl^- efflux (Fig. 11). However, this was only the case for cells grown on permeable substrates (Formvar-film covered grids or Transwell inserts) but not for cells grown on impermeable substrate (glass or plastic cover slips) (Ring *et al.*, 1995).

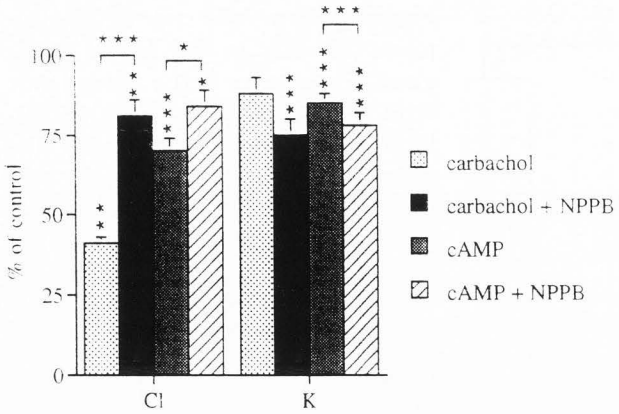

Figure 7. Primary cultures of human sweat gland coil cells (Mörk *et al.*, 1995). Carbachol stimulation results in a loss of about 60% of the intracellular Cl, and this loss can be greatly reduced by NPPB. cAMP causes only about 30% loss of Cl, also this loss can be reduced by NPPB. The significance of differences is given by asterisks (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). Asterisks directly over the bar refer to differences between treatment and control, asterisks over brackets refer to differences between stimulated cells and cells stimulated in the presence of NPPB. The data are based on 3-5 experiments with 10-15 cells analyzed in each experiment; mean and standard error are given.

Tumor cell lines

The cell line T84 is derived from an adenocarcinoma. Both cAMP and ionomycin (which increases the intracellular Ca^{2+} concentration) cause Cl^- and K^+ efflux from T84 cells (Fig. 12).

Discussion

Many of the types of experiments described in this study could not, or only with great difficulty, be carried out in humans or animals *in situ*. The difficulties include controlling the concentration of the drug *in situ*, getting the drug to the correct location, systemic effects, and possible risks for the volunteers. As alternatives to *in situ* studies it can be attempted to use a variety of *in vitro* systems: slices, isolated glands, primary cultures, and cell lines.

The advantage of the tissue slice system is that it is technically simple. The data on the pancreas slices show that this system performs relatively well: although there are some initial changes in elemental concentration (increased Na and Cl, decreased K), the concentrations stabilize during the incubation in KRB. Similar results were obtained for the submandibular gland (Hongpaisan and Roomans, 1995). In that study it was also shown

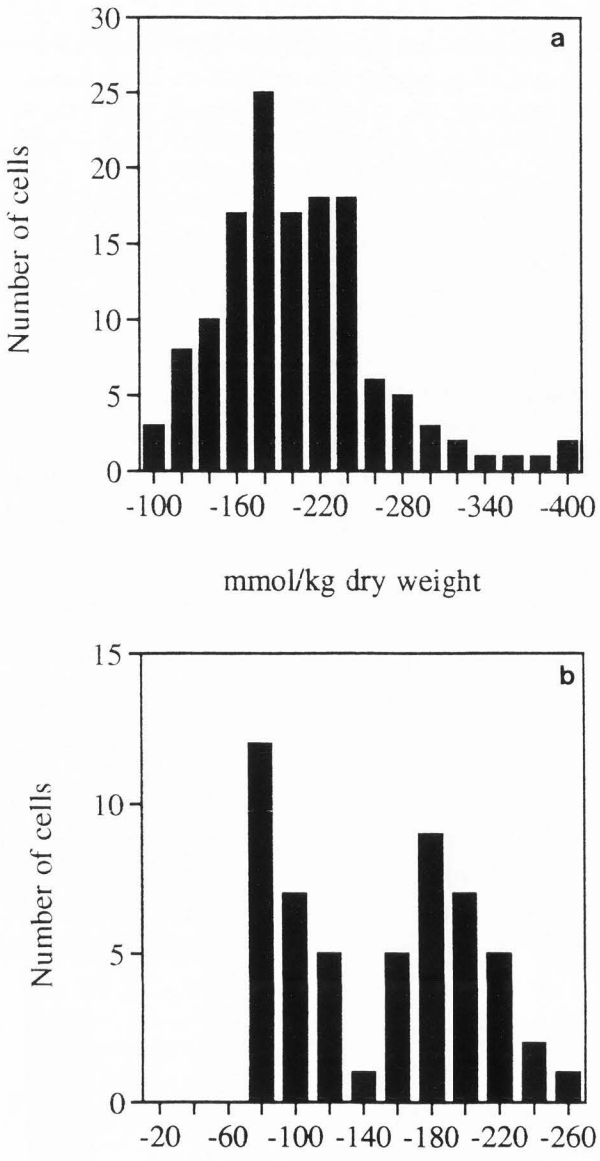

Figure 8. Primary cultures of human sweat gland coil cells (Mörk *et al.*, 1995). Frequency distribution of the Cl concentration in unstimulated cells (a) and in the cAMP-stimulated cells (b). About half of the cells lose about 60% of their Cl whereas the remaining half does not appear to lose any Cl. This results in an about 30% loss of Cl for the population as a whole as seen in Figure 7.

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that the acinar cells in pancreas and submandibular gland react in the same way to cholinergic stimulation as the cells *in situ*. However, the liver (in common with brain tissue, Hongpaisan and Roomans, 1995) shows more extensive changes in elemental composition when slices

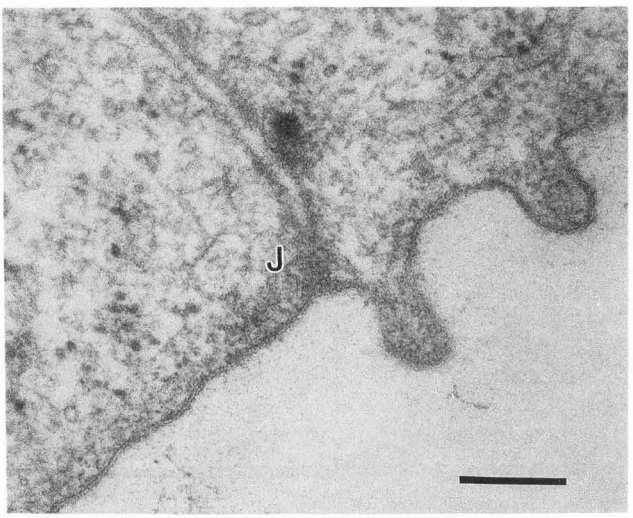

Figure 9. TEM micrograph of a primary culture of uterine epithelial cells, showing a junctional complex between cells (J). Bar is 1 μm.

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are incubated in KRB buffer. The disadvantage of slices is that diffusion of oxygen and nutrients is limited, resulting in decreased activity of ATPases such as the Na^+-K^+-ATPase and the Ca^{2+}-ATPase. The resulting elemental changes are typical for this kind of damage.

It is of interest that the elemental content of the cells is preserved best when the slices are stored in a medium with a K/Na ratio closer to the intracellular ratio (instead of in a solution resembling the extracellular environment). This method has been used in some biochemical studies (Elliott, 1969) and is, e.g., used in the storage of tissue for transplantation (Wahlberg *et al.*, 1986). Similarly, Sjöstrand (1992) has suggested that it should be possible to reduce the so-called "infarct-reflux" damage to heart tissue by perfusion with K-rich instead of Na-rich fluids. Exchanging NaCl for K gluconate results in a high intracellular K/Na ratio; it is not unlikely that K^+ ions diffuse into the cell from the extracellular medium. On the other hand, chloride ions slowly diffuse out of the cells into the gluconate medium. Morphological studies show, however, that despite the "normal" intracellular K/Na ratio, significant oedema occurs even in K gluconate medium (Hongpaisan and Roomans, 1995), and it is likely that the cell membrane, under the conditions of the experiment, is not in full control of the ion fluxes.

Also during isolation of acini from the rat submandibular gland and ducts or secretory coils, respectively, from the human sweat glands, marked ionic changes take place, especially in the sweat glands. With regard to the submandibular gland acini, it appears possible to

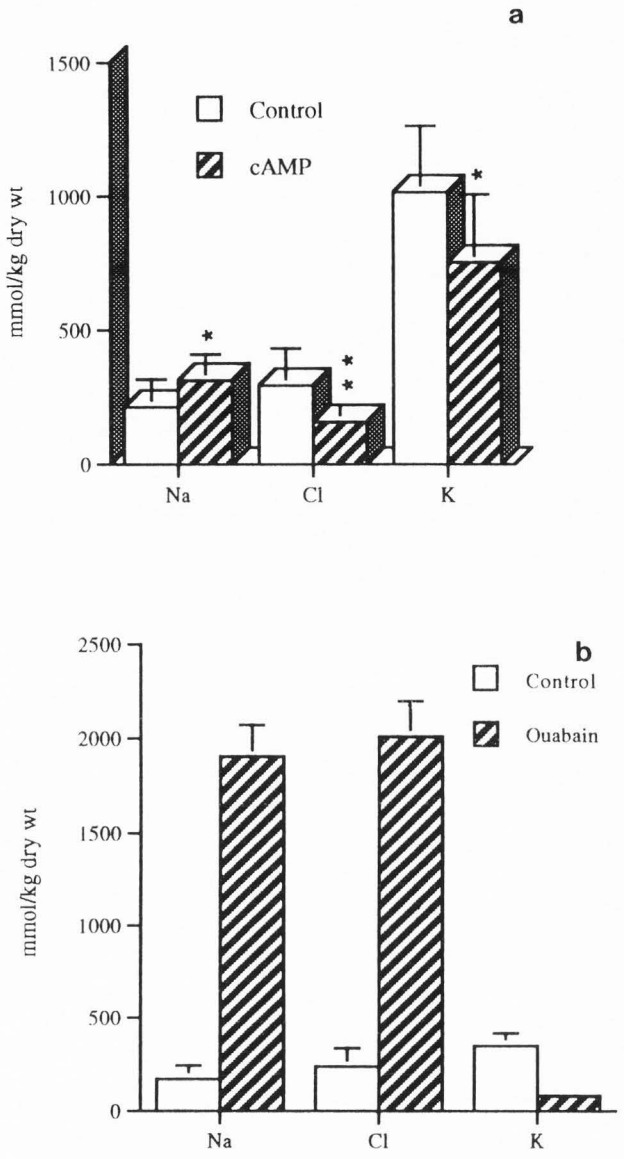

Figure 10. (a) Effect of cAMP (5 mM, 5 min), (b) effect of ouabain (50 μM, 5 min) on the elemental composition of cultured uterine epithelial cells; mean $\pm$ standard deviation (n=25), data in mmol/kg dry weight. The data are based on 25 spectra per treatment. Significant differences are indicated by * ($p<0.01$), ** ($p<0.05$) and *** ($p<0.001$).

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have a cell preparation that in its ionic content resembles the *in situ* elemental content, and reacts in a similar way to a cholinergic stimulus, even though a transient increase of the Na and Cl concentration takes place during the isolation procedure. It is likely that this preparation can be relatively easily used for *in vitro* experiments. With regard to the human sweat glands, the situation is much more difficult. The increase in Na and Cl, and the decrease in K are very marked in the coil

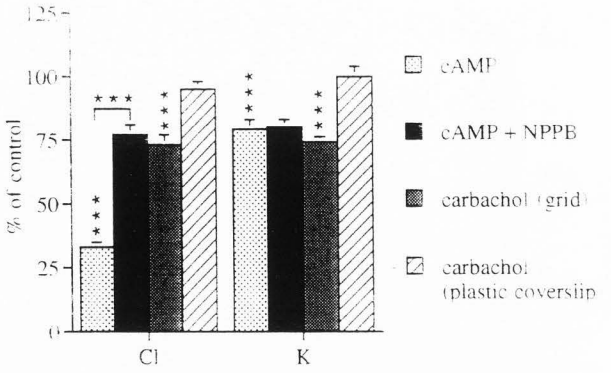

Figure 11. Immortalized human sweat gland cell line NCL-SG3 (Mörk *et al.*, 1996a; Ring *et al.*, 1995). Stimulation with cAMP results in a considerable loss of Cl (data in % of unstimulated cells). This loss can be reduced by the chloride channel blocker NPPB. Stimulation by cAMP also leads to a loss of K. On cells cultured on Formvar-film covered titanium grids, carbachol induced loss of Cl and K, but this was not the case in cells cultured on an impermeable substrate, such as a plastic coverslip. The significance of differences is given by asterisks (*, $p<0.05$; **, $p<0.01$; *, $p<0.001$). Asterisks directly over the bar refer to differences between treatment and control, asterisks over brackets refer to differences between stimulated cells and cells stimulated in the presence of NPPB. The data are based on 4-6 separate experiments, with a total of 20-80 cells for each treatment. Mean and standard error are given.

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and even more pronounced in the duct. It appears that the changes in the duct are mostly non-reversible, and this may be the explanation for the fact that it was difficult to prepare primary cultures of duct cells. The coil cells are evidently damaged to a lesser extent, and coil cells can be more easily cultured.

The sweat gland can be functionally divided into a secretory coil, where the primary sweat is produced under cholinergic control with Ca^{2+} as a second messenger, and a duct where Cl^- and Na^+ are reabsorbed under β-adrenergic control with cAMP as a second messenger. It is of interest that the specificity of activation of chloride efflux in the different parts of the sweat gland is retained during the isolation, but that it is lost in the primary cultures of sweat gland cells, where both cAMP- and Ca^{2+} activated chloride channels are present, at least in part of the cells. Analysis of a cell population by X-ray microanalysis provides a unique opportunity to demonstrate a heterogeneity in the cell population (Von Euler *et al.*, 1993). In this particular experiment it can be seen that about half of the cells does not react to stimulation with cAMP, but that the other half of the cells reacts and loses about the same relative amount of

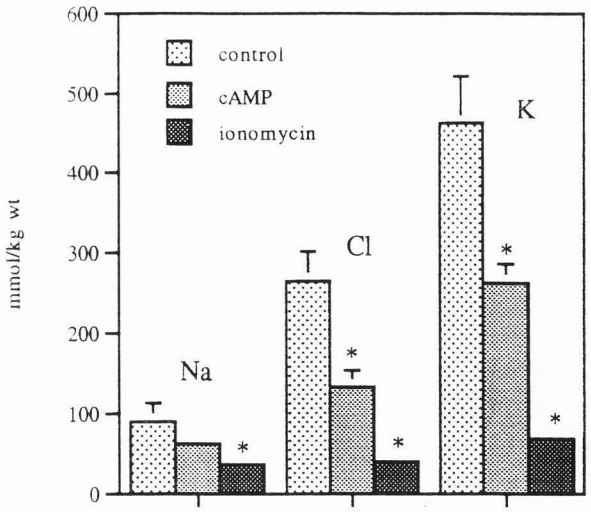

Figure 12. Effect of stimulation with cAMP and ionomycin on cultured T84 cells. Data in mmol/kg dry weight, mean and standard error (number of measurements 35 in each group, two separate experiments), significance of differences indicated by an asterisk (*). Differences were considered significant for $p<0.05$.

Cl compared to cholinergic stimulation. Also in the immortalized NCL-SG3 sweat gland cell line (Lee and Dessi, 1989) which was thought to originate from duct cells, both cAMP- and Ca^{2+}-activated chloride channels have been demonstrated (Mörk *et al.*, 1996a; Ring *et al.*, 1995). Using the patch-clamp technique, Mörk *et al.* (1996a) could show that increasing the cellular Ca^{2+} concentration by calcium ionophores activated chloride channels, but that carbachol had no effect. This was in apparent contradiction with the data obtained by X-ray microanalysis. Patch-clamp experiments are, however, carried out with cells cultured on an impermeable substrate. When the X-ray microanalysis experiments were repeated on cells cultured on some types of impermeable substrate, no effect of carbachol could be seen, whereas cells cultured on permeable substrates consistently showed activation of Cl^- efflux by carbachol. These results presumably indicate that Ca^{2+}-activated chloride channels are indeed present in NCL-SG3 cells (despite the fact that these cells are of ductal origin), but that the cholinergic receptor is on the side of the cells touching the substrate and cannot be reached by cholinergic agonists when the cells are grown on impermeable substrates. When cells are grown on permeable substrates, the receptors are more accessible, and the cells can be stimulated by cholinergic agonists to secrete chloride. cAMP-activation of Cl^- efflux can, however, be obtained both in cells grown on permeable and in cells grown on impermeable substrates (Mörk and

Roomans, 1993). In principle, it is not uncommon for epithelial cells to have mechanisms for both a Ca^{2+}-activated and a cAMP-activated chloride secretion, as is shown in the experiments on T84 cells where both cAMP and ionomycin (presumably via an increase in intracellular free Ca^{2+}) cause chloride efflux. *In situ*, in general, one of the mechanisms appears to predominate, e.g., in the colonic epithelium, cAMP-induced chloride efflux predominates. Why this is not exactly the same in the cultured counterparts is a very interesting question.

Relatively little is known about ion transport mechanisms in the uterine epithelium. Matthews *et al.* (1993) have proposed that Na^+ absorption by this epithelium plays an important role in the regulation of the composition of the uterine fluid. It is well known that female patients with cystic fibrosis have a decreased pregnancy rate, which is suspected to be associated with altered quality of the cervical mucus and altered ion concentrations in the uterine fluid (Brugman and Taussig, 1984). Since it is known that the basic defect in cystic fibrosis is a defective cAMP-regulated chloride channel, it is not unreasonable to suspect that the cells of the uterine epithelium possess a cAMP-activated chloride channel. The results of the X-ray microanalysis bear this out.

Acknowledgements

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Discussion with Reviewers

K. Izutsu: With regard to the sweat gland duct, it is stated that there is an inwardly directed Cl^- channel in the apical membrane, and an outwardly directed Cl^- channel in the basolateral membrane, and that together these channels effect Cl^- resorption from the primary sweat. If the composition of the primary sweat is isotonic, how can passive forces cause an influx at one surface, and an efflux at the other? Is the membrane potential different at the two surfaces?

Authors: The accepted model for NaCl uptake from the sweat gland duct (Quinton, 1990) 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. 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, and Cl^- moves passively across both the apical and the basolateral membrane down electrochemical gradients. The chloride channel CFTR (cystic fibrosis transmembrane regulator) has been shown to be present in both apical and basolateral membranes of sweat duct cells (Cohn *et al.*, 1991; Denning *et al.*, 1992).

H.Y. Elder: I do not agree that your results obtained with the primary culture of sweat gland coil cells indicate specificity being lost during culture. In the secretory coil of human sweat glands cholinergic and adrenergic α and β pathways are well known to exist (Sato, 1977; Bovell *et al.*, 1989).

Authors: Indeed, we accept the evidence that coil cells are sensitive to β-adrenergic activation, but the references you quote show that the role of β-adrenergic activation is only minor. The effect was not sufficient to be seen in our experiments on isolated coils. In the experiment on cultured cells, the effects of the Ca^{2+}-induced (cholinergic) and the cAMP-induced (β-adrenergic) pathway appeared to be in the same order of magnitude. Possibly we should qualify our statement to say that we do not mean absolute specificity but rather the clear predominance of one system over the other.

K. Izutsu: The KRB contained no bicarbonate, yet it was oxygenated with 95% O_2 and 5% CO_2. Was the pH of this solution stable over a long time?

Authors: In the incubation experiments, the tissue slices were superfused with KRB at a rate of 2 ml/min, to mimic circulation that would provide oxygen and nutrients and clear the waste products produced by the cells. Moreover, the KRB used in this study contained HEPES (Good *et al.*, 1966), which is an effective hydrogen buffer. The pH was constant over the incubation time.

K. Izutsu: Incubation was stopped by a 4 sec rinse in ammonium acetate or in distilled water. Were the sweat glands sensitive to hypotonic conditions? Many cell types would undergo regulated volume changes under these conditions or would increase their cytosolic Ca^{2+} concentrations. Was there any evidence for any of these changes, or perhaps morphological findings of cell swelling?

K. Zierold: Have you found changes of intracellular dry

Table 1. Elements in nucleus and cytoplasm in submandibular gland acini *in situ* and at the end of the isolation procedure.

	gland *in situ*		isolated acini	
	nucleus	cytoplasm	nucleus	cytoplasm
Na	80 ± 7	74 ± 6	106 ± 7	72 ± 6 ***
P	603 ± 33	418 ± 27 ***	433 ± 14	253 ± 14 ***
Cl	151 ± 7	150 ± 7	126 ± 7	107 ± 8
K	427 ± 27	354 ± 19 *	252 ± 10	216 ± 12 *

Data given as mean and standard error, about 40 measurements in each group. Significant differences between nucleus and cytoplasm are indicated by * ($p<0.05$) and *** ($p<0.001$), respectively.

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mass or water portion depending on experimental conditions?

Authors: We realize that rinsing the cells with ammonium acetate or distilled water can induce artefacts. The ammonium acetate is (about) isotonic and no volume changes would therefore be expected, but the possibility of pH changes cannot be excluded. On the other hand, washing with distilled water could induce osmotic effects. For the sweat gland cell line, we determined that there were no significant differences between the washing fluids, and the same was previously shown for fibroblasts (Roomans, 1991; text reference). It should be realized though, that we measure freeze-dried cells: any (transient) changes in water content of the cells during rinsing (or as a result of other experimental conditions) will not be apparent after freeze-drying. Damage such as bursting of the cells was not observed. No significant changes in elemental composition after rinsing with ammonium acetate or distilled water, respectively, were observed. It should also be taken into consideration that controls and experimentally treated cells were rinsed in the same way, and that minor artefacts caused by the rinsing procedure therefore would cancel out.

H.Y. Elder: Some of the cryofixation methods selected would seem to be well short of the best that could have been applied, e.g., the brass clamps used to freeze the tissue slices, or the endometrial primary cultures frozen by plunging into liquid nitrogen. Why were these methods selected in preference to proven better methods? Did you examine the ice crystal artefact sizes in the analyzed tissues? What probe diameters were employed and what was the relation of these to the phase separation artefacts found?

Authors: X-ray microanalysis of the incubated tissue slices was carried out on 16 μm freeze-dried cryosections, in which the analyzed volume is not less than 8-10 μm due to scattering of the electron beam in the tissue, even if the initial probe is narrow. Ice crystal remnants are well below this size. Also because sectioning is carried out at relatively high temperatures (-30°C), there is no need to use the most sophisticated freezing techniques. Because of their small size, cell cultures are easy to cryofix and again, in the analysis of cultured cells the analyzed volume comprises a large part of the cells, and ice crystals are small in relation to the probe volume.

H.Y. Elder: You mention oedema in the context of tissue slices. Have you measured the degree of swelling and/or correlated it with any of the imposed ionic changes?

Authors: We have so far only qualitatively shown oedema in micrographs (Hongpaisan and Roomans, 1995); a more quantitative study is under way. Quantitative data on oedema in different storage fluids on a macroscopic scale have been published by Wahlberg *et al.* (1986) (text reference).

K. Izutsu: The finding that submandibular acini have significantly altered K^+ concentrations is an interesting and important finding in that it indicates that significant changes in K^+ concentrations occur during isolation of acini. Hence, the limit of these preparations should be borne in mind when performing experiments with dispersed acini.

Authors: It appears that it is not uncommon for *in vitro* preparations to have different Na, K and Cl concentrations compared to tissue *in situ*. This is not only valid for isolated submandibular acini, but also for many, if not all, other tissues. Possibly, insufficient attention has been paid to this in the past. However, this does not mean that it is impossible to use *in vitro* preparations.

R. Wróblewski: When working with tissue slices or with cultures from dissected organs, one is always

wondering what happens during the phase which we call for incubation or stabilization. Several morphological studies have shown rather extensive changes at the morphological level. Still, cells in the slices and in the organ cultures do react to different types of stimuli several days after they have been removed from the body. Could you comment on the following issues: (a) How quick is the diffusion of the stimulus in a slice of 1 mm in diameter in the tissue slices of the organs you have been investigating (depth versus time)?; (b) Do you expect any differences in the elemental content of mobile ions in the nucleus and cytoplasm after incubation for 2-3 hours in the incubation buffer?

Authors: We cannot give an exact answer to your question about the diffusion rate of the stimulus in tissue slices, since we have not measured this parameter. We analyzed cells within 240 μm of the surface; the stimulus could reach the cells either by diffusion or by propagation via gap junctions. It is evident that diffusion becomes less of a problem with thinner *in vitro* samples, and that therefore e.g., isolated submandibular gland acini are more suitable as an experimental system than a slice of submandibular gland. We think that in general, the nuclear membrane is freely permeable to Na^+, Cl^- and K^+, and that this does not change during incubation. In the isolated submandibular gland acini, where we have measured both nucleus and cytoplasm, the nucleus had (somewhat) higher concentrations of Na, Cl. K and P than the cytoplasm; this was the case both in the *in situ* gland and at the end of the incubation (Table 1).

K. Zierold: Are the intracellular elemental contents compiled in the diagrams shown, distributed homogeneously in the cells? Have you observed intracellular gradients, storage or binding sites?

Authors: Measurements at the subcellular level were only carried out in the isolated sweat glands and in the submandibular gland *in situ* and in the isolated acini. In the sweat gland we could not find significant differences in elemental concentrations between nucleus and cytoplasm, except for P, which was higher in the nucleus. Results for the submandibular gland are shown in Table 1. A more detailed study of the submandibular gland *in situ* is in press (Mörk *et al.*, 1996b).

H.Y. Elder: You quote Na/K ratios of up to 16 from the isolated sweat glands and imply that these exceptionally high ratios could be correlated with the preparative state. You indicate that such ratios are not simply indications of moribund cells since such cells could subsequently be cultured (though rarely the duct cells with the highest ratios). However, other authors (e.g., Zierold *et al.*, 1994) have taken Na/K ratios of higher than about 0.3 to indicate poor preparative technique and damaged physiological state. Could you comment further?

Authors: There is no question that a high Na/K ratio indicates a damaged physiological state. However, our data, e.g., from the submandibular gland acini, show that cells can recover from a temporary rise of the intracellular Na/K ratio, and the isolated acini have a Na/K ratio of 0.4. Also, within a population of cells with a mean Na/K ratio of 16, there are cells with an even higher and cells with a lower Na/K ratio value. It is not unlikely that the cells that manage to survive and grow, belong to the group with relatively low Na/K ratios in this population.

K. Zierold: You report that the elemental content of cells in tissue slices is preserved best when the slices are stored in a medium of high K/Na ratio. However, under these conditions a high intracellular K/Na ratio can be expected even in cells with completely destroyed membranes! Do you have any other indications for preservation of the vitality of cells incubated in a medium with high K content?

J. Beesley: The authors state that the elemental content of the cells is preserved best when the slices are stored in a medium with a K/Na ratio close to the intracellular ratio instead of a solution resembling the extracellular environment and suggest that under the conditions of the experiment it is likely that the cell membrane is not in full control of the ion fluxes. Would this condition have any adverse effect on the reaction of cells to stimuli?

Authors: We agree that under these experimental conditions the high intracellular K/Na ratio is no evidence for proper functioning of the membrane or even of the vitality of the cell. However, the fact that storage of tissue in solutions with a high K/Na ratio experimentally has been found to work well during organ transplants indicates that tissues can retain their viability in solutions with a high K/Na ratio. Whether the reaction of the tissue to stimuli would be affected by the extracellular K/Na ratio would depend on the tissue and the stimulus under investigation. Certainly all reactions to stimuli that are dependent on membrane potential or influx of Na^+ would be affected by changes in the extracellular fluid. Therefore, we would not recommend carrying out stimulation experiments in a medium with a high ratio, but it would be interesting to test whether cells that have been stored for some time in a medium with high K/Na ratio could be transferred to extracellular fluid with a low K/Na ratio, and if they would perform better than cells kept all the time in fluid with a low K/Na ratio. This will be the subject of further experiments.

K. Izutsu: The ability to demonstrate a heterogeneity in

the cell population is also a unique application of the microanalysis technique. Hence, experimenters who perform studies using cell suspensions should be aware of possible complications arising from working with a heterogeneous population of cells.

Authors: We agree with this comment, and would like to add that the same is valid for e.g., Ussing chamber experiments with cell monolayers.

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