Intracellular Calcium Store and Transport of Elements in Acinar Cells of the Salivary Gland Determined by Electron Probe X-ray Microanalysis

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Abstract Electron probe X-ray microanalysis using freshly frozen hydrated and dried thin sections of dog submandibular gland was performed to determine the distribution of elements and water in the acinar cells of resting and stimulating states. The results obtained are as follows: (a) The secretory granules contained high concentrations of Ca and S while high concentrations of K and P were present in the cytoplasm and/or nucleus of acinar mucus cells of the gland in the resting state. (b) With pilocarpine stimulation, the concentration of Ca increased in the cytoplasm and decreased in the secretory granules, while there was an increase in the concentration of Na and Cl in both the cytoplasm and secretory granules of the cells. (c) The local dry-mass fractions of acinar cells, estimated by comparing the continuum radiation of X-ray spectrum from the frozen hydrated sections with that from the frozen dehydrated sections, were approximately 20 and 33% in the cytoplasm and secretory granules of resting acinar cells, respectively, and each value was not significantly altered under conditions of stimulation having a tendency to decrease slightly. Therefore, the passive Na and Cl influx and the cytoplasmic Ca flowed in from extracellular spaces and released from secretory granules, an intracellular calcium store, by secretory stimulation probably triggers the passive or active Na and Cl extrusion and consequently the osmotic water flux from the basal part of acinar cells to the secretory granules and the lumen, as well as the serial exocytosis of the granules in the luminal side of the acinar cells.

Key Words: electron probe X-ray microanalysis, dog salivary gland, pilocarpine stimulation, electrolyte transport, intracellular calcium store.

Saliva is primarily secreted in the acini of the salivary glands and is followed by a secondary process of reabsorption in the duct system (Young and Martin, 1982).
Electron microscope observations revealed that the acinar cells of these glands occupy the bulk of the cytoplasm in the form of secretory granules. It is considered that with nerve stimulation, the contents of the secretory granules of the acinar cells, amylase, mucin, and several ions as well as water, are secreted into the glandular lumen by an exocytotic mechanism (Nakagaki et al., 1978). Recent studies (Nielsen and Petersen, 1972) on the secretory process in the acinar cells of the salivary gland using tracers such as $^{45}$Ca have drawn attention to the distribution of calcium and other ions in these cells and to the intracellular transport of ions in the secretory stimulating state of the glands.

We carried out electron probe X-ray microanalysis of freshly frozen dried and hydrated sections to determine the elemental distribution and concentration in the acinar cells of the salivary gland in the resting and stimulating states, with special reference to the ionic content of cytoplasm and secretory granules, using the standard albumin frozen thin sections and the calibration curves for elemental concentration-peak/back ratio (Moreton et al., 1974; D"orge et al., 1978). Preliminary reports have been made elsewhere (Sasaki et al., 1979, 1980).

**METHODS**

*Electron probe X-ray microanalysis.* Small pieces of submandibular gland were excised from adult dogs anesthetized with sodium thiopentobarbiturate (30–40 mg/kg) given intravenously. The specimens from the glands of normal and low Na, Cl (Na 95, Cl 85 mM/kg water) extracellular conditions in the resting or secretory stimulating states (pilocarpine 1 mg/adult dog, 1 min after intravenous injection), were mounted in Krebs-Hensel solution containing 20% bovine serum albumin (BSA), on copper specimen holders, and rapidly frozen by pressing against the wall of a copper block which had been pre-cooled to $-196^\circ$C in liquid nitrogen. Frozen sections were cut into 100–200 nm at $-130^\circ$C with glass knives on a Frozen Thin Sectioner of Porter-Blum MT-2 ultramicrotome, placed on pre-cooled gold or copper grids covered with collodion film and flattened on the grids to be stamped with liquid nitrogen-chilled, polished copper rods. Both gold and copper grids were used because the Cu L peak and the Au M peak interfered with the Na peak and P and S peaks in the energy dispersive spectrum of X-ray microanalysis, respectively. In some cases, nickel and titanium grids were also used. For X-ray microanalysis of frozen dried sections, the grids with frozen thin sections were transferred to a liquid nitrogen-chilled glass vial and transported in liquid nitrogen to an FTS freezing-drier (FTS Systems, Inc., Stone Ridge, N. Y., U. S. A.) where the frozen thin sections were dried at $-85^\circ$C by pumping at $10^{-8}$–$10^{-4}$ Torr overnight. The grids with frozen dried thin sections were carbon-coated and loaded onto a cold stage of a Hitachi H-500 transmission electron microscope modified to accept a Kevex Si (Li) X-ray detector (Kevex Corp., Burlingame, Calif., U. S. A.) inter-
faced with Kevex 5100 or 7000 multichannel analyzer. The probe currents used ranged from $10^{-8}$ to $10^{-10}$ A and the probe diameter was 100 nm. The detecting time for obtaining X-ray spectra was 100 sec. For the X-ray microanalysis of frozen hydrated sections, immediately after the sectioning of frozen thin sections, the grids with frozen sections were transferred in the cryochamber of the Frozen Thin Sectioner of the ultramicrotome to a cooling specimen holder of Hitachi H-500 electron microscope (H5001C), which had been pre-cooled to $-130^\circ$C with liquid nitrogen. The tip of the cooling specimen holder with frozen hydrated sections was withdrawn inside a metal tube pre-cooled with liquid nitrogen and then transported in liquid nitrogen to the cold stage of electron microscope to avoid the absorption of moisture from the atmosphere (GUPTA et al., 1977). The X-ray microanalysis was performed at $-130^\circ$C as described above, and then inside the column of the electron microscope, the specimens were warmed and dehydrated by charging an electric current and by heating the tip of the cooling specimen holder. The X-ray microanalysis of frozen dehydrated sections was also performed to determine the values of the local dry-mass fractions of the cells (GUPTA et al., 1977, 1978). The measurement of elemental concentrations in the spots of the specimens was performed according to the methods reported by other workers (SOMLYO et al., 1977; GUPTA et al., 1977; DÖRGE et al., 1978), using the characteristic peak X-ray continuum ratio of the energy dispersive spectrum obtained. The concentration was read off the graph of the elemental concentration-peak/back ratio, applying the peak/back ratios of the four elements (Ca, K, Na, Cl) from the X-ray spectra of acinar mucous cells in the resting and secretory stimulating state to the standard curve of the graph which was made using X-ray spectra from the standard albumin frozen thin sections containing various concentrations of elements and bovine serum albumin. The concentration of the extracellular space of acinar mucous cells which was immersed with Krebs-Henseleit solution containing 20% albumin, was also read off the graph, and our quantitative X-ray microanalysis was always checked up. Calculation of elemental concentrations was performed by means of special utility programs for a few kinds of microcomputer.

**Conventional electron microscopy.** The specimens from animals in the resting state were fixed successively in glutaraldehyde and osmium, dehydrated in ethanol and embedded in Epon 812. After ultrathin sectioning with an ultramicrotome, the specimens were stained with uranyl acetate and lead citrate and observed under an electron microscope at an accelerating voltage of 100 kV.

**RESULTS**

**Electron microscopy**

Figure 1 shows the transmission electron micrographs of a conventionally fixed and embedded ultrathin section (Fig. 1A) and freshly frozen dried sections (Fig. 1B, C) of resting dog submandibular glands. The acinus of the gland is com-
Fig. 1. Electron micrographs of conventionally Epon 812 embedded and freshly frozen dried thin sections of dog submandibular glands in the resting state. A: a conventional transmission electron micrograph of an acinus of submandibular gland. The micrograph shows that the serous (Se) and myoepithelial cells (My) lie on the basal side of mucous cells (Mu) which surround the glandular lumen (L) of acinus and are occupied in the bulk of cytoplasm by secretory granules (G). ×2,700. Bar, 10 µm. B: a scanning transmission electron micrograph of a frozen dried thin section cut from the freshly frozen sub-
posed of serous cells, myoepithelial cells making up the demilune at the basal parts of acinus and mucous cells surrounding the glandular lumen. The mucous cells are occupied in the bulk of the apical cytoplasm by numerous secretory granules, some of which have been discharged into the glandular lumen by an exocytotic mechanism. Relatively electron dense small granules are also seen in the serous cells which are located in contact with the basolateral plasma membrane of mucous cells (Fig. 1A). The scanning transmission electron microscope images of freshly frozen dried sections of submandibular glands are shown in Fig. 1B and C. The structural details in the image are consistent with those seen under a conventional electron microscope (Fig. 1B). The contrast is good and the major cellular features, even the membranes of secretory granules of mucous cells are clearly evident (Fig. 1C).

**X-ray microanalysis of frozen dried sections**

The X-ray spectra from areas in the cytoplasm, secretory granules, nucleus and 20% BSA immersed baso-lateral extracellular spaces of mucous cells in the resting state are shown in Fig. 2a, b, c, and d. The spectra show the presence of a large concentration of K and P in the cytoplasm and relatively high concentrations of Ca, K, Cl, S, and Na in the secretory granule of the cell. Our X-ray microanalysis of the nucleus and the mitochondrial matrix presented similar spectra to that seen in the cytoplasm, except that the nucleus contained a larger quantity of P (Fig. 2c). The Na peak in the spectrum of secretory granule was displaced slightly toward the Mg peak of a higher X-ray energy level. This spectrum (Fig. 2b) shows that the secretory granules do contain some Mg. In our X-ray spectra of the extracellular space, high Na and Cl peaks were present (Fig. 2d). The X-ray spectra from areas in the cytoplasm and secretory granules of mucous cells in the stimulating state of normal and low Na, Cl extracellular conditions are shown in Fig. 3a, b, c, and d. The spectra from the glands of normal extracellular condition show the presence of the Ca, Cl, and Na peaks in addition to the K and P peaks in the cytoplasm, and the K, Cl, S, Na, and small Ca peaks in the secretory granule of the cell. These elements are also present in the glandular lumen and/or exocytotic granules of mucous cell, which have discharged their contents into the lumen (Fig. 3a, b). The spectra from the glands of low Na, Cl extracellular condition show a smaller peak and a much larger peak of Ca in the cytoplasm and secretory granule,
respectively and the smaller peaks of Na and Cl in both cytoplasm and secretory granules to compare with those from the glands of normal extracellular condition (Fig. 3c, d).

**Estimation of local dry-mass fractions**

Since the main body of our measurements is concerned with the sections and standards in the dried states, the primary results are actually dry-weight concentration. However, they may be designated as suggested by GUPTA et al. (1977). Dry-weight concentration $c_d$ is converted to wet-weight concentration $c_w$ by means of the equation

$$c_w = f_d c_d$$

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Fig. 2. Representative energy dispersive X-ray spectra of cytoplasm, secretory granule, nucleus and extracellular space of acinar mucous cells of the resting gland. The specimens were prepared as in Fig. 1B and C. X-ray spectra were taken with a beam of 75 kV, probe diameter 100 nm and probe current 0.1–1 nA for 100 sec livetime. Extraneous small peaks of Au M and Si, which originate in the gold grid and the X-ray detector are not subtracted in the spectra. a: X-ray spectrum of cytoplasm. Note the high K and P peaks in the cytoplasm of acinar mucous cell. The small Na and Cl peaks are also present in the spectrum. b: X-ray spectrum of secretory granules. Note the relatively high Ca, K, Cl, and S peaks and the Na peak in the secretory granules of acinar mucous cell. c: X-ray spectrum of nucleus. Note the high K and the much higher P peaks in the nucleus of acinar mucous cell. The small Cl peak is also present in the spectrum. d: X-ray spectrum of extracellular space. The large Na and Cl peaks are seen in the extracellular space of acinar cells. The small Ca and K peaks and the S peak are also found in the spectrum.
where \( f_d \) is the local dry-mass fraction. The local dry-mass fractions of acinar cells of salivary gland were estimated by comparing the continuum radiation of spectrum from the frozen hydrated sections \((-130^\circ C)\) with that from the frozen dehydrated sections. The scanning transmission electron micrographs of a frozen hydrated section revealed few details of the cellular structure (Fig. 4A). Figure 4B shows that the image contrast in the section enhanced gradually while the specimen was warmed from \(-130^\circ \) to \(-80^\circ C\) and dehydrated by charging an electric current and by heating the tip of the cooling specimen holder, and dramatic improvement in contrast and spatial resolution occurred after the complete removal of water at 0°C and the secretory granules of acinar cells were clearly identifiable as shown in Fig. 1. Figure 5 shows the X-ray spectra of the cytoplasm and secretory granules of mucous cells from frozen hydrated sections (Fig. 5a and b) and dehydrated sections.
The local dry-mass fractions were $20.3 \pm 3.2$ S.D. % ($n=10$) and $32.7 \pm 4.1$ S.D. % ($n=10$) in the cytoplasm and the secretory granules of resting acinar mucous cells, respectively. Each value was not significantly altered in the stimulating state having a tendency to decrease slightly.

Measurements of elemental concentrations

The standard curves for elemental concentration peak/back ratio, were made using X-ray spectra from the standard albumin frozen thin sections containing various concentrations of elements (K, Ca, Na, Cl) and 5, 20, or 35% bovine serum albumin (Fig. 6). There was a linear relationship between the peak/back ratio of the various elements and elemental wet concentrations, and the slopes of lines in

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the graphs of respective elements became steeper as the dry-mass fractions of frozen sections decreased. The concentrations of Na, Cl, K, and Ca in the cytoplasm and secretory granules of acinar mucous cells in the resting and secretory stimulating state, as estimated by the method described above using frozen dried sections of standard albumin solution (20% BSA for cytoplasm and 33% BSA for secretory granule) and calibration curves for elemental concentration-peak/back ratio, are shown in Table 1. The Na, Cl, and Ca concentrations in the cytoplasm as well as the Na and Cl in the secretory granules increased, while the K in the cytoplasm and the Ca in the granule decreased when the gland was stimulated.

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DISCUSSION

The concentrations of K in the cytoplasm shown in Table 1 and Fig. 2 are in good agreement with estimations made by other workers (POULSEN and OAKLEY, 1978) and our independent experiments by the ion-selective microelectrode technique. In our microelectrode studies, the value of the K concentration in the cytoplasm of acinar cells of dog submandibular gland was 121 ± 17 S.D. mmol/kg cell water, in the resting state and such decreased in the ACh stimulating state. The ion-selective microelectrode responds directly to ionic activities, while in the electron probe X-ray microanalysis, the values refer to the elemental concentration. Therefore, it is reasonable that there should be minor discrepancies between the two different estimations. In our electron probe X-ray microanalysis, the estimations of the elemental concentrations of the extracellular space in the resting

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Table 1. X-ray microprobe measurements of elemental concentrations in the cytoplasm and secretory granules of acinar mucous cells in a resting state and a secretory stimulating state (pilocarpine).

<table>
<thead>
<tr>
<th></th>
<th>Cytoplasm</th>
<th>Secretory granule</th>
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<tbody>
<tr>
<td>Resting state</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na</td>
<td>25±12</td>
<td>36±17</td>
</tr>
<tr>
<td>Cl</td>
<td>21±11</td>
<td>67±24</td>
</tr>
<tr>
<td>K</td>
<td>115±37</td>
<td>55±14</td>
</tr>
<tr>
<td>Ca</td>
<td>—</td>
<td>49±16</td>
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| Stimulating state (pilocarpine) | |
| Na                             | 57±19             |
| Cl                             | 54±21             |
| K                              | 76±26             |
| Ca                             | 9± 5              |

Stimulating state (pilocarpine) | 74±31             | 87±29             | 71±24             | 32±15             |

Mean value ± S.D. of mean mm/kg wet weight (n=22). The concentration was read off the graph of elemental concentration-peak/back ratio, which was made using X-ray spectra from the standard albumin frozen thin sections containing 20% (for measurements in cytoplasm) and 33% (for measurements in secretory granules) bovine serum albumin and various concentrations of elements (Ca, K, Cl, Na), applying the peak/back ratios from the X-ray spectra of acinar mucous cells in a resting state and a secretory stimulating state (pilocarpine 1 mg/adult dog, 1 min after intravenous injection) to the standard curves of the graph. The scintillation counts of Cu L peak and K Kβ peak were subtracted from those of the Na and Ca peaks, respectively. Data represent mean ± S.D. obtained from 22 separate experiments (22 representative spectra).

state (immersed with Krebs-Henseleit solution containing 20% BSA: spectrum, Fig. 2d), are as follows; Na 112±29 S.D. mmol, Cl 135±24 S.D. mmol, K 8±5 S.D. mmol, Ca 3±2 S.D. mmol/kg wet weight (n=10). As these values are also quite reasonable, our microprobe technique is quantitatively valid. The P concentration is high in the cytoplasm and nucleus, as shown in Fig. 2. This is probably due to the nucleic acid content of the ribosome and/or chromatin, although the contribution by the other organic and inorganic phosphate cannot be ruled out. The high concentration of S in the secretory granules shown in these figures may be due to sulfated compounds such as proteoglycan, and secretory proteins such as amylase and mucin. The evidence that the concentration of Cl in the secretory granules (Fig. 2) is relatively high, is also in good agreement with the data estimated by the ion-selective microelectrode technique suggesting that Cl is sequestered by the vesicles and/or granules within the cells of amphibian kidney tubules (Fujimoto and Kubota, 1976).

The secretory granules in the resting state (Fig. 2b) contained a relatively high content of Ca. Most of this seemed to be in a bound form because the level is too high for free Ca. Experiments using a flame photometric procedure showed that rat submandibular glands as well as saliva contain relatively large amounts of Ca (12±5.0 S.D. mmol/kg·wet tissue, rat submandibular gland). The significance of the evidence that the secretory granules of acinar mucous cells of these subman-

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dibular glands contain Ca is not clear. One possibility is that the accumulated Ca as well as S in the secretory granules may be concerned with the condensation and/or aggregation of secretory protein, as suggested by Palade (1975) and other workers (Wallach and Schramm, 1971). On the other hand, the migration of secretory granules from basal to apical cytoplasm of acinar cells, and exocytosis of granules into the lumen in the apical portion of the cell and these being associated with the contractile proteins such as microtubules, microfilaments, and myosin in the cytoplasm around the secretory granules (Nakagaki et al., 1978), as in the case of posterior silk gland cells (Sasaki and Tashiro, 1976a, b), may require an appropriate concentration of calcium ion which is leaking out passively from the secretory granule, an intracellular calcium store. Moreover, the membrane of secretory granules may contain a calcium pump such as Ca$^{2+}$, Mg$^{2+}$-dependent ATPase, and it has been proposed that an ATP-dependent calcium sequestration system analogous to the well studied sarcoplasmic reticulum of skeletal muscle is present in microsome or Golgi derived vesicle membrane isolated from dog salivary gland or rat liver cells (Watson et al., 1974; Chakravarty and Holm Nielsen, 1980; Hodson, 1978). It has also been suggested that the endoplasmic reticulum of L-cells and macrophages has a calcium sequestering system and contains a rel-

Fig. 7. Schematic drawing of the transport of solute and water in the acinar cell of salivary gland at the secretory stimulating state. It is suggested that the cytoplasmic calcium flowed in from extracellular space and released from secretory granules by the secretory stimulation with ACh or pilocarpine could bring the passive sodium and chloride influx and small potassium outflux in the baso-lateral plasma membrane (BPM), and those induce sodium and chloride extrusion and therefore the osmotic water flux from basal part of acinar cells to the secretory granules and/or glandular lumen, as well as the serial exocytosis of the granules in the luminal side of acinar cells. The Na-K ATPases which maintain the electrochemical potential gradient in the resting state of acinar cells are located in baso-lateral plasma membrane (circles). BPM: baso-lateral plasma membrane.
transported relatively high concentration of calcium ion in the cisternae and that such is probably involved in the oscillatory membrane potential change and cell motility (Henkart and Nelson, 1979; Okada et al., 1977). It has been reported that the Goblet cells of the ileum contain such calcium reservoirs (Gupta and Hall, 1978) and that most of the radioactive Ca enters the saliva with 5-HT stimulation from prelabelled Calliphora salivary gland cells which have intracellular calcium reservoirs (Berridge and Lifke, 1979).

We also found that with pilocarpine stimulation there was an increase in the Na, Cl, and Ca concentrations in the cytoplasm as well as in the Na and Cl concentrations of the secretory granules. The data that this increase became smaller, were also obtained in our perfusion experiments where the glands were incubated in the medium containing low concentrations of Na and Cl. These evidences suggested that the increase and/or influx of cytoplasmic Na and Cl by stimulation could bring about an increase in a sodium-calcium exchange process in the membrane of the secretory granules, an intracellular calcium store, and the extrusion of Na and Cl into the secretory granules and lumen, as well as the increase of cytoplasmic Ca. It is also suggested that the Ca out-Na in exchange and the Ca in-Na out exchange occur in the secretory granules of acinar cells at stimulating and resting states, respectively. Another possibility is that the cytoplasmic Ca flowed in from extracellular space and released from the granules by stimulation (Nielsen and Petersen, 1972; Nishiyama and Petersen, 1974), could affect the Na channel on the basolateral plasma membrane of acinar cells and induce a passive Na and Cl influx, and this influx enhances an active Na and Cl extrusion in the apical portion of the cells by a Na (Cl) pump which may be ouabain insensitive and different from Na-K ATPase distributed on the basolateral plasma membrane maintaining the electrochemical potential gradient of the acinar cell in the resting state (Imai, 1974; Nakagaki et al., 1978).

The Na and Cl flux, thus transported in the acinar cells could form the local osmosis in the granules and lumen (Diamond and Bossert, 1968; Gupta and Hall, 1979), and the osmotic water flux from the basal part of acinar cells to the granules and the lumen, while the cytoplasmic Ca induces the serial exocytosis of the granules in the luminal side of acinar cells, as proposed electrophysiologically and electron microscopically for pancreatic acinar cells (Kanno, 1972; Petersen and Iwatsuki, 1978) (Fig. 7). Therefore, the secretory granules which function as an intracellular calcium store in the acinar cells, similar to the sarcoplasmic reticulum of skeletal muscle may be involved in the coupling of the secretory stimulation and intracellular transport or secretion of ions such as Na and Cl, water, and secretory proteins in the acinar cells of the salivary gland (Douglas and Poisner, 1963).

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