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Electron Probe X-Ray Microanalysis of Cellular Ions in the Eccrine Secretory Coil Cells during Methacholine Stimulation

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Summary. Intracellular concentrations of Na, K, Cl ([Na], [K] and [Cl], respectively) and other elements were determined in isolated monkey eccrine sweat secretory coil cells using quantitative electron probe X-ray microanalysis of freeze dried cryosections. The validity of the methodology was partially supported by qualitative agreement of the X-ray microanalysis data with those obtained by micro-titration with a helium glow spectrophotometer. [Na], [K] and [Cl] of the cytoplasm were the same as those in the nucleus in both clear and dark cells. [Na], [K], and [Cl] of the clear cells were also the same as those of the dark cells at rest and after stimulation with methacholine (MCh), suggesting that these two cell types behave like a functional syncytium. MCh stimulation induced a pharmacologically specific, dose-dependent decrease in [K] and [Cl] (as much as 65%), and a 3.7-fold increase in [Na]. In myoepithelial cells, a similar change in [Na] and [K] was noted after MCh stimulation although the decrease in [Cl] was only 20%. The MCh-induced change in [Na], [K] and [Cl] was almost completely inhibited by removal of Ca^{2+} from the medium. 10^{-4} M bumetanide inhibited the MCh-induced increase in [Na], reduced the decrease in [K] by about 50%, but slightly augmented the MCh-induced decrease in [Cl]. 10^{-4} M ouabain increased [Na] and decreased [K] as did MCh; however, unlike MCh, ouabain increased [Cl] by 56% after 30 min of incubation. Thus the data may be best interpreted to indicate that Ca-dependent K efflux and (perhaps also Ca-dependent) Cl efflux are the predominant initial ionic movement in muscarinic cholinergic stimulation of the eccrine sweat secretory coils and that the ouabain-sensitive Na pump plays an important role in maintenance of intracellular ions and sweat secretion.

Key Words eccrine sweat gland · ouabain · bumetanide · X-ray microanalysis · acetylcholine · potassium · sodium

Introduction

In humans and primates, the secretory portion of the eccrine sweat gland is made up of three types of cells (clear or secretory, dark and myoepithelial cells) and elaborates nearly isotonic primary sweat in response to cholinergic stimulation (Sato & Sato, 1987*a*). Although the ionic mechanism of sweat secretion has been partially clarified in recent years (Sato & Sato, 1987*a*,*b*; Saga & Sato, 1988), further understanding of its mechanism is hampered by our lack of knowledge of the intracellular ionic concentrations of Na, K and Cl in the secretory or clear cell before and after stimulation with secretagogues. Likewise, knowledge of the change in intracellular electrolyte concentration in the dark cells may provide insight into the pharmacology and function of these cells, which are assumed to be involved in secretion of mucous or glycoproteins (Yanagawa, Yokozeki & Sato, 1986).

At present, three methods are commonly used to determine intracellular electrolyte concentrations: namely, (i) intracellular ion-sensitive glass microelectrodes, (ii) electron probe X-ray microanalysis and (iii) micro-titration of tissue electrolytes in acid extracts of the tissue. Each technique has advantages and disadvantages. Considering the difficulties in puncturing secretory cells with a single barreled conventional glass microelectrode (Sato, 1986), the use of double barreled ion-sensitive glass microelectrodes does not appear to be a practical approach at present. Determination of extractable cellular ions by helium glow spectrophotometry or electrical titration is also cumbersome and is incapable of identifying the cell types that are mainly responsible for the change in tissue electrolyte contents or studying ion concentrations in different intracellular compartments. Although the electron probe X-ray microanalysis is also technically demanding and is not without methodological limitations, it is instrumental in obtaining a qualitative measure of intracellular ions in different subcellular compartments in different cell types (Saubermann, 1986; Smith & Cameron, 1987). In the present study, we employed X-ray microanalysis to determine [Na], [K], [Cl] and other elemental contents of the cytoplasm and nucleus in all three cell types and in the dark cell granules before and after

stimulation with MCh in the presence and absence of the inhibitors of sweat secretion. A limited attempt was also made to test the qualitative validity of our X-ray microanalysis data by comparing them with the values of tissue [Na] and [K] as determined by helium glow spectrophotometry.

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, 1973). Skin biopsy specimens, approximately 4 × 8 mm, were repeatedly obtained from the palms of 12 monkeys tranquilized with a mixture of Ketalar (Parke-Davis) and Innovar (Janssen). In each animal, 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, and 5 glucose. 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 (at 37°C) for varying periods of time. After incubation, the secretory coils were picked up by holding the periglandular collagen fibers (but not the gland itself) with a pair of sharp forceps, quickly blotted of the attached incubation medium, equilibrated in 7% bovine serum albumin (BSA) in KRB (mounting medium) for a few seconds by gently shaking the glands in the medium, and were quickly placed on a small piece of Millipore filter disc secured on top of a specimen pin (holder).

In a series of experiments where the extracellular standard solution was used to derive intracellular ion concentrations, 20% BSA in KRB was used as mounting medium. (The cellular electrolyte values so obtained were comparable to those observed when 7% BSA in KRB was used as mounting medium.) After the excess periglandular mounting medium was quickly blotted, the specimen pin was mounted upside down on a wooden sliding rod fabricated according to Christensen (1971). The secretory coils on the pin were instantaneously frozen by sliding down the pin on the rod and pressing the tissue against the polished flat surface of a copper block continuously cooled with liquid nitrogen (Christensen, 1971). 0.2 μm thick cryosections were cut with a glass knife mounted on a Reichert Ultracut E microtome in a FC-4 cryochamber (kept at −140°C). The sections were placed on a titanium grid (75 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 either at −100°C and 10^{-7} torr overnight in a Balzer 301 freeze fracture apparatus or at −60°C and 10^{-3} torr in an Emscope FD500 freeze drier. Since there were no differences in morphology and tissue electrolyte content between the two freeze drying methods, the results were lumped together. Freeze dried sections were carbon coated in a high vacuum evaporator and stored in a desiccator for X-ray microanalysis at a later time, usually within a week. Under this storage condition, the amount and subcellular distribution of tissue electrolytes remained unchanged, which was demonstrated by a second analysis of the same sections after several months of storage.

X-Ray Microanalysis

Energy-dispersive X-ray microanalysis was performed using a Hitachi H-600 electron microscope in a scanning transmission mode equipped with a 30-mm Kevex Si/Li X-ray detector (Kevex Corp., Foster City, CA). 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 4.0 nA, beam diameter 50 nm and counting live time 100 sec. X-ray spectra were quantitated by Hall's method (Hall, 1979), which is based on the fact that the ratio of a characteristic elemental peak count to the continuum count is proportional to the elemental concentration. 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). The concentration of each element in the tissue was computed from the linear calibration curve of the peak/continuum ratios *vs.* concentrations of that element in the standard solutions (also containing 20% BSA). The computer software for the Kevex detector is designed (and partially 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, which was mainly due to escape peaks from Ti and the other elements in the grid and Formivar, was less than 5% of that when tissue was present and there were no detectable peaks for Na, K, Cl, Ca, S, and P. The actual background count with the tissue on the film should be much smaller than the above background count (because fewer electrons actually reach the film when the tissue is present) and is effectively negated when the elemental concentrations are calculated from the linear standard curve. Although the use of thin sections (i.e., 0.2 μm), which were used to improve resolution of the electron microscopic image for precise localization of the electron beam, slightly decreased the peak/background ratio, the possible error arising from the present method may be negligibly small. The fact that the peak/background ratio increased linearly over the range of the elemental concentrations in the external standard further supports the validity of the present methodology. The elemental concentrations will be slightly underestimated in the dark cell granules whose tissue dry mass fraction is slightly higher than that of the clear or dark cell cytoplasm. Two to three sites in each subcellular compartment (e.g. cytoplasm, nucleus, etc.) were analyzed in each cell and averaged as a single data point. Three to six cells could be analyzed in each tissue.

When continuum counts were directly compared to study the difference of dry mass in the cytoplasm of different cell types, only those sections that contained all the three cell types in the same section were selected. The beam current, determined at the level of specimen, was usually constant during each analysis. Furthermore, the sequence of microanalysis was randomized relative to different cell types and subcellular compartments in each experiment to minimize the experimental biases. Concentrations of elements were expressed simply as mmol/kg dry mass, and no attempts were made in the present study to estimate the actual concentrations on the wet weight basis.

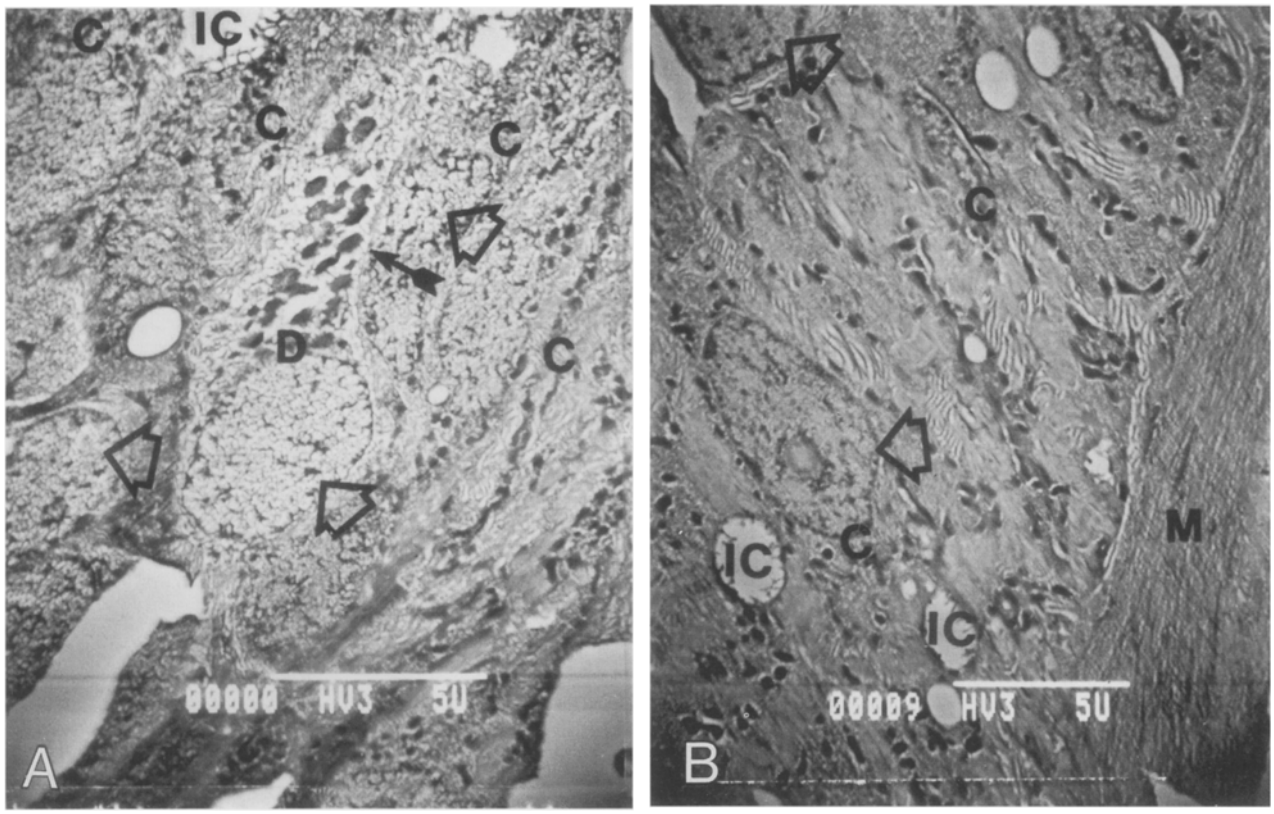

Fig. 1. Scanning transmission electron micrographs of nonfixed freeze dried cryosections of the isolated eccrine secretory coil. The sections are about 0.2 μm thick. Bar = 5 μm. *C*, clear cell; *D*, dark cell; *IC*, intercellular canaliculi; *M*, myoepithelial cell; thin arrow, dark cell granules. Some of the nuclei are indicated by thick arrows. *A* shows both dark and clear cells, whereas *B* shows a myoepithelial cell and many clear cells. The mosaic pattern in the nucleus in the center of *A* (which may be interpreted as due to ice crystal formation) was not a constant finding. Note a similar mosaic pattern is not found in either adjacent nuclei in the same section, or in Figs. 1–8

Determination of Tissue [Na] and [K] by Microtitration with a Helium Glow Spectrophotometer

In an attempt to test the validity of our X-ray microanalysis data, a limited effort was made to microtitrate the total extractable [Na] and [K] in the secretory coil segments before and after MCh stimulation. The method was modified from that of Sudo and Morel (1984), which was used for determination of tissue electrolytes in isolated nephrons. We took advantage of the fact that the secretory coil lumen is very narrow or collapsed (especially when short segments are isolated and incubated as in the present study) so that the contribution of the luminal (extracellular) electrolytes to the total tissue electrolytes is minimal and constant before and during MCh stimulation. Furthermore, the tissue volume can be easily estimated by measuring its diameter (range 60–75 μm) and the tubular length (500 to 850 μm) on the Polaroid pictures of the gland taken before preincubation. A short segment of the secretory coil was first preincubated in KRB for 5 min at 37°C in the concavity of a glass slide placed in a moist chamber kept at 5% CO_2/95% O_2, then transferred to a new incubation medium (containing appropriate drugs to be studied) in an adjacent concavity. After incubation, the coil was picked up by suction on a small sieve (400 mesh copper grid secured by epoxy glue, about 1 mm in diameter) secured at the open end of a pipette tip. The periglandular ions were easily removed by quickly immersing the sweat gland held at the tip of the pipette in a cold isotonic Tris buffer (pH 7.4, Tris-HCl 150 mM, $MgCl_2$ 1.2 mM, and $CaCl_2$ 1.0 mM) under constant suction for 1 sec because, as will be shown in Fig. 11, the extracellular Na was nearly completely removed already after 0.5 sec of vigorous washing and the tissue [K] and [Na] remained unchanged until 5 sec (of vigorous washing). The secretory tubule was quickly transferred to a 100-nl droplet of 10% trichloroacetic acid (TCA) in hydrated mineral oil in a moist chamber, and Na and K were extracted overnight at 4°C. [Na] and [K] in the TCA extract was determined by helium glow spectrophotometry using standard solutions prepared in 10% TCA and taking other precautions recently elaborated by us (Sato & Sato, 1987*a*).

Results

X-Ray Microanalysis

As shown in Fig. 1, the fine morphology of the sweat gland was well preserved in freeze dried cryosections with minimum structural damage. All three types of cells in the secretory coil were easily

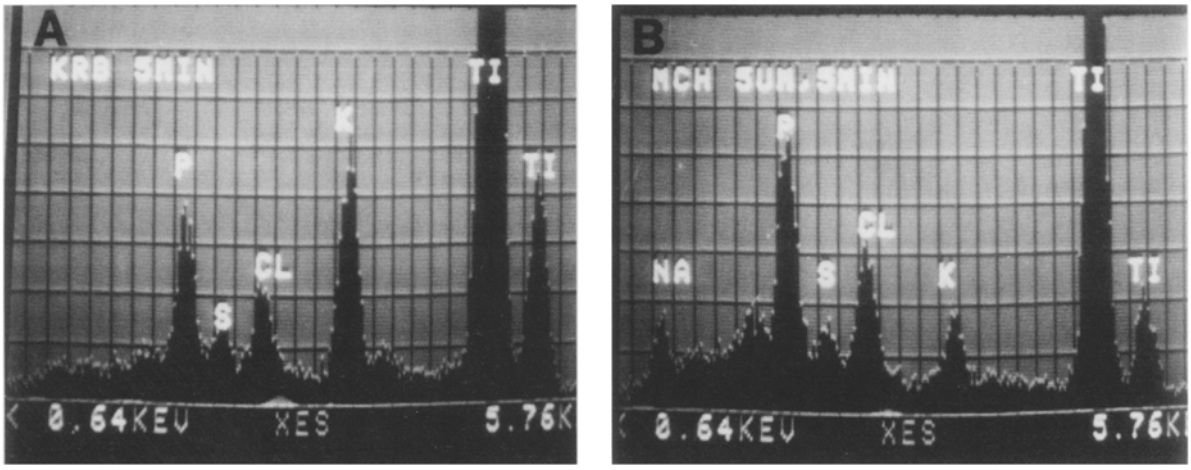

Fig. 2. Illustrative examples of X-ray spectra obtained from the cytoplasm of a resting (incubated in KRB, *A*) and a MCh-stimulated (at 5×10^{-6} M for 5 min) secretory cell (*B*). Note the increase in the height of the Na peak and the decrease in the height of the K peak after MCh stimulation (*B*)

Table. Concentrations of elements in the cytoplasm, nucleus and dark cell granules in unstimulated sweat secretory coil cells

	Na	K	Cl	P	S	Ca
Clear cell						
Cytoplasm	81 ± 10	547 ± 20	277 ± 10	372 ± 14	98 ± 2	12 ± 2
Nucleus	55 ± 3	566 ± 15	244 ± 20	421 ± 17	76 ± 4	17 ± 7
p	NS	NS	NS	NS	NS	NS
Dark cell						
Cytoplasm	86 ± 13	517 ± 15	286 ± 18	350 ± 16	75 ± 5	10 ± 7
Nucleus	88 ± 14	558 ± 11	248 ± 9	360 ± 8	90 ± 4	11 ± 2
p	NS	NS	NS	NS	NS	NS
Granules	73 ± 14	254 ± 11	251 ± 4	238 ± 10	128 ± 3	85 ± 7
Myoepithelial cell						
Cytoplasm	80 ± 7	416 ± 12	279 ± 6	298 ± 17	153 ± 8	12 ± 2
Nucleus	83 ± 11	414 ± 24	246 ± 9	369 ± 19	121 ± 7	13 ± 3
p	NS	NS	$P < 0.01$	$P < 0.05$	$P < 0.05$	NS

Concentrations are given as mmol/kg dry mass ± SEM of from 8 to 17 cells. *p*, significance calculated by Student's *t* test, unpaired and double tailed (for the cytoplasm and nucleus in each cell type). NS, $P > 0.05$.

discerned in the cryosections because dark cells line along the lumen and contain electrondense dark cell granules whereas clear (or secretory) cells locate around the intercellular canaliculi. Myoepithelial cells are filled with characteristic smooth muscle fibers and are present at the basal side of the tubule (Fig. 1*B*). In all cell types, cell boundary, nuclear envelope, mitochondria and dark cell granules were clearly distinguished in the image on the CRT.

In targeting the electron beam on the clear cell cytoplasm, efforts were made to select the most homogeneous perinuclear areas; however, the X-ray emission spectra from the more vesicular areas of cytoplasm (presumably endoplasmic reticulum or Golgi apparatus) was not significantly different from that of the relatively homogeneous perinuclear areas (*not illustrated*). Illustrative X-ray spectra from the clear cell cytoplasm before (panel *A*) and after (panel *B*) stimulation with 10^{-6} M MCh were shown in Fig. 2. The spectrum from the unstimulated clear cell shows the predominance of P, Cl and K, whereas the Na peak was inconspicuous. The Ti peak is most likely due to the grid. In the MCh-treated secretory cells (at 10^{-6} M for 5 min), the Na peak became more prominent and the K peak less prominent than in control.

In the Table the elemental concentrations of the cytoplasm are compared with those of the nucleus

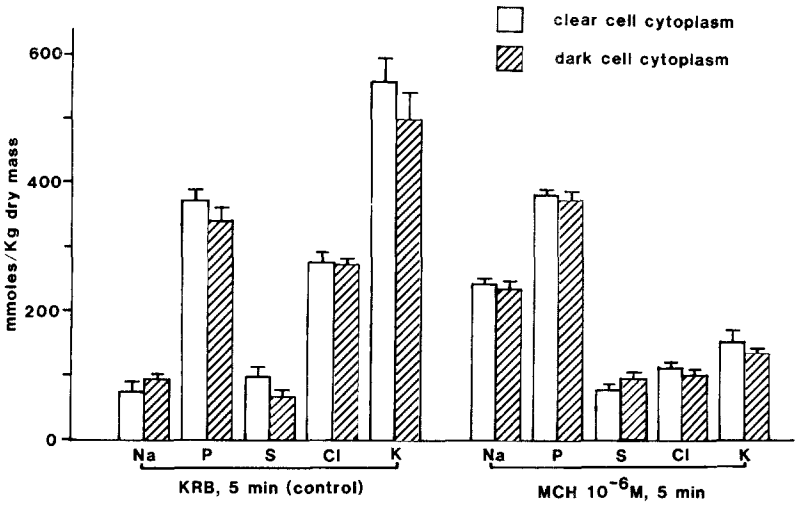

Fig. 3. Effect of MCh on elemental concentrations in clear and dark cell cytoplasm as determined by X-ray microanalysis. Each column is the mean ± SEM mmol per kg dry mass from 12 to 17 cells incubated for 5 min with or without 10^{-6} M MCh. No significant difference was seen in elemental concentrations between clear and dark cells in either control or stimulated glands ($P > 0.05$, by Student's t test, unpaired one tail) except in [S] in control glands ($P > 0.05$)

in all three cell types (i.e., clear, dark and myoepithelial) of unstimulated secretory coils (after 5 min of pre-incubation in KRB at 37°C). A considerable similarity was noted in the elemental composition between the cytoplasm and nucleus in both clear and dark cells in the resting state (Table) as well as after stimulation with MCh (not included in the Table).

Interestingly, the dark cell granules were characterized by low [K] and high [S] and [Ca]. In the myoepithelial cell cytoplasm, [K] was also slightly lower than in clear and dark cells but the concentrations of other elements were rather comparable with those in clear and dark cells. The relatively low [K] in the dark cell granules and the myoepithelial cell may be partially due to their high tissue dry mass fractions (e.g., 670 ± 34 counts per 100 sec of dry mass continuum for the clear cell cytoplasm, 670 ± 16 for the dark cell cytoplasm, 714 ± 21 for dark cell granules and 870 ± 41 for the myoepithelial cell cytoplasm) and thus the smaller water content, which reduces the ratio of elemental peak count to the continuum count and underestimates the true elemental concentrations.

As illustrated in Fig. 3, both clear and dark cells showed an identical change in [Na], [K] and [Cl] after stimulation with MCh, suggesting that these cells behave like a functional syncytium. Myoepithelial cells also responded to MCh by increasing its cytoplasmic [Na] and decreasing [K] (Fig. 4) but, interestingly, the decrease in [Cl] was only 20%.

The time course of the change in cytoplasmic [Na], [K] and [Cl] after stimulation with 10^{-6} M MCh is shown in Fig. 5. Upon stimulation there was a drastic decrease in intracellular [K] and [Cl], i.e., from 560 to 125 and from 275 to 110 mmol/kg, respectively, and an increase in [Na] from 75 to 273

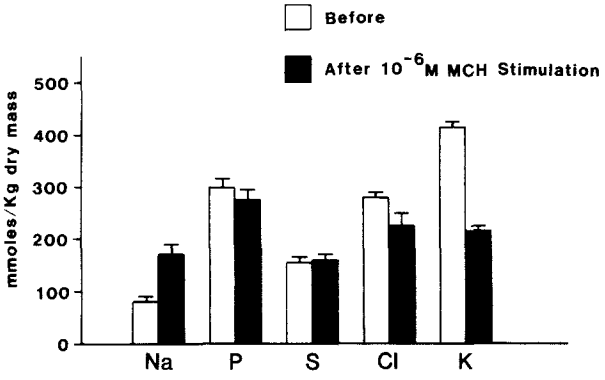

Fig. 4. Effect of MCh on elemental concentrations in myoepithelial cell cytoplasm as determined by X-ray microanalysis. Each column is the mean ± SEM of from 7 to 11 cells

mmol/kg. The cytoplasmic electrolyte concentrations, however, plateaued after 2 to 3 min MCh-stimulation and remained unchanged thereafter for as long as 60 min. In the insert to Fig. 5 are shown two curves, the upper curve representing [Na] + [K] and the lower curve [Cl], which were replotted from the main panel of the same Figure. As can be seen, the wide separation of the two curves indicates the presence of a large anion deficit (i.e., the [Na]+ [K] − [Cl]) of about 300 mmol/kg, at rest as well as after MCh stimulation. Furthermore, the magnitude of the change in the sum of cations ([Na] + [K]) after MCh stimulation was the same as that of [Cl], suggesting a net loss of cellular electrolytes, most likely KCl (because cellular [Na] has increased) and, if so, the resultant cell shrinkage.

The pharmacological specificity of the effect of MCh on cellular ion concentrations is best shown by the recovery of [Na], [K] and [Cl] to the pre-stimulation levels after the addition of 5×10^{-7} M

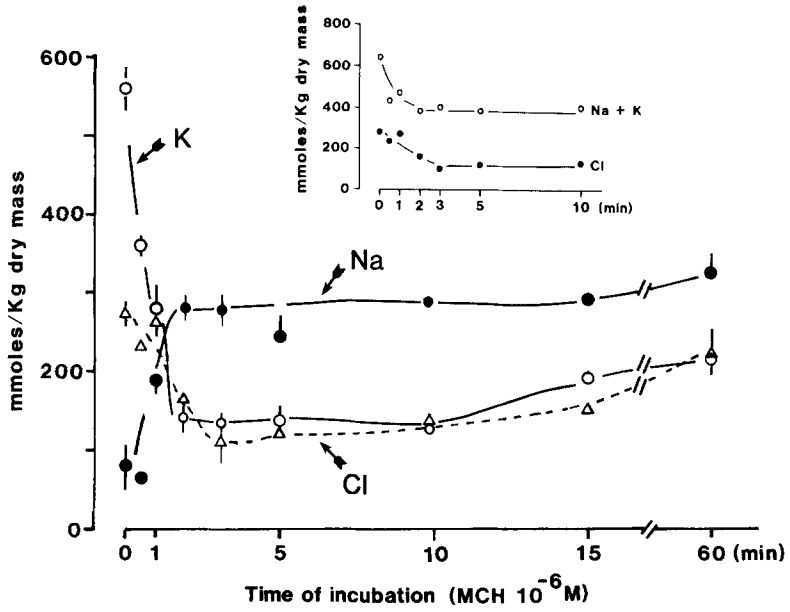

Fig. 5. Time course of the effect of 10^{-6} M MCh on cytoplasmic Na, K and Cl concentrations in the secretory cell cytoplasm as determined by X-ray microanalysis. Each plot is the mean ± SEM of from 7 to 17 cells. In the insert, the sum of [Na] + [K] is compared wtih [Cl] over the incubation period of up to 10 min. Note that the two curves show a parallel change

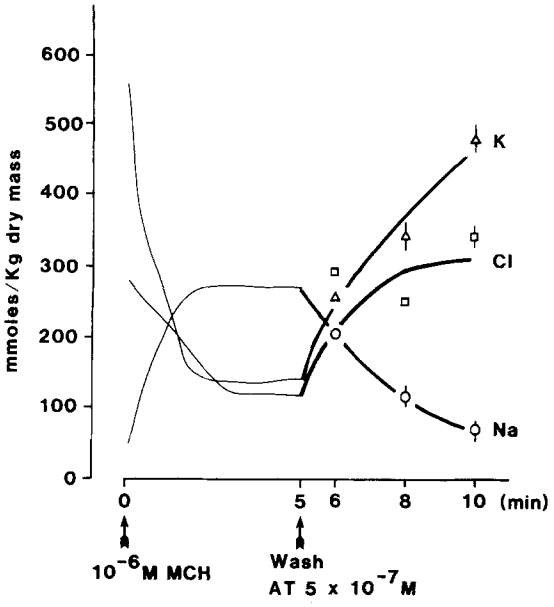

Fig. 6. Reversibility of MCh-induced change in [Na], [K] and [Cl] by atropine (AT). After 5 min of incubation with MCh, 5×10^{-7} M AT was added to the medium. The curves before addition of AT (i.e., 0–5 min) are replotted from Fig. 5. Each plot is the mean ± SEM of from 6 to 8 cells

atropine (Fig. 6). Likewise, in the presence of 5×10^{-7} M atropine, 10^{-6} M MCh showed no effect on intracellular [Na], [K] and [Cl] for as long as 1 hr of incubation ($n = 6$, *not illustrated*). The dose response to MCh of the change in intracellular [Na], [K], and [Cl] also showed saturation kinetics (Fig. 7) similar to that of [MCh] *vs.* sweat rate in vitro (Sato & Sato, 1981*a*).

In a preliminary attempt at elucidating the mechanism of the MCh-induced change in intracellular [Na], [K] and [Cl], we studied whether some of the manipulations known to inhibit sweat secretion alter the effect of MCh on cellular electrolyte concentrations. First, the effect of Ca-free medium (which completely inhibits cholinergic sweat secretion in vitro (Sato & Sato, 1981*b*) was studied on intracellular [Na], [K] and [Cl]. As seen in Fig. 8, reduction of extracellular $[Ca]_{free}$ to the nanomolar range (by addition of 0.2 mM EGTA to the nominally Ca-free KRB) almost completely abolished the effect of MCh. It is of interest to note that Cl gradually decreased to near the control level over the 15-min period in the Ca-free medium. Its significance is not certain, however, because at 5 min the decrease in $[Cl]_i$ is very small and prolonged stimulation with MCh in the Ca-free medium irreversibly impairs the viability of the glands (Sato & Sato, 1981*a*). As expected, inhibition of Na-K exchange pump with 10^{-4} M ouabain decreased [K] and increased [Na] over the 30 min of incubation (Fig. 9). However, unlike MCh which decreased [Cl], ouabain increased it slowly over the course of incubation. Bumetanide, a widely used inhibitor of a Na-K-2Cl cotransporter (Palfrey, Feit & Greengard, 1980) inhibits MCH-induced sweating in vitro at high concentrations (e.g., 10^{-4} M) and abolishes the MCh-induced lumen-negative potential difference in the isolated sweat secretory coil (Sato & Sato, 1987*b*).

In the present study, secretory coils were incubated with 10^{-4} M bumetanide in the presence and absence of 5×10^{-6} M MCh. In the presence of bumetanide (but without MCh), [Na] remained un-

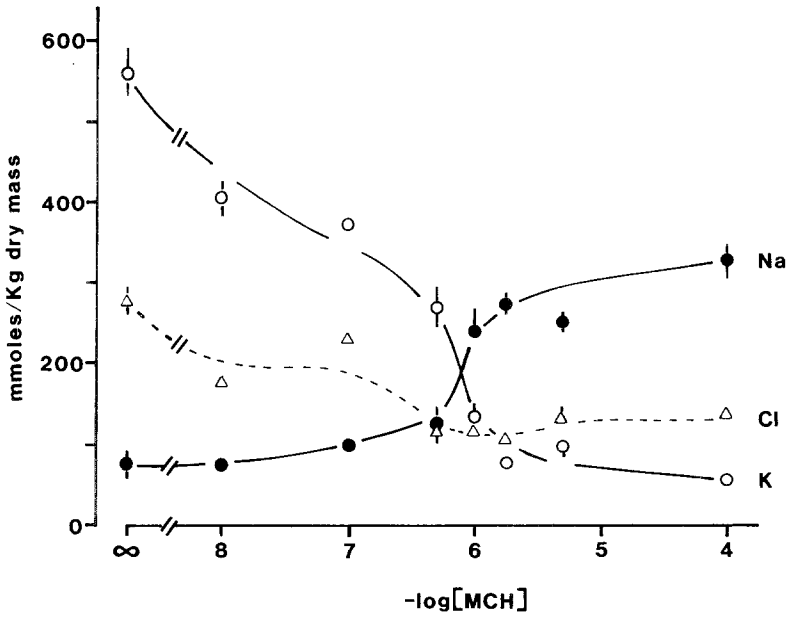

Fig. 7. Dose response to MCH of cytoplasmic concentrations of Na, K and Cl as determined by X-ray microanalysis. Isolated sweat glands were incubated in various concentrations of MCh for 5 min before freezing. Each plot is the mean ± SEM of from 12 to 18 cells

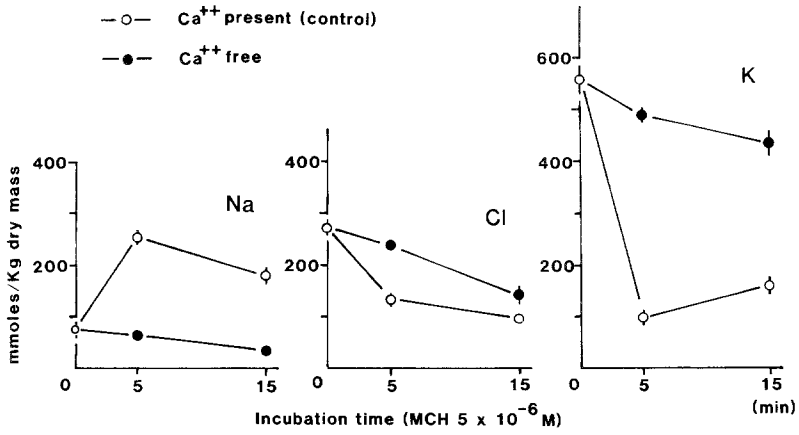

Fig. 8. Effect of Ca-free medium on MCH-induced change in cytoplasmic [Na], [K] and [Cl]. For the Ca-free system, isolated sweat glands were preincubated in Ca-free KRB (also containing 0.2 mM EGTA, which reduces $[Ca]_{free}$ to below 4 nM (Saga et al. 1988) for 5 min and at time zero 5×10^{-6} M MCh was added. Each plot is the mean ± SEM of from 15 to 17 cells

changed but [K] and [Cl] changed by less than 15% (Fig. 10). Puzzlingly enough, the MCh-induced decrease in [K] was decreased by 50% in the simultaneous presence of bumetanide. In contrast, the MCh-induced decrease in cellular [Cl] was slightly enhanced ($P < 0.001$). Furthermore, bumetanide completely abolished the increase in [Na] normally seen with MCh alone.

Determination of Tissue [Na] and [K] by Helium Glow Spectrophotometry

One of the technical difficulties involved in determining the tissue electrolytes by acid extraction and microtitration is to eliminate contamination of cellular electrolytes by extracellular ions by effectively washing the tissue without losing intracellular ions. Figure 11 shows that the present method is instrumental in removing the extracellular ions without significantly losing intracellular Na and K as seen by the fact that [K] decreased from 110 to 104 mM during the first vigorous washing in Tris buffer for 0.5 sec while total [Na] dropped from 207 (most of which represents the extracellular Na) to 32 mM. Furthermore, between 0.5 and 5 sec of vigorous washing, neither [Na] and [K] changed significantly. Thus in studying the effect of 5×10^{-6} M MCh on total extractable tissue [Na] and [K], we washed the tissue for 1 sec. The ionic concentrations in the secretory coil were conveniently expressed as millimoles per liter of glandular volume determined photometrically (Sudo & Morel, 1984), recognizing that the cellular volume may be somewhat overestimated (because it also includes some periglandular extracellular space, the small luminal space and the space occupied by the structural proteins) and thus the actual cellular ionic concentra-

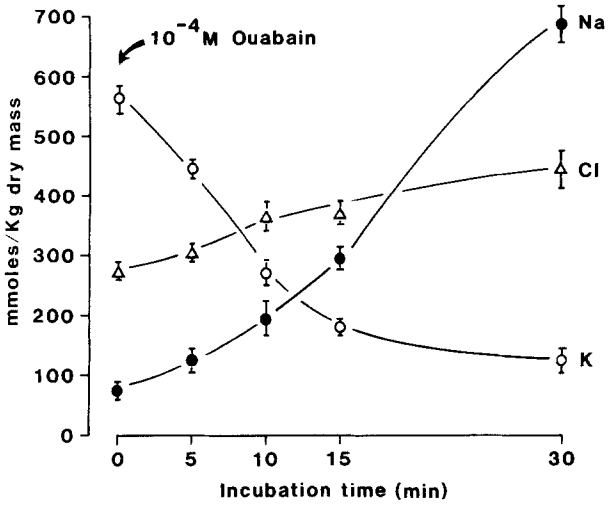

Fig. 9. Time course of the effect of 10^{-4} M ouabain on cytoplasmic [Na], [K] and [Cl] in secretory cells as determined by X-ray microanalysis. Ouabain was added at time 0. Each plot is the mean ± SEM of from 12 to 17 cells

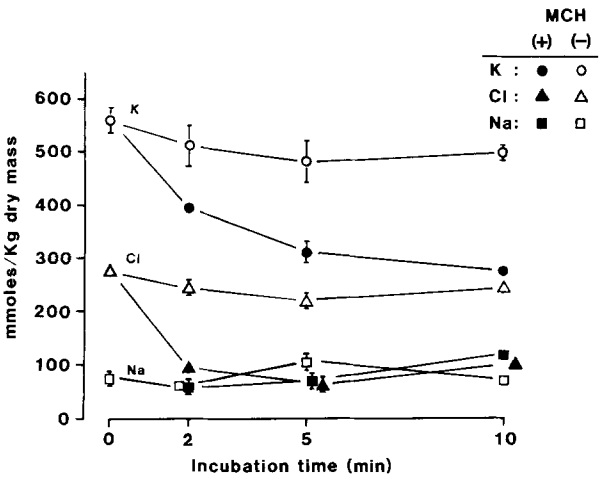

Fig. 10. Effect of 10^{-4} M bumetanide on MCH-induced change in cytoplasmic [Na], [K] and [Cl]. After preincubation in KRB for 5 min, 10^{-4} M bumetanide alone (empty symbols) or bumetanide plus 5×10^{-6} M MCh (solid symbols) was added to the medium. Each plot is the mean ± SEM of from 7 to 17 cells

tions underestimated. Nevertheless, as shown in Fig. 12, MCh decreased [K] from 92 to 32 mM and increased [Na] from 35 to 59 mM during the 5 min of incubation, with the time course similar to that observed with X-ray microanalysis (*see* Fig. 5). As expected, 10^{-6} M atropine added at 5 min partially reversed the MCh-induced cellular electrolyte changes.

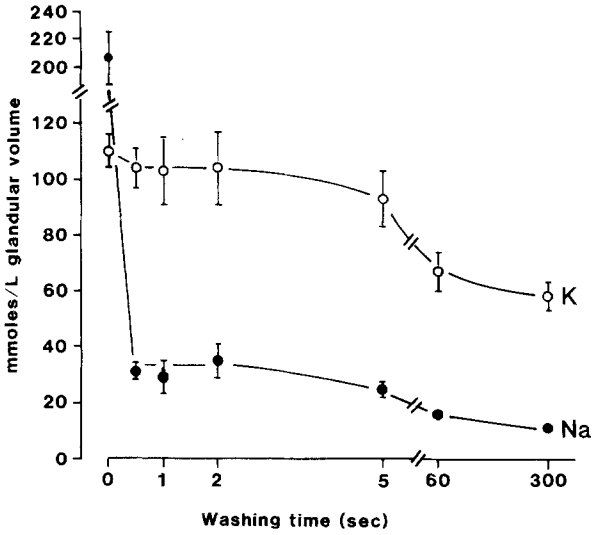

Fig. 11. Effect of washing time on tissue contents of Na and K as determined by microtitration with a helium glow spectrophotometer. The plots at time zero are the glands taken out of KRB and the periglandular medium simply removed by suction on the sieve at the tip of a suction pipette (*see* text for details of methodology). All other glands were vigorously washed by immersing the sweat glands at the tip of the suction pipette in isotonic Tris buffer under constant suction for the indicated periods. Na and K concentrations were calculated based on the assumption that the extracellular volume is zero. Each plot is the mean ± SEM of six secretory coil segments

Discussion

Subcellular and Cellular Distribution of Various Elements

Although various analytical approaches have been employed for electron probe X-ray microanalysis of cryosections, the Hall's (1979) continuum normalization method is perhaps the most widely used (Sasaki et al., 1983; Izutsu et al., 1985; Izutsu & Johnson, 1986; Saubermann, 1986). To the best of our knowledge, the present study is the first to employ X-ray microanalysis for determination of intracellular elemental concentrations in the isolated eccrine sweat secretory coils.

Recapitulating the present result, we found that both perinuclear cytoplasm and nucleus of both clear and dark cells contain similar concentrations of Na, P, S, Cl, Ca and K in unstimulated and MCh-stimulated conditions, suggesting either that clear and dark cells behave like a functional syncytium or that both clear and dark cells are endowed with similar cholinergic receptor function and/or membrane transport activity. It also appears that the nu-

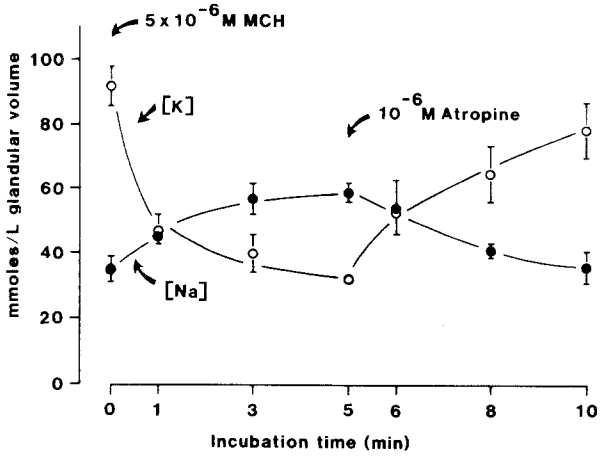

Fig. 12. The time course of the effect of 5×10^{-6} M MCh on total glandular Na and K contents as determined by microtitration with a helium glow spectrophotometer. Five minutes after MCH stimulation, 10^{-6} M atropine was added to the incubation medium. [Na] and [K] were conveniently expressed as millimoles per liter of glandular volume determined photometrically (*see* Materials and Methods for details). Each plot is the mean ± SEM of from 5 to 7 secretory coils

clear membrane is freely permeable to [Na], [K] and [Cl]. Like the secretory granules of the salivary glands, dark cell granules of the sweat gland contained higher [S] and [Ca] and lower [K] than did the cytoplasm. In the myoepithelial cells, concentrations of all the elements are about 30% lower. However, this may be partially due to the fact that the myoepithelial cells have higher dry mass fractions than in clear and dark cell cytoplasm and thus the true elemental concentrations are somewhat underestimated (Sauberman, 1986; Smith & Cameron, 1987). The possible origins of tissue S, P and Ca have been speculated in previous reports by others (Nakagaki et al., 1984; Izutsu & Johnson, 1986) and will not be repeated here.

Changes in [Na], [K] and [Cl] during Stimulation with MCh

The present data have clearly shown that MCh decreased intracellular [K] and [Cl] and increased [Na] to the same extent in both clear and dark cells and to a somewhat smaller extent in myoepithelial cells. That these MCh-induced ionic changes are not simply due to experimental artifacts such as cell damage during tissue processing is readily shown because the effect of MCh was dose dependent and inhibitable by atropine. Furthermore, MCh-induced ionic changes were nearly completely inhibited in a Ca-free medium, which also inhibits cholinergic sweat secretion (Sato & Sato, 1981*b*). Other potential sources of artifact, such as contribution of cytoplasmic vesicles containing extracellular fluid or biased scanning of the cytoplasm-containing plasma membrane infoldings also containing extracellular fluid, can be readily ruled out because during MCh stimulation [Cl] also decreased, rather than increased, and because the nucleus showed the same electrolyte concentrations as the cytoplasm before and after MCh stimulation.

As expected, the only experimental condition where cytoplasmic electrolyte concentrations approached those of extracellular medium was when the secretory coils were poisoned with ouabain for 30 min. The fact that the change in [Na] and [K] as determined by helium glow spectrophotometry qualitatively agreed with that of X-ray microanalysis further supports the validity of our methodology and the results. The decrease in cytoplasmic [K] and the increase in [Na] during cholinergic stimulation have also been reported in the rat parotid gland (Izutsu & Johnson, 1986), the dog submandibular acini (Sasaki et al., 1983), and the dog pancreatic acini (Nakagaki et al., 1984). However, in contrast to the sweat secretory coils, [Cl] was increased by cholinergic stimulation in these exocrine acini.

One of the most surprising observations in the present study is that as much as 65% (by microtitration) or 75% (by X-ray microanalysis) of cellular K left the cell during the first 3 min of stimulation with 10^{-6} M MCh (which usually yields the maximal sweat rate; Sato & Sato, 1981*a*). As shown in the insert to Fig. 5, the sum of [Na] and [K] decreased by 220 mmol/kg dry mass (or 30% of the control level) and [Cl] decreased in parallel, suggesting that it was mainly KCl that left the cell. If so, the net KCl efflux should result in cell shrinkage unless other ions or osmotically active solutes simultaneously accumulate in the cell to keep the cellular osmolarity constant.

We have previously reported that the sweat secretory coil contracts vigorously upon MCh stimulation (Sato, 1977; Sato, Nishiyama & Kobayashi, 1979) and concluded that it is due to myoepithelial contraction. Photographic analysis of tubular contraction suggests that the tubular volume can easily decrease by 30% (Sato, 1977) during MCh stimulation. Thus, it remains unknown whether the observed net KCl efflux is the result of mechanical compression of cells by myoepithelial contraction or whether cell shrinkage is the consequence of primary KCl efflux during the maximal cholinergic stimulation or a combination of the two. If cell

shrinkage indeed occurs during MCh stimulation, then the continuum normalization method underestimates (thus overestimates the change in) cellular ionic concentrations during MCh stimulation because of the resultant increase in dry mass fractions and the decrease in cell water volume. Thus we tentatively surmise that the true magnitude of the decrease in [KCl] during 10^{-6} M MCh stimulation may not have been as much as 75% but was actually around 65% as determined by the microtitration method.

Interpretation of the Cellular Ionic Changes in Relation to the Ionic Mechanism of Sweat Secretion

At present, the ionic mechanism of cholinergic sweat secretion is not well understood. However, some of the indirect observations suggest that Na-K-2Cl cotransport mechanism (Geck & Heinz, 1986) may be at least in part involved in cholinergic sweat secretion. For example, we observed the inhibitory effect of loop diuretics (although at high concentrations) on sweating in vitro (Sato & Sato, 1987*b*), predominantly basolateral localization of paranitrophenyl phosphatase activity (equivalent of Na-K-ATPase) (Saga & Sato, 1988), a high K permeability of the basolateral membrane (Sato, 1986), a lumen negative potential (Sato & Sato, 1982), the depolarization of luminal membrane potential during MCh stimulation presumably due to the increase in Cl permeability (Sato, 1986), and secretion of Cl across the epithelial wall against the electrochemical potential gradient (Sato & Sato, 1987a). It should be noted, however, that the Na-K-2Cl cotransport model calls for complete recycling of Na and K across the basolateral membrane and thus does not explain the massive KCl efflux and a 3.7-fold increase in [Na] during MCh stimulation in the sweat secretory cells. How, then, should we interpret the observed MCh-induced ionic changes relative to the proposed ionic mechanism of sweat secretion?

In the present study, the actual intracellular concentrations of three major ions, Na, K and Cl, were not directly determined on the wet weight basis (because Hall's continuum normalization method was used and because the possibility of cell shrinkage cannot be ruled out during MCh stimulation). However, we can still estimate the range of cellular [Na], [K] and [Cl] under two different assumptions. First, we assume that the sum of the major cations, Na and K, is always isotonic at 155 mM inside and outside the cell, and second, the dry mass fraction in the cytoplasm is constant and is equivalent to 20% BSA before and after MCh stimulation (thus ignoring the consequence of possible cell shrinkage; concentrations estimated according to the second assumption are shown in parentheses). Then the estimated cytoplasmic ionic concentrations in mM are Na = 17 (17.5), K = 138 (140) and Cl = 68 (68.8) before stimulation and Na = 103 (70), K = 52 (35) and Cl = 41 (27.5) after stimulation with 10^{-6} M MCh. As expected, [Na] and [K] (on the basis of glandular volume) determined by the microtitration method (Figs. 11 and 12) are different from the above values because the actual cytoplasmic fluid volume may be at least 20 to 30% smaller than the total glandular volume and because the extracellular volume that includes both the basal membrane infoldings and luminal as well as intercellular canalicular space (containing fluid of predominantly plasma-like composition) could occupy another 10–15%.

If the electrically neutral Na-K-2Cl cotransporters indeed occur at the basolateral membrane, the sum of the chemical potential gradient for Na, K, and Cl is 2.8 mV (1.2 mV) at rest and 7.7 mV (49.5 mV) after MCh stimulation in favor of coupled NaCl/KCl influx into the cell [in theory, the electrical potential difference of about −70 mV across the basolateral membrane, which depolarized by only a few mV by the steady state of MCh stimulation (Sato, 1986), should not affect the transport of ions carried by electrically neutral cotransporters]. On the other hand, if the electrically neutral Na-Cl cotransporter is involved, the sum of chemical potentials for NaCl influx is 73 mV (72 mV) at rest and 39.2 mV (59.8 mV) down the gradient during MCh stimulation.

Although both transporters have favorable chemical potential gradients (irrespective of assumptions for estimating cellular ionic concentrations), the Na-Cl cotransporter appears to have a more favorable gradient than the Na-K-2Cl cotransporter. However, in the Na-Cl model, the driving force decreases during MCh stimulation. At the luminal membrane, the electrochemical gradients for Cl is 52.7 mV (52.0 mV) at rest and 37.7 mV (20.3 mV) during MCh stimulation in favor of Cl movement from the cell interior into the lumen [assuming luminal sweat Cl concentrations of 146 mM during MCh stimulation and 127 mM at rest (Sato & Sato, 1987*a*) and the apical membrane potential of 70 mV at rest and 62 mV during MCh stimulation (Sato, 1986)]. These electrochemical gradients favor the

conductive movement of Cl across the apical membrane, which is one of the requirements for the cotransport models.

Inhibition of transport processes by loop diuretics, bumetanide or furosemide, is generally regarded as supportive evidence for the involvement of Na-K-2Cl cotransporters (Palfrey et al., 1980; Palfrey, Silva & Epstein, 1984). In the present study, 10^{-4} M bumetanide alone caused a very small decrease in [K] and [Cl] without a change in [Na] in unstimulated secretory coils (Fig. 10). This is not inconsistent with the notion that the Na-K-2Cl cotransporter is involved in maintenance of resting cellular electrolytes because in the absence of Na-K-2Cl influx into the cell, cell may lose K and Cl while Na may not change in the absence of its influx by the cotransporter. However, when K-influx mediated by the cotransporter is inhibited by bumetanide, MCh-induced K efflux should be enhanced (or at least unchanged). Puzzlingly enough, however, the opposite has been observed: bumetanide reduced the MCh-stimulated K-efflux by about 50% (Fig. 11). This unexpected effect of bumetanide was also observed when MCh-induced K efflux from the secretory coils was determined with an extracellular K electrode (Saga, Sato & Sato, 1988). On the other hand, the inhibition by bumetanide of the MCh-induced increase in [Na] is consistent with the notion that Na influx is mediated by bumetanide-sensitive cotransport process(es).

Other requirements of membrane transport for the cotransport model include basolateral Ca-dependent K channels and apical Ca or cAMP-dependent Cl channels. The present observation that MCh-induced K and Cl efflux is strictly dependent on $[Ca]_{free}$ in the medium (Fig. 8) is indirectly supportive of the involvement of Ca-dependent K and Cl channels. Furthermore, our recent report that intracellular [Ca], as determined by quin 2, increases during MCh stimulation in a pharmacologically specific manner (Sato & Sato, 1988) also supports such a thesis. Thus, although the present analysis of cytoplasmic ionic concentrations by X-ray microanalysis cannot determine whether bumetanide-sensitive cotransporter(s) are indeed involved, it has provided initial data on the overall ionic movement during muscarinic cholinergic stimulation in the sweat gland and will be useful for further studies on the ionic mechanism of sweat secretion.

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