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Intracellular Ion Concentrations and Cell Volume During Cholinergic Stimulation of Eccrine Secretory Coil Cells

T. Takemura, F. Sato, K. Saga, Y. Suzuki, and K. Sato
Department of Dermatology, Marshall Dermatology Research Laboratories, University of Iowa College of Medicine, Iowa City, Iowa, 52242

Summary. Methacholine (MCh)-induced changes in intracellular concentrations of Na, K, and Cl ($[Na]_i$, $[K]_i$, and $[Cl]_i$, respectively) and in cellular dry mass (a measure of cell shrinkage) were examined in isolated monkey eccrine sweat secretory coils by electron probe X-ray microanalysis using the peripheral standard method. To further confirm the occurrence of cell shrinkage during MCh stimulation, the change in cell volume of dissociated clear and dark cells were directly determined under a light microscope equipped with differential interference contrast (DIC) optics. X-ray microanalysis revealed a biphasic increase in cellular dry mass in clear cells during continuous MCh stimulation; an initial increase of dry mass to 158% (of control) followed by a plateau at 140%, which correspond to the decrease in cell volume of 37 and 29%, respectively. The latter agrees with the MCh-induced cell shrinkage of 29% in dissociated clear cells. The MCh-induced increase in dry mass in myoepithelial cells was less than half that of clear cells. During the steady state of MCh stimulation, both $[K]_i$ and $[Cl]_i$ of clear cells decreased by about 45%, whereas $[Na]_i$ increased in such a way as to maintain the sum of $[Na]_i$ + $[K]_i$ constant. There was a small (12–15 mM) increase in $[Na]_i$ and a decrease in $[K]_i$ in myoepithelial cells during stimulation with MCh. Dissociated dark cells failed to significantly shrink during MCh stimulation. The decrease in $[Cl]_i$ in the face of constant $[Na]_i$ + $[K]_i$ suggests the accumulation of unknown anion(s) inside the clear cell during MCh stimulation. While the decrease in $[K]_i$ and $[Cl]_i$ may be instrumental in facilitating influx of ions via Na-K-2Cl cotransporters, the functional significance of MCh-induced cell shrinkage remains unknown.

Key Words eccrine sweat gland · cell volume · X-ray microanalysis · acetylcholine · potassium · sodium

Introduction

Previous studies from our laboratory suggest that cholinergic stimulation of eccrine secretory cells is associated with efflux of intracellular KCl and an influx into the cells of Na (Saga, Sato & Sato, 1988; Saga & Sato, 1989). However, the extent to which the cytoplasmic ionic concentrations change during stimulation with methacholine (MCh) remains unknown. For example, using the continuum normalization method (Hall, 1979) of X-ray microanalysis, we observed that, during stimulation with MCh in vitro, intracellular K and Cl concentrations ($[K]_i$ and $[Cl]_i$, respectively) in clear cells decreased as much as 65%. Also, $[Na]_i$ increased 3.7-fold in clear cells (Saga & Sato, 1989) when concentrations of ions are expressed on the basis of the cellular dry mass, i.e., mmol/kg dry wt. It is puzzling, however, that the sum of $[K]_i$ and $[Na]_i$ (mmol/kg dry mass) also decreased by about 35% within 2 min of MCh stimulation, and persisted as long as MCh stimulation continued (Saga & Sato, 1989). Since the cell interior most likely remained isosmotic to the extracellular fluid before and after MCh stimulation, we speculated that the apparent decrease in the sum of the two major intracellular cations (i.e., $[K]_i$ + $[Na]_i$ mmol/kg dry mass) could have been caused either by accumulation of (as yet unknown) osmotic solutes in the cytoplasm during stimulation or by MCh-induced cell volume shrinkage (and thus an increased dry mass per unit cell volume). Thus the present study was designed to directly test the latter possibility that cholinergic stimulation is associated with cell shrinkage in eccrine secretory cells. We first employed the X-ray microanalysis using the peripheral standard method (Dörge et al., 1978; Rick et al., 1987) which allows not only the change in cellular dry mass but the actual concentrations (mM) of cellular ions to be determined. We then made a limited attempt at directly determining the cell volume using collagenase-dissociated secretory coil cells under a light microscope.

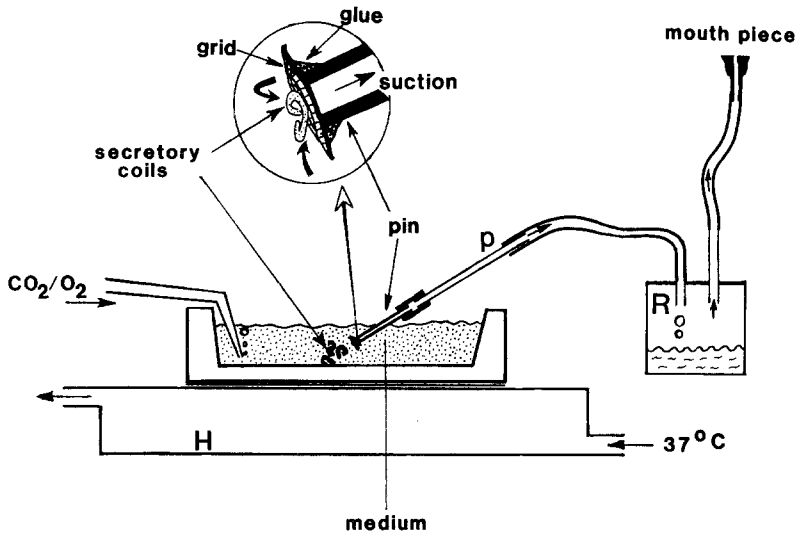

Fig. 1. A modified specimen pin for holding a single segment of the secretory coil, rapidly replacing the external medium, or for holding a droplet of standard solution used for calibration of X-ray yields. The head of the pin is an ordinary grid (for electronmicroscopy) epoxy glued to the shaft of a hollow brass tubing, 2.5 mm in diameter and 2.5 cm long (*inset*). *P*, pipet to hold the pin with a short piece of silicone tubing; *H*, heating table; *R*, reservoir for suction fluid

Materials and Methods

Preparation of Sweat Glands for X-Ray Microanalysis

Preparation of isolated rhesus monkey palm sweat glands is essentially the same as described previously (Sato & Sato, 1981). Skin biopsy specimens, approximately 4 × 8 mm, were repeatedly obtained from the palms of 15 monkeys tranquilized with a mixture of Ketalar (Parke-Davis) and Innovar (Janssen). In each monkey, skin biopsy was done at intervals no shorter than 4 weeks, and the areas of skin adjacent to previous biopsy sites were avoided. The excised tissue was blotted of blood, sliced into several pieces, and immediately washed in several changes of cold (about 10°C) modified Krebs-Ringer bicarbonate solution (KRB) containing (in mM): 125 NaCl, 5 KCl, 1.2 $MgCl_2$, 1.0 $CaCl_2$, 25 $NaHCO_3$, 1.2 NaH_2PO_3, 5.5 glucose, and 7% bovine serum albumin (BSA). The pH of this medium was 7.48 at 37°C when gassed with a mixture of 5% CO_2/95% O_2. Single sweat glands were isolated under a stereomicroscope using sharp forceps in a dissection chamber kept at 14°C. The secretory coils were separated from the ducts and were preincubated for 5 min in KRB at 37°C before incubation in experimental media. After incubation in KRB containing 10^{-5} M MCh for varying periods of time, the secretory coils were picked up and held on the head of a specially constructed specimen pin (Fig. 1) under gentle suction and placed in the external standard solution (KRB containing 20 or 30% BSA and 10^{-5} M MCh) for 2 sec while applying a constant negative pressure through the mouthpiece. The new specimen pin had the same dimensions as the original pin except that the solid shaft was replaced by a hollow brass tubing (2.5 mm in diameter and 2.5 cm long) and the pinhead was replaced by a copper or tungsten grid for electron microscopy (400 mesh, 3 mm in diameter). This modification enabled us to pick up and hold a small piece of tissue, such as an isolated segment of the secretory coil, on the pinhead and to rapidly replace the periglandular fluid when a gentle suction is applied and the medium allowed to flow past the glands through the grid (Fig. 1). When the suction is stopped, a small amount of extracellular standard solution remains attached to the gland. The secretory coil on the pin was instantaneously frozen by plunging it into liquid propane cooled by liquid nitrogen (−180°C).

Cryomicrotomy and X-Ray Microanalysis

The frozen secretory coil (with a small amount of periglandular peripheral standard solution attached) on the tissue pin was mounted on a Reichert Ultracut E microtome in a FC-4 cryochamber (kept at −140°C) and 0.5–1 μm thick cryosections were cut with a cold, dry glass knife. The cryosections were placed on a nickel grid (100 mesh) coated with carbon-stabilized Formvar and were flattened on the grid by pressing with the polished end of a cooled metal rod. The sections were freeze dried for 14 hr at −60°C and 10^{-3} torr in an Emscope FD500 freeze drier and gradually warmed up to room temperature under a vacuum. Freeze-dried sections were stored in a desiccator and used for X-ray microanalysis usually within a week. The cellular ionic concentrations thus determined on days 2 or 3 were not significantly different from those determined on days 6 or 7 (*not illustrated*), suggesting that the cellular ion concentrations remain unchanged during storage as discussed in a previous study (Saga & Sato, 1989).

In a previous study (Saga & Sato, 1989), 0.2-μm thick sections were used to obtain a high resolution of the ultrastructure at the expense of the reduced sensitivity for quantitation of cellular ions. However, in sections thicker than the 0.5 μm as used in the present study, the ultrastructural resolution had significantly decreased so that the dark cell granules as well as dark cells could no longer be identified because the cytoplasm of all the cell types were dark and no distinction could be made between mitochondria and small dark cell granules when cytoplasm appeared granular (*see* Fig. 2). In contrast, clear cells (which are located immediately inside the myoepithelium) and the myoepithelial cells (which are fusiform and are arranged in the outermost layer of the tubule) could still be identified (Fig. 2, right panel). Thus in the present study, only clear and myoepithelial cells have been used for X-ray microanalysis.

Energy dispersive X-ray microanalysis was performed using a Hitachi H-600 electron microscope in a scanning transmission

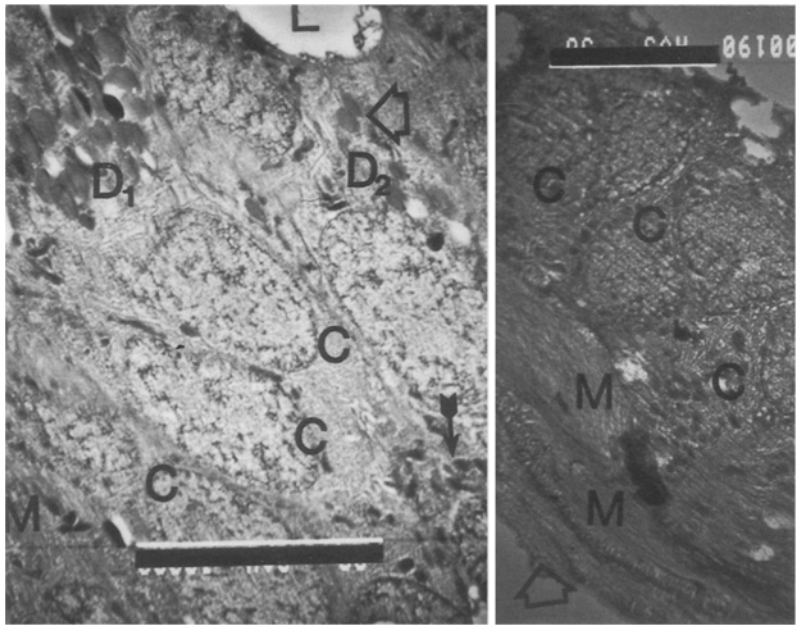

Fig. 2. Scanning transmission electron micrographs of nonfixed freeze-dried cryosections of an isolated eccrine secretory coil. Left panel: In this thin section, less than 0.3-μm thick, three types of cells can be identified. Right panel: In 1-μm sections, which are used for quantitation of cellular ions in the present study, dark cells are extremely difficult to identify although both myoepithelial cells and clear cells could be localized by their general location inside the tubule. Bar = 5 μm. *C*, clear cell; D_1, dark cell with abundant dark cell granules; D_2, dark cell with a few small dark cell granules; *L*, lumen; *M*, myoepithelial cell; thin arrow, mitochondria in a clear cell; large empty arrow, small dark cell granules (left panel) and the thin layer of periglandular Ringer's solution (right panel)

mode equipped with a 30-mm Kevex Si/Li X-ray detector (Kevex, Foster City, CA) (Fig. 3). X-ray signals were stored and analyzed in a Kevex 7000 multichannel analyzer. To reduce specimen contamination, the cold trap and cold finger of the microscope were left on all the time. Accelerating voltage was 75 kV, beam current 3.5 nA, beam diameter 50 nm, and counting live time 100 sec. Characteristic peak counts were obtained within 150 eV energy range of each peak. Continuum counts were measured in the energy region of 1.34–1.64 keV (Somlyo, Shuman & Somlyo, 1977). Cl concentration, on a wet weight basis (mmol/kg wet weight or mM), in the cytoplasm was calculated according to Dörge et al. (1978) from the concentration of Cl in the peripheral standard solution ($[Cl]_{pst}$ = 134.4 mM) multiplied by the ratio of the peak Cl counts in the cytoplasm (PC_{Cl}) to that of the peripheral standard in the same cryosection (PC_{Clpst}), i.e., 134.4 mM · PC_{Cl}/PC_{Clpst}, assuming that the thickness of the cryosection is uniform. The cytoplasmic [K] was calculated from the equation 134.4 mM · (PC_K/PC_{Clpst}) · (a_{Cl}/a_K), where PC_K is the peak K count in the cytoplasm and a_{Cl}/a_K is the difference in the X-ray yield between Cl and K determined using separate standard solutions containing either 20 or 30% BSA and different concentrations of Cl and K (Dörge et al., 1978). [Na] was calculated in the same fashion. Since there were no differences in the calculated tissue electrolyte contents between 20 and 30% BSA in the peripheral standard solutions and since the cellular dry mass fraction may be between 20 to 30%, the results obtained using the two standard solutions were lumped together. The computer software for the Kevex detector is designed (and particularly modified by the University of Iowa electron microscopy facility director, K.C. Moore) so that the background continuum is automatically subtracted from the peak count. The background continuum count in the absence of tissue on the grid was less than 5% of that when tissue was present and there were no detectable peaks for Na, K, Cl, Ca, S, and P. Three or four sites in the cytoplasm were analyzed in each cell and averaged as a single data point. Two or three cells could be analyzed in each tissue sample.

Preparation of Dissociated Secretory Coil Cells

Dissociated cells have been prepared according to Sato and Sato (1988) with modifications. Briefly, a minimum of 100 to 200 isolated whole sweat glands were first incubated with 0.75 mg/ml type I collagenase in Krebs bicarbonate buffer also containing 10 mM HEPES buffer (HEPES-KRB, pH 7.48) for 15 min then thoroughly washed in fresh (collagenase-free) HEPES-KRB. This brief collagenase digestion made the subsequent manual separation of the secretory coils from the ducts considerably easier. Isolated secretory coils were incubated in KRB containing 0.1 mM Ca, 0.75 mg/ml type I collagenase and 100 μg/ml DNAase I (all from Sigma) for 5 min at 37°C. The coils were then incubated for 10 min in two changes of 500 μl Ca- and Mg-free KRB containing 2 mM EGTA. A second enzyme digestion was performed for 30 to 60 min in KRB containing 0.1 mM Ca, 1.25 mg/ml collagenase (or 0.125 mg/ml TPCK-treated trypsin from Sigma), penicillin and streptomycin mixture (30 U/ml), and 100 μg/ml DNAase. The cells were dispersed from the digested glands by repeatedly passing them through a sieve, which was a tungsten grid for electron microscopy (3-mm o.d., 100 mesh) epoxy glued to a disposable plastic pipette tip. Dispersed cells were subsequently passed through a finer sieve (200–300 mesh) and centrifuged at 900 × *g* for 2 min. The cells were then suspended in KRB containing 4% bovine albumin. The electron microscopy of the dispersed cells obtained in this way showed more than 60% secretory cells, less than 30% dark cells and about 10% myoepithelial cells. The mixed dispersed cells thus prepared were used for cell volume analysis because different cell types can be identified by fluorescent microscopy (e.g., autofluorescent lipofuscin granule in the clear cell) or by differential interference contrast (DIC) (dark cell granules for the dark cell; characteristic appearance of the myoepithelial cell; characteristic appearance of lipofuscin granules for clear cells). More than 95% of these cells excluded trypan blue.

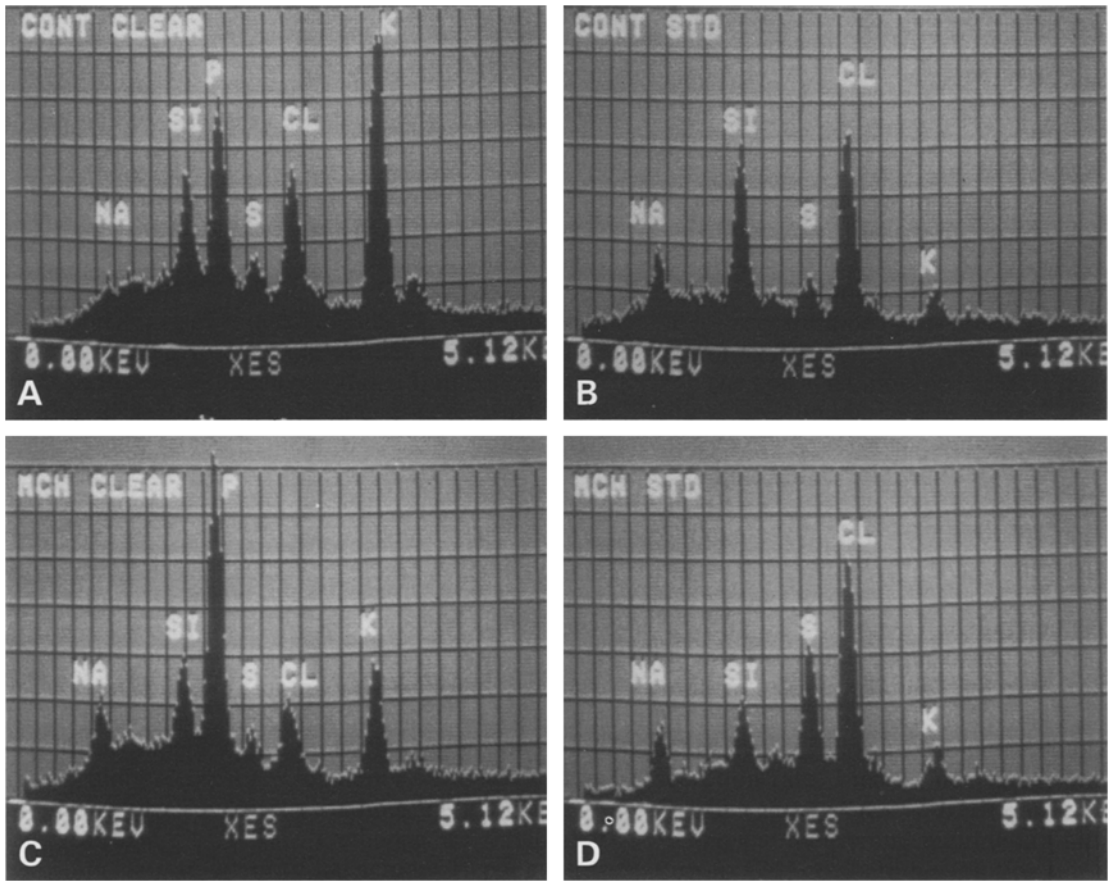

Fig. 3. Illustrative examples of X-ray spectra obtained from the cytoplasm of a resting (incubated in KRB, panel *A*) and a MCh-stimulated (at 10^{-5} M for 2 min) clear cell (panel *C*). (*B*) and (*D*) are the spectra of periglandular standard solutions of the glands *A* and *C*, respectively. Note the similarity in Na, K, and Cl peaks between *B* and *D*. Also note the decrease in the height of K and Cl peaks in *C* as compared with those in *A* and the increase in the height of the Na peak in *C* as compared with that in *A*

Cell Volume Analysis by Light Microscopy with Differential Interference Contrast (DIC)

Figure 4 shows schematic illustrations of the perfusion chamber. The method of constructing the chamber is detailed in the legend. In order to coat the coverglass with poly-L-lysine (M_r 10–40K, Sigma), 5 μl of its solution (1 mg/ml H_2O) was placed in the marked center of the cover glass and allowed to dry. The slide was washed in distilled water for 5 sec and air dried. A sample of 2–3 μl of cell suspension was placed on the polylysine-coated area and left for 30 sec. The cover glass was inverted (with cells on the undersurface), placed on the lower chamber so that the cells were in the center of the slit-like perfusion groove, sealed with silicone grease, and further secured with dental wax. When KRB (used as perfusate) was infused into the slit, unattached cells were lost. Polylysine coating was adjusted (by changing the duration of washing) so that a sufficient number of cells were loosely attached to the cover glass. Temperature of the Olympus BH microscope was adjusted so that the center of the chamber was set at 37°C. The cells were viewed and photographed using a 100× oil immersion objective, a DIC condenser system (1.25×), and a 15× photomicrograph eyepiece (a total magnification of 1875×). Focus was frequently adjusted so as to obtain the optical section (with DIC) at the maximal diameter of the cell. In preliminary experiments, we confirmed that the decrease of cell diameter in the horizontal direction is comparable to that in vertical direction (*not shown*). Thus, relative cell volumes were calculated from the average horizontal diameter alone during the experiment. Myoepithelial cells were not studied because they are not spherical in shape (Sato et al., 1989); thus, cell volume could not be determined with sufficient accuracy.

Results

X-Ray Microanalysis

Since the background continuum count (or Bremsstrahlung) is directly proportional to the cellular dry mass (Dörge et al., 1978), the relative continuum count was compared before and after stimulation

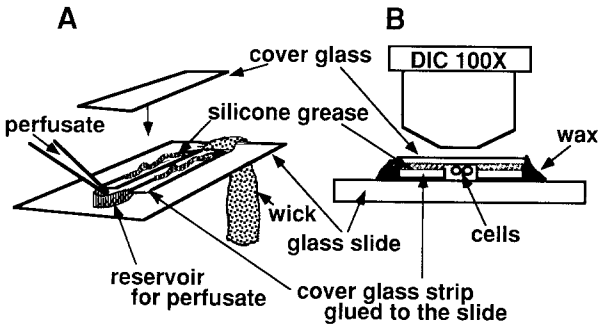

Fig. 4. Schematic illustrations of a perfusion chamber for cell volume analysis by light microscopy. (*A*) A sketch of the chamber created on a glass slide, 5 × 7 cm. Two strips of cover glass, 3.5 × 30 × 0.13 mm were epoxy glued to the glass slide. The 3-mm wide groove between the two strips accommodates the cells and serves as a flow chamber for the perfusate when a polylysine-coated cover glass, 10 × 30 mm, with cells attached to its undersurface is mounted and sealed with silicone grease (Dow Corning vacuum grease) and further secured by dental wax. (Polysciences). A semicircular plastic ring glued to the slide serves as a reservoir for the perfusate. Perfusate is continuously infused into the slit-like flow chamber or pipetted into the reservoir. The perfusate emerging from the opposite end of the flow chamber is continuously removed with a wick. (*B*) A schematic illustration of the cross section of the chamber. The chamber is mounted on a thermostated stage (at 37°C) of an Olympus BH upright microscope with a 100× differential interference (DIC) contrast optical system

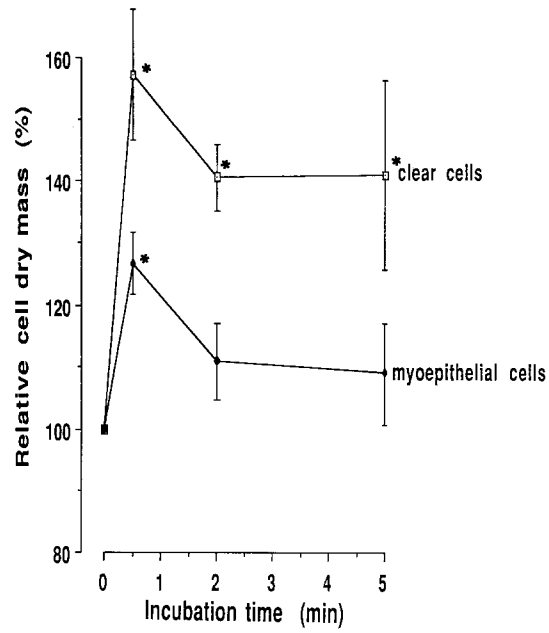

Fig. 5. Time course of change in cytoplasmic dry mass in clear and myoepithelial cells after MCh stimulation as determined by X-ray microanalysis. Time zero is prestimulation control. Each plot is the mean ± SE of from 17 to 19 cells. Cellular dry mass normalized by the peripheral standard containing either 20 or 30% BSA is compared before (taken as 100%) and after stimulation. *, $P < 0.001$ (Student's t test); $P < 0.05$ for the other myoepithelial plots. All the clear cell plots are significantly ($P < 0.01$) higher than the corresponding myoepithelial plots

with 10^{-5} M MCh. As seen in Figure 5, cytoplasmic dry mass in clear cell cytoplasm increased to 158% of control within 30 sec of MCh stimulation and plateaued at around 140% thereafter, suggesting that clear cells shrink by 37% [100(1–100/158)%] and 29% during cholinergic stimulation. Cellular dry mass also increased in myoepithelial cells after MCh stimulation (to the peak of 126 and 110% at the plateau) but to a lesser extent than in clear cells.

Figure 6 shows that the change in $[Na]_i$, $[K]_i$, and $[Cl]_i$ after stimulation with 10^{-5} M MCh occurred concurrently with the change in cell volume shown in Fig. 5. Within 2 min of stimulation with MCh, $[K]_i$ and $[Cl]_i$ decreased from 124 to 69 mM (44% decrease) and from 92 to 55 mM (40% decrease), respectively, and $[Na]_i$ increased from 28 to 83 mM (3.6-fold increase). The MCh-induced change in cytoplasmic electrolyte concentrations persisted thereafter ($P > 0.4$). In contrast to our previous study (Saga & Sato, 1989), the sum of $[Na]_i + [K]_i$ (in mM, the upper curve) remained unchanged during MCh stimulation. Nevertheless, $[Cl]_i$ decreased, suggesting that some other unknown anions (e.g., HCO_3^-, lactate, PO_4^-, polyanions(?)) may be simultaneously accumulated inside the cell to maintain the neutrality of charges.

In the myoepithelial cell cytoplasm, $[Na]_i$, $[K]_i$ and $[Cl]_i$ were all slightly lower than those of clear cells (Fig. 7), which could be explained by the higher dry mass continuum and thus smaller water content in the myoepithelial cells than in clear cells (Saga & Sato, 1989). $[K]_i$ decreased by 15 mM (from 116 before to 101 mM at 2 min of MCh stimulation) but $[Na]_i$ increased 12 mM (from 23 to 35 mM) so that the sum of $[Na]_i + [K]_i$ was unchanged ($P > 0.5$) during the same period. $[Cl]_i$ did not change for the first 2 min but gradually decreased by 18 mM between 2 and 5 min of MCh stimulation.

Cell Volume Analysis by Light Microscopy

Figure 8 shows illustrative DIC pictures of dissociated clear cells before (Fig. 8*1*) and after 2-min application of 10^{-5} M MCh. (Fig. 8*2*). Approximately 10% reduction in diameter (which corresponds roughly to 30% reduction in cell volume) is well within the sensitivity of the present method. Within 1 min after switching to 10^{-5} M atropine (AT), cell diameter returned to the prestimulation level. The results of

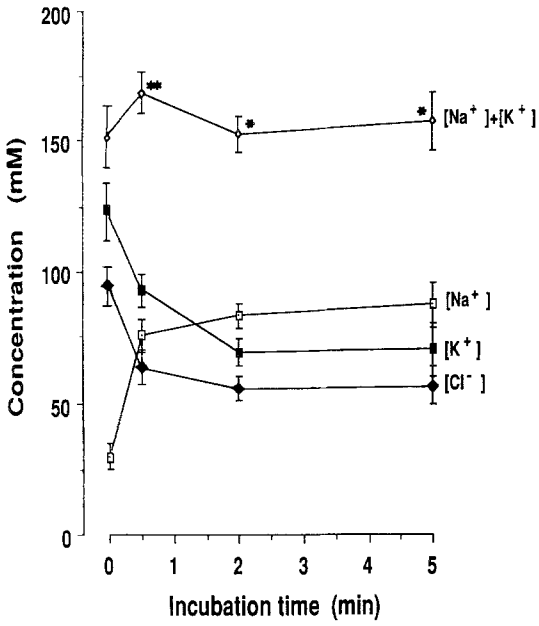

Fig. 6. Time course of change in $[Na]_i$, $[K]_i$, and $[Cl]_i$ in clear cells after MCh (10^{-5} M) stimulation as determined by X-ray microanalysis. Time 0 is unstimulated control. *, $P > 0.05$ against control; **, $0.05 > P > 0.01$. Each plot is the mean ± SE of from 13 to 19 cells

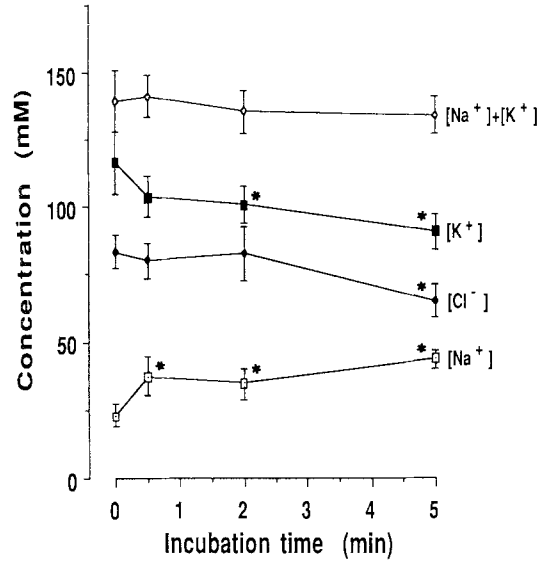

Fig. 7. Effect of MCh on $[Na]_i$, $[K]_i$, and $[Cl]_i$ in myoepithelial cell cytoplasm as determined by X-ray microanalysis. Each plot is the mean ± SE of from 16 to 20 cells. Time 0 is prestimulation control. *, $P < 0.05$ against respective controls

14 similar experiments are shown in Fig. 9. In all the clear cells studied, the calculated cell volume decreased (the average decrease was 29%). It is of interest that the two different methods of cell volume analysis, namely X-ray microanalysis of intact secretory tubule and light microscopy of dissociated clear cells, yielded an identical value for cell volume shrinkage at 2 min of MCh stimulation, i.e., 29%. After washout with AT, nine of those cells that had remained attached to the cover glass recovered to near the original cell volume. It is puzzling that none of the dark cells studied shrank in response to MCh. The average cell volume decrease of the dark cells was only 4% reduction ($P = 0.093$) (*not illustrated*).

Discussion

The major goals of the present study were to clarify that cholinergic stimulation of the eccrine secretory portion is associated with significant cell volume decrease and to determine to what extent cytoplasmic $[Na]_i$, $[K]_i$ and $[Cl]_i$ concurrently change during the maximal cholinergic stimulation. We thus employed two experimental approaches: X-ray microanalysis using the peripheral standard method (Dörge et al., 1978) to estimate actual concentrations of cellular ions (i.e., in mM) and light microscopic determination of cell volume of dissociated secretory coil cells using DIC optics and a conventional microscope camera. A new tissue pin was designed to facilitate rapid replacement of extracellular media from the incubation medium to the one containing protein-rich peripheral standard solutions immediately before shock freezing the tissue for subsequent X-ray microanalysis. A new, inexpensive perfusion chamber was also constructed for light microscopic observation of immobilized dissociated cells for cell volume analysis.

The X-ray microanalysis technique we employed in our previous study (Saga & Sato, 1989), i.e., the continuum normalization method (Christensen, 1971; Hall, 1979), is still widely used because it is relatively simple, but it is not an ideal method for tissues whose cytoplasmic dry mass (or background continuum as revealed by X-ray microanalysis) changes during cellular activity (e.g., cell volume changes during stimulation with drugs). The continuum normalization method is also unsuitable where elemental concentrations need to be expressed on a wet wt basis (i.e., mmol/kg wet wt). Nevertheless, the study (Saga & Sato, 1989) provided qualitative evidence suggesting that MCh stimulation of eccrine secretory coils is associated with dramatic changes in $[Na]_i$, $[K]_i$ and $[Cl]_i$ in all three types of cells, namely clear, dark, and myoepithelial cells.

It is puzzling, however, that the sum of the concentrations of two major intracellular cations in clear cells, expressed on a per kg dry wt basis, i.e., $[Na]_i$

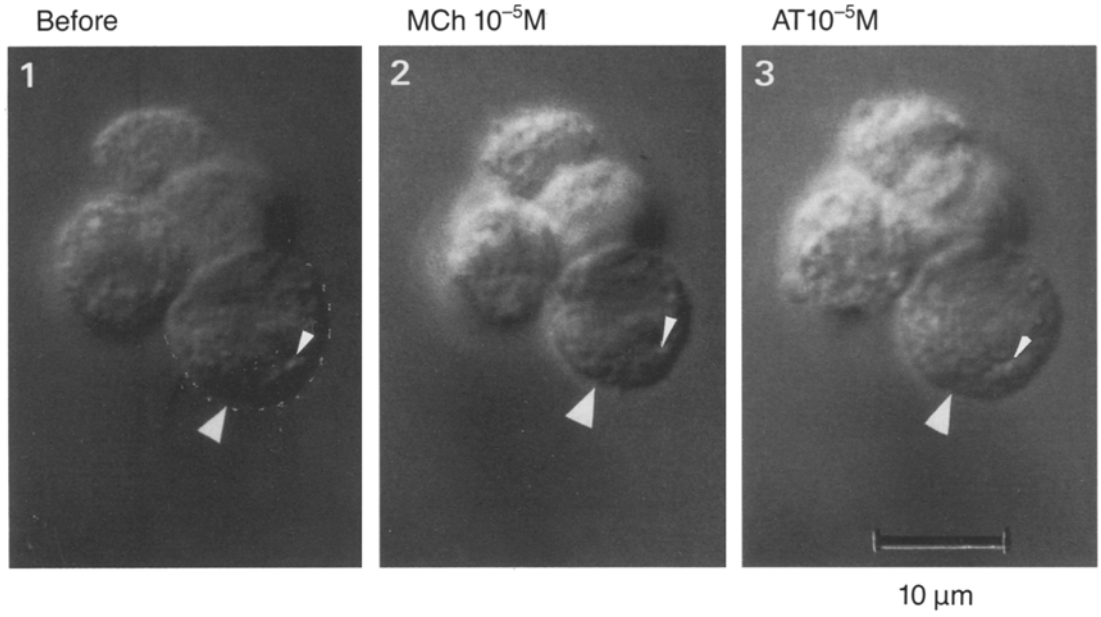

Fig. 8. MCh-induced cell shrinkage in dissociated eccrine clear cells. In this illustrative experiment, the cell indicated by an arrow is focused so that the largest diameter can be optically sectioned by DIC optics. The cell diameter was determined in two directions and averaged for calculation of cell volume assuming that the cell was spherical. *1*, the cell border was highlighted with an interrupted line. *1*, before; *2*, clear cells stimulated by continuous perfusion of MCh (10^{-5} M) in KRB; *3*, the same cells after switching the perfusate to 10^{-5} M atropine in KRB. The bar indicates 10 μm. The slender arrow inside the cell points to a lipofuscin granule

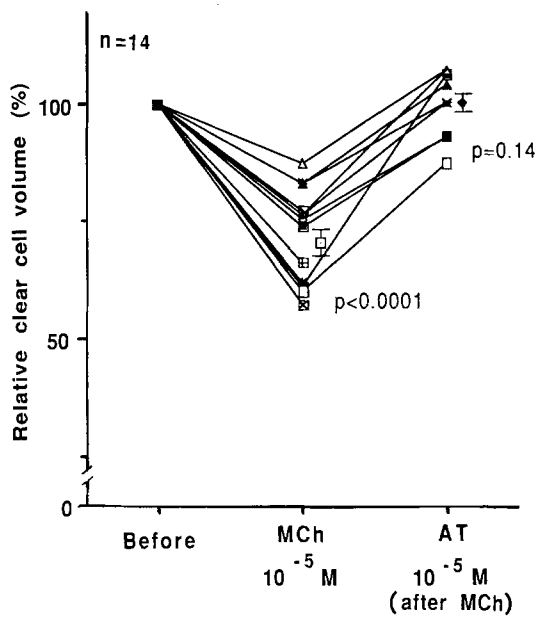

Fig. 9. Effect of MCh on cell volume in 14 dissociated clear cells. The experimental protocols are the same as in Fig. 8. The result of each experiment is shown by a line. *P* values are calculated relative to control (100%). In each experiment, cell volume is expressed relative to the control cell. *n*, the number of cells studied

+ $[K]_i$ per kg dry wt, decreased by 30 to 35% after stimulation with MCh. We theorize that the observed decrease in the sum of $[Na]_i$ + $[K]_i$ (per kg dry wt) may have been caused by the increase in dry wt per unit area (the very artifact arising from continuum normalization method) due to MCh-induced cell shrinking rather than to the true decrease in $[Na]_i$ + $[K]_i$ (in mM), with or without concomitant accumulation of other osmotic solutes. X-ray microanalysis with the peripheral standard method not only allows determination of $[Na]_i$, $[K]_i$, and $[Cl]_i$ (in mM) in each cell type, irrespective of the presence or absence of cell shrinking, but also allows direct determination of the change in cellular dry mass, which is a measure of the change in cellular volume. In fact, the observed result (Fig. 5) indicates that MCh stimulation of clear cells is associated with an increase in cellular dry mass, suggesting that cell volume decrease has occurred during stimulation with MCh. Such an increase in cytoplasmic dry mass is biphasic, i.e., the initial overshoot reaches as high as 158% of control at 30 sec of MCh stimulation followed by a plateau at around 140% from 2 to 5 min, which corresponds to a decrease in cytoplasmic volume of 37 and 29%, respectively. Interestingly, this value (29%) agrees with the relative cell shrinkage in dissociated clear cells of 29% (mean) at 2 min of MCh stimulation as determined by light microscopy (*see* Fig. 9).

The cellular dry mass in myoepithelial cells increased to only 127% at 30 sec and about 110% during the plateau of MCh stimulation (*see* Fig. 5), which corresponds to 21 and 9% of the cell volume decrease, respectively. Thus it appears that myoepithelial contraction (Sato, 1977) is not associated with a drastic decrease in cellular volume. The small magnitude of cell volume change in myoepithelial cells may be partially caused by the fact that myoepithelial cells have higher cellular dry mass per unit cell volume and thus less cell water content than clear cells (Saga & Sato, 1989).

In light of our previous data that $[Na]_i$, $[K]_i$, and $[Cl]_i$ changed in both clear and dark cells during MCh stimulation to a similar extent (Saga & Sato, 1989), the observed sluggish response of dissociated dark cells to MCh (only 4% decrease in cell volume, $P = 0.093$) is somewhat unexpected. Some of the possibilities that account for this disagreement include: (*i*) dark cells are more sensitive to enzymatic digestion than clear cells and have lost their cholinergic sensitivity during cell preparation; (*ii*) dark cells form a functional syncytium with clear cells in intact tissues, thereby responding to MCh stimulation in the previous study (Saga & Sato, 1989); (*iii*) dark cells are filled with dark cell granules and thus less cytoplasmic water is available for cell shrinkage; and (*iv*) the change in $[Na]_i$, $[K]_i$, and $[Cl]_i$ in dark cells occurs in the absence of a change in cell volume. Unfortunately, in the present study, the use of thicker (0.5–1 μm) cryosections for the peripheral standard method (Dörge et al., 1978) compromised our ability to accurately identify dark cells in cryosections.

Although the present study has uncovered some of the novel cellular events that accompany cholinergic stimulation of eccrine secretory coil cells, these observations alone created more questions relative to the ionic mechanism of eccrine sweat secretion. We have recently suggested (Saga & Sato, 1988, 1989; Sato, 1986; Sato & Sato, 1987*a*,*b*; Sato et al., 1989) that the major ionic mechanism of cholinergic sweating may involve, among others, Na-K-2Cl cotransporters (Geck & Heinz, 1986). Although the observed decrease in $[K]_i$ and $[Cl]_i$ in clear cells may provide a favorable chemical potential for the cotransporters (thereby facilitating the influx of Na, K, and Cl into the cell), it is obvious that the original cotransport model does not necessarily require both the observed change in cellular ions and the decrease in cell volume that occur during MCh stimulation. The mechanisms by which the observed changes in cellular ions occur during MCh stimulation are also unknown. It appears that MCh-induced cell shrinking is the result of the net K_i and Cl_i efflux through their respective channels, activated in a Ca-dependent fashion (Saga & Sato, 1989; Sato, 1986; Sato & Sato, 1988, 1989). It should be noted that the MCh-induced net KCl efflux and the resultant cell shrinking (by about 30% due to outflux of water accompanying KCl efflux) alone does not adequately explain the observed decrease in $[K]_i$ and $[Cl]_i$ (if KCl simply leaves the cell isotonically, there should be minimal change in $[K]_i$ and $[Cl]_i$). Also, it does not adequately account for the concomitant increase in $[Na]_i$ because the cell shrinkage itself may contribute to an increase in $[Na]_i$ by only 30%, i.e., $[Na]_i$ actually increased threefold (from 28 before to 83 mM after 2-min MCh stimulation). We surmise that the additional Na-influx may be partially explained by activation of Na-K-2Cl cotransporters because bumetanide minimized the MCh-induced increase in $[Na]_i$ (Saga & Sato, 1989). It is interesting to note that while $[K]_i + [Na]_i$ remained unchanged during MCh stimulation, $[Cl]_i$ decreased from 92 mM at rest to 55 mM (a decrease of 40%) at 2 min of MCh stimulation, indicating that other unknown anion(s) may accumulate inside the cell to maintain electrical neutrality. At present the anion(s) presumably involved in filling the above anion deficit is (are) unknown. However, we speculate that HCO_3^-/CO_2 may play a role, at least in part, because K efflux (which may also be coupled to movement of Na as well as Cl) from the secretory coil is drastically reduced in the absence of HCO_3^- in the medium or in the presence of inhibitors of parallel exchangers (Kang, Saga & Sato, 1988).

The functional significance of MCh-induced cell shrinkage, as well as its ionic mechanism, remains to be studied. It is of interest to note Foskett and Melvin (1989) observed that cholinergic stimulation is associated with cell shrinkage and the increase in the cytosolic Ca^{2+} concentration in dissociated rat salivary acinar cells. It is tempting to speculate therefore that the intracellular ionic mechanisms may be similar between the eccrine clear cells and the salivary acinar cells.

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References

Christensen, A.K. 1971. Frozen thin sections of fresh tissue for electron microscopy, with a description of pancreas and liver. *J. Cell Biol.* **51**:772–804

Dörge, A., Rick, R., Gehring, K., Thurau, K. 1978. Preparation of freeze-dried cryosections for quantitative X-ray microanalysis of electrolytes in biological soft tissues. *Pfluegers Arch.* **373**:85–97

Foskett, J.K., Melvin, J.E. 1989. Activation of salivary secretion: Coupling of cell volume and $[Ca^{2+}]_i$ in single cells. *Science* **24(4912):**1582–1585

Geck, P., Heinz, E. 1986. The Na-K 2Cl cotransport system. *J. Membrane Biol.* **91:**97–105

Hall, T. 1979. Biological x-ray microanalysis. *J. Microsc.* **117:**145–163

Kang, W.H., Saga, K., Sato, K. 1988. Effects of transport inhibitors and ions on K^+ efflux from monkey eccrine secretory coils during cholinergic stimulation. *Clin. Res.* **36:**660*A*

Rick, R., Beck, F.X., Dörge, A., Thurau, K. 1987. Intracellular ion concentrations in the frog cornea epithelium during stimulation and inhibition of Cl secretion. *J. Membrane Biol.* **95:**229–240

Saga, K., Sato, K. 1988. Ultrastructural localization of Na,K-ATPase in the monkey palm eccrine sweat gland. *J. Histochem. Cytochem.* **36:**1023–1030

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

Saga, K., Sato, F., Sato, K. 1988. K^+ efflux from the monkey eccrine secretory coil during the transient of stimulation with agonists. *J. Physiol. (London)* **405:**205–217

Sato, K. 1977. Pharmacology and function of the myoepithelial cell in the eccrine sweat gland. *Experientia* **33:**631–633

Sato, K. 1986. Effect of methacholine on ionic permeability of basal membrane of the eccrine secretory cell. *Pfluegers Arch.* **407(Suppl. 2):**S100–S106

Sato, K., Kang, W.H., Saga, K., Sato, K.T. 1989. Biology of sweat glands and their disorders. 1. Normal sweat gland function. *J. Am. Acad. Dermatol.* **20:**537

Sato, K., Sato, F. 1981. Pharmacologic responsiveness of isolated single eccrine sweat glands. *Am. J. Physiol.* **240:**R44–R51

Sato, K., Sato, F. 1987*b*. Nonisotonicity of simian eccrine primary sweat induced in vitro. *Am. J. Physiol.* **252:**R1099–R1105

Sato, F., Sato, K. 1987*a*. Effect of periglandular ionic composition and transport inhibitors on Rhesus monkey eccrine sweat gland function in vitro. *J. Physiol. (London)* **393:**195–212

Sato, K., Sato, F. 1988. Relationship between Quin 2-determined cytosolic $[Ca^{2+}]$ and sweat secretion. *Am. J. Physiol.* **254:**C310–C317

Sato, K., Sato, F. 1989. Characteristics of Na, K, and Cl channels in the clear cell membrane of the eccrine sweat gland as studied by the patch clamp techniques. *Clin. Res.* **39:**369*A*

Somlyo, A.V., Shunan, H., Somlyo, A.P. 1977. Elemental distribution in striated muscle and the effects of hypertonicity: Electron probe analysis of cryosections. *J. Cell Biol.* **74:**828–857

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