CONFORMATIONAL CHANGES IN ZYMGEN GRANULES
FROM THE PAROTID GLANDS INDUCED BY ATP
AND A LOW CONCENTRATION OF CALCIUM

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In preceding papers (1, 2), we reported that acetylcholine, excess K+ or noradrenaline
in the presence of Ca++ caused secretion of amylase from slices of parotid glands and re-
lease of amylase from zymogen granules, consisting mainly of stored amylase. Moreover,
ATP and a low concentration of Ca++ were also important in the process of secretion.
Accordingly, there is some secretory process inside the cell which is regulated by Ca++. It has also been reported that the release of catecholamine from chromaffin granules
(3-5) and of acetylcholine from synaptic vesicles were caused by ATP (6). Oka et al. (7)
also suggested that the release of catecholamine from chromaffin granules is associated
with a change in structure of the granules.
In the present work, we investigated the effect of a low concentration of Ca++ on the
structure of zymogen granules to clarify the relation between release of amylase from the
granules and their change in structure.

MATERIALS AND METHODS

Preparation of zymogen granules and supernatant
Parotid glands of Sprague-Dawley rats were homogenized in ice-cold 0.25 M sucrose
and zymogen granules were isolated by the method of Schramm and Dannon (8). The
granules were suspended in 0.25 M sucrose at a concentration of about 2 mg protein per
1.0 ml for the experiments.
The supernatant was prepared as described previously (1).
Incubation of granules
A volume of 0.3 ml of granule suspension was added to prewarmed medium con-
taining 111.1 mM KCl, 3.33 mM MgCl2, 22.2 mM sucrose, 11.1 mM Tris-HCl buffer (pH
7.4) and other additions as listed in the tables and figures, in a total volume of 2.7 ml. The
mixture was then incubated at 37°C for 5 minutes in a 3 ml cuvette and changes in optical
density of the mixture at 540 m/~ were measured using a spectrophotometer (Hitachi Perkin-
Elmer 139 UV-VIS). The initial optical density was about 0.400 at 540 m/. Results are
expressed as percentages of the optical density at 0 time.

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Osaka.
In experiments on the changes in distribution of phospholipids, 0.9 ml of granule suspension was added to 8.1 ml of the reaction medium.

**Sonication of granules**

A mixture of 0.9 ml of granule suspension and 8.1 ml of 0.25 M sucrose was ultrasonicated at 10 KC per second for 5 minutes.

**Osmotic treatment of granules**

A mixture of 0.9 ml of granule suspension and 8.1 ml of distilled water was stored in an ice bath for a certain period.

**Density gradient fractionation of granules**

Volumes of 0.5 ml of incubated suspensions of granules were layered on 4.5 ml sucrose density gradients of 1.5 to 1.925 M prepared using a density-gradienter (Hitachi, type D-GKU) and centrifuged at 25,000 rev/min for 3 hours in a swinging-bucket rotor (Hitachi, RPS40-A). Fractions were collected by cutting the tube into 0.5 cm wide pieces, using a tube slicer (Hitachi, type 40P-TSI) and each fraction was adequately diluted with distilled water to lyze the granules and then used for estimation of enzyme activities and protein.

**Estimations of amylase and adenosine triphosphatase (ATPase) activities and protein**

Amylase activity (EC 3.2.1.1) and protein were estimated by the method of Fuwa (9) and Lowry et al. (10), respectively. The basic medium for estimation of ATPase activity (EC 3.6.1.4) was as follows: MgCl₂ 2 mM, ATP 2 mM, Tris-HCl buffer (pH 7.4) 40 mM and 0.5 ml of enzyme solution in a total volume of 2.0 ml. After incubation for 30 minutes at 37 °C, the reaction was stopped by addition of 0.5 ml of 50% trichloroacetic acid. The amount of the inorganic phosphate liberated during the incubation was determined by the method of Takahashi (11).

**Estimation of phospholipids**

Granule suspensions after incubation, sonication or treatment under hypotonic conditions were centrifuged at 10,000 × g for 20 minutes and the supernatants were recentrifuged at 100,000 × g for 60 minutes. The two precipitates obtained by these centrifugation treatments were suspended in small volumes of the reaction medium and made up to 9.0 ml. Phospholipids extracted from supernatants and precipitates by the method of Folch (12) were estimated by the method of Bartlett (13). About 95% of the phospholipid present in the initial suspension was recovered in the particular fraction.

**Procedure for ultramicroscopy**

Rat parotid glands collected in ice-cold Krebs Ringer Tris medium consisting of 121.3 mM NaCl, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 3.0 mM CaCl₂, 16.5 mM Tris-HCl buffer (pH 7.4) were cut into small cubes and fixed with 2% OsO₄ containing 0.1 M S-collidine buffer (pH 7.4). Zymogen granules were precipitated by centrifugation and fixed with 2% OsO₄ containing 50 mM cacodylate buffer (pH 7.4) and 130 mM sucrose. For incubation of the zymogen granules, an equal volume of 6% glutaraldehyde containing 130 mM sucrose, 0.5 mM EDTA and 10 mM cacodylate buffer (pH 7.4) to that of the reaction medium was added and the mixture was stood for 30 minutes at room temperature.
and then centrifuged at 100,000 \( \times \) g for 60 minutes. The precipitate was then fixed over night at 0\(^\circ\) C with 2\% OsO\(_4\) containing the other additions listed above. The fixed materials were dehydrated by passage through a series of increasing concentrations of ethanol and then propylene oxide and embedded in Epon 812. Ultrathin sections were prepared with a microtome (Portar-Blum, model MT-2) and stained with uranylacetate and lead citrate and were examined under an electron microscope (Hitachi HU-7S, at 50 KV.).

Chemicals used

The following chemicals were used: Glycoletherdiamine tetraacetic acid (EGTA), ethylenediamine tetraacetic acid (EDTA), adenosine monophosphate disodium salt (AMP), adenosine diphosphate disodium salt (ADP), adenosine triphosphate disodium salt (ATP-2Na), adenosine triphosphate tris salt (ATP).

RESULTS

Effect of ATP on the turbidity of suspension of zymogen granules

Addition of ATP caused a reduction in turbidity of a suspension of zymogen granules (Fig. 1-a). When the mixture contained no MgCl\(_2\), addition of ATP alone had no effect on the turbidity. The optimum concentration of ATP for this effect under our conditions was 3 mm and then the optimum molar ratio of ATP to Mg\(^{2+}\) was about one, as for the release of amylase from the granules (Fig. 2).

The presence of the supernatant of the mixture had no effect on the reduction in turbidity caused by ATP-Mg\(^{2+}\) (Fig. 1-b). Moreover, neither ADP nor AMP could replace

![Fig. 1. Effect of ATP on the turbidity of suspension of zymogen granules.](image)

Granules were incubated in the absence (Fig. 1-a) and presence (Fig. 1-b) of the supernatant at 37\(^\circ\) C for 10 minutes.

Final concentrations: 100 mm KCl, 3 mm MgCl\(_2\), 3 mm ATP, 20 mm sucrose, 0.5 mm EGTA, 10 mm Tris-HCl buffer (pH 7.4), 200 \( \gamma \)/ml protein of the supernatant and 200 \( \gamma \)/ml protein of the granules. Optical density of the mixture were measured at 540 nm. Results were expressed as percentages of the optical density at 0 time.

Open circles: no ATP. Solid circles: 3 mm ATP. Solid squares: 3 mm ATP with 0.5 mm EGTA.
FIG. 2. Effect of ATP concentration on the turbidity of suspension of zymogen granules.
Granules were incubated in the absence of the supernatant. Other conditions were described in Fig. 1.
Open squares: 1.5 mm ATP.
Solid squares: 3.0 mm ATP.
Open circles: 4.5 mm ATP.
Solid circles: 6.0 mm ATP.

FIG. 3. Effect of AMP, ADP and ATP on the turbidity of suspensions of zymogen granules.
Granules were incubated in the absence of the supernatant. Other conditions were described in Fig. 1.
Solid squares: 3 mm AMP-2Na.
Open circles: 3 mm ADP-2Na.
Solid circles: 3 mm ATP-2Na.

ATP, as shown in Fig. 3.

Reduction in turbidity caused by ATP-Mg\textsuperscript{2+} was similar in Na\textsuperscript{+} and K\textsuperscript{+} media.

Effect of temperature on the reduction in turbidity of isolated zymogen granules caused by ATP-Mg\textsuperscript{2+}
When granules were incubated at 10°C, ATP-Mg\textsuperscript{2+} caused no reduction in turbidity, but reduction was marked at 25°C or 37°C (Fig. 4-a, b, c). Thus the reduction in turbidity caused by ATP-Mg\textsuperscript{2+} was dependent on the temperature.

Effect of EGTA on the turbidity of suspension of zymogen granules
On incubation of granules in medium containing both ATP-Mg\textsuperscript{2+} and EGTA, no reduction in turbidity was observed with or without the supernatant (Fig. 1-a, b). However, when EGTA was added to the reaction mixture after preincubation for 2.5 or 5 minutes with ATP-Mg\textsuperscript{2+}, EGTA was not inhibitory, as shown in Fig. 5.

The above results suggest that Ca\textsuperscript{2+}, probably present in the mixture as a contaminant, is necessary for the reduction in turbidity caused by ATP-Mg\textsuperscript{2+}, but that after some changes in the structure of the granules had occurred, the changes were not stopped by addition of EGTA and removal of Ca\textsuperscript{2+}.

Effect of different concentrations of Ca\textsuperscript{2+} on the turbidity of suspensions of zymogen granules
Addition of concentrations of less than \(2 \times 10^{-4} \text{ M CaCl}_2\) to medium containing 3 mm ATP, 3 mm MgCl\textsubscript{2}, 0.5 mm EGTA and 200 \(\mu\text{g}/\text{ml}\) protein of the supernatant, did not affect the turbidity, but concentrations of more than \(4 \times 10^{-4} \text{ M}\) caused marked reduction in turbidity (Fig. 6).
When the degree of reduction in turbidity of the zymogen granule suspension was plotted against the concentration of CaCl₂, an S-shaped curve was obtained. It seems that free Ca²⁺ has an effect on the granules and that the effective concentration for this is less than 10⁻⁵ M.

**Effect of the supernatant on reduction in turbidity of suspensions of zymogen granules caused by Ca²⁺**

Addition of 8 × 10⁻⁴ M CaCl₂ to medium containing 3 mM ATP, 3 mM MgCl₂ and

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**Fig. 4. Effect of temperature on the reduction in the turbidity of suspensions of zymogen granules by ATP-Mg²⁺.**

Incubations were carried out at 10°C (a), 25°C (b) and 37°C (c) in the absence of the supernatant. Other conditions were described in Fig. 1.

Open circles: no ATP.
Solid circles: 3 mM ATP.

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**Fig. 5. Effect of EGTA on the reduction in the turbidity of suspensions of zymogen granules by ATP-Mg²⁺.**

Granules were incubated in the absence of the supernatant. Other conditions were described in Fig. 1.

Open circles: 3 mM ATP-Mg²⁺ and 0.5 mM EGTA.
Solid circles: 3 mM ATP-Mg²⁺.
Open squares: 0.5 mM EGTA added after preincubation for 2.5 or 5 minutes with 3 mM ATP-Mg²⁺.

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**Fig. 6. Effect of CaCl₂ concentrations on the turbidity of suspensions of zymogen granules.**

Granules were incubated in the presence of the 200 μg/ml protein of the supernatant, 3 mM ATP-Mg²⁺ and 0.5 mM EGTA. The CaCl₂ concentrations were 10⁻⁵, 5 × 10⁻⁶, 10⁻⁶, 2 × 10⁻⁶, 4 × 10⁻⁶, 8 × 10⁻⁶ and 10⁻⁵ M. Other conditions were described in Fig. 1.

The incubation periods were: open squares, 2.5 minutes; solid squares, 5 minutes; open circles, 7.5 minutes and solid circles, 10 minutes.
FICG. 7. Effect of the supernatant on the reduction in turbidity of suspensions of zymogen granules by Ca$^{2+}$.

Granules were incubated in the absence (open circles) and presence (solid circles) of the supernatant at a concentration of 200 μg/ml protein.

The CaCl$_2$, EGTA and ATP-Mg$^{2+}$ concentrations were $8 \times 10^{-4}$ M, 0.5 mM and 3 mM, respectively.

Other conditions were described in Fig. 1.

0.5 mM EGTA, had no effect on the turbidity of the granule suspension. However, in the presence of the supernatant this concentration of CaCl$_2$ caused marked reduction in turbidity (Fig. 7). The supernatant was ineffective when denatured by heating for 60 minutes at 60°C or for 30 minutes in a boiling water bath (Fig. 8). The presence of ATP-Mg$^{2+}$ in medium containing the supernatant was also necessary for the effect of Ca$^{2+}$ on the granules, because on omission of ATP, CaCl$_2$ had no effect (Fig. 9). Thus a heat labile protein-like substance in the supernatant is involved in the action of Ca$^{2+}$ on the granules in the presence of EGTA and ATP-Mg$^{2+}$.

**Effects of different concentrations of the supernatant on the reduction in the turbidity of suspensions of zymogen granules by Ca$^{2+}$.**

On incubation of the granules in medium containing 3 mM ATP, 3 mM MgCl$_2$, 0.5 mM EGTA, $8 \times 10^{-4}$ M CaCl$_2$ and supernatant at protein concentrations of 10, 50, 100 and 200 μg/ml. CaCl$_2$ caused 45, 66, 70 and 75% reduction in turbidity, respectively, relative to that observed on omission of the supernatant (Fig. 10).
FIG. 9. Effect of ATP on the turbidity of suspensions of zymogen granules in the presence of Ca\(^{2+}\) and supernatant. The medium contained 3 mM MgCl\(_2\), 0.5 mM EGTA, 8 \(\times\) 10\(^{-4}\) M CaCl\(_2\), 200 \(\gamma/mL\) protein of the supernatant and other constituents. Other conditions were described in Fig. 1. Solid circles: 3 mM ATP. Open circles: no ATP.

FIG. 10. Effect of different concentrations of supernatant on the reduction in the turbidity of suspensions of zymogen granules by Ca\(^{2+}\). The medium contained 0.5 mM EGTA, 3 mM ATP-Mg\(^{2+}\), 8 \(\times\) 10\(^{-4}\) M CaCl\(_2\) and other constituents. The supernatant was used at concentrations of 10, 50, 100 or 200 \(\gamma/mL\) protein. Other conditions were described in Fig. 1. Incubation period: open circles, 5 minutes; solid circles, 10 minutes.

TABLE 1. Changes in the distribution of phospholipids in suspensions of zymogen granules after various treatments.

| Treatment               | Sup. (%) | Ppt. (%) |
|-------------------------|----------|----------|
| Control                 | 7.3      | 92.7     |
| ATP-Mg\(^{2+}\)         | 35.7     | 94.3     |
| ATP-Mg\(^{2+}\) + EGTA | 7.0      | 93.0     |
| Osmotic shock           | 5.0      | 95.0     |
| Sonication              | 32.0     | 68.0     |

Incubation: A mixture of 0.9 ml of the granule suspension and 8.1 ml of the reaction medium was incubated in the absence of the supernatant for 10 minutes at 37°C.

Osmotic shock: A mixture of 0.9 ml of the granule suspension and 8.1 ml of distilled water was stored in an ice bath for a certain period.

Sonication: A mixture of 0.9 ml of the granule suspension and 8.1 ml of 0.25 M sucrose was ultrasonicated at 10 KC per second for 5 minutes.

Phospholipids were extracted from supernatant and precipitate separated from the mixture described above by the centrifugation. Values are expressed as percentages of the total phospholipid in the mixture.
This experiment confirms that the supernatant is necessary for the effect of Ca\(^{2+}\) on the granules in the presence of ATP-Mg\(^{2+}\).

**Changes in the distribution of phospholipids in the suspension of zymogen granules after various treatments**

Zymogen granules were incubated in medium containing 3 mm ATP or 3 mm ATP, 3 mm MgCl\(_2\) and 0.5 mm EGTA. Then the mixtures were centrifuged and phospholipids were separated from the supernatant. These amounted to 7.3 or 7% of those in the whole suspension, respectively. However, after incubation with ATP and MgCl\(_2\), the lipids amounted to 26% of those in the whole suspension. The total amount of phospholipid in the whole suspension did not change on incubation in the different media.

No significant change in the distribution of phospholipids in the suspension of granules was observed after osmotic treatment, but sonication caused a change similar to that observed on incubation of granules with ATP-Mg\(^{2+}\) (Table 1).

The phospholipids in the supernatant and precipitate after incubation with ATP-Mg\(^{2+}\) were analyzed by thin layer chromatography. No changes in the components were observed.

**Density gradient centrifugation of isolated zymogen granules**

Zymogen granules in 0.25 M sucrose were layered on a sucrose density gradient. On centrifugation the zymogen granules became concentrated in a lower portion of the tube since protein, amylase and Mg-ATPase activities were mainly found in the lower part of the tube, as shown in Fig. 11-a.

**Fig. 11.** Density gradient centrifugation of isolated zymogen granules.

Granules were not incubated (a), incubated for 10 minutes with ATP-Mg\(^{2+}\) (b) or with ATP-Mg\(^{2+}\) and EGTA (c) in the absence of the supernatant. Other conditions were described in Fig. 1. 0.5 ml of not incubated or incubated suspension of granules was layered on 4.5 ml sucrose density gradient of 1.5 to 1.925 M and centrifuged at 25,000 rev/minutes for 3 hours in a swinging-bucket rotor. Fractions were collected by cutting the tube into 0.5 cm with pieces, using a tube slicer. Results were expressed as percentages of the total.
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After incubation in medium containing 3 mm ATP and 3 mm MgC\textsubscript{2}, granules remained in the top of the tube on centrifugation (Fig. 11-b). Furthermore, after incubation in medium containing ATP-Mg\textsuperscript{2+} and EGTA granules behaved similarly to unincubated granules on centrifugation (Fig. 11-c).

These results suggest that change in the structure of the granules is associated with reduction in turbidity of the suspension.
d) Granules after incubation for 10 minutes with 3 mM ATP-Mg$^{2+}$ and 0.5 mM EGTA (x 12,000)
Granules after incubation for 10 minutes with 3 mM ATP-Mg\(^{2+}\), 0.5 mM EGTA, 
8 \times 10^{-3} \text{M CaCl}_2 and 200 \mu l protein of the supernatant (\times 12,000)

**Fig. 12.** Electronmicroscopic appearance of zymogen granules.
Granules were incubated under conditions described in Fig. 1.
Scale : 1 \mu, Mt : mitochondria, Mf : membrane fragment, Long arrow : membrane like structure, Short arrow : small particles.

**Electronmicroscopic observation of zymogen granules**

In resting acinar cells in the parotid glands, zymogen granules appear ultramicroscopic-ally as dense, round, osmiophilic bodies of about 1.0 \mu diameter (Fig. 12-a). Granule incubated in medium containing ATP-Mg\(^{2+}\) and EGTA or no ATP (Fig. 12-c), appeared similar to those in resting cells or to unincubated granules (Fig. 12-b). But granules incubated with ATP-Mg\(^{2+}\) or with ATP-Mg\(^{2+}\), EGTA, supernatant and CaCl\(_2\), changed in structure: their shape was lost almost completely, and many small and osmiophilic particles of about 100 \AA\ diameter and membrane-like structures appeared (Fig. 12-d).

These observations suggest that zymogen granules are split into many small particles of about 100 \AA\ diameter and membranous structures when the turbidity of the granule suspension is greatly reduced.

**DISCUSSION**

This study was on the action of a low concentration of Ca\(^{2+}\) on the structure of zymogen granules in relation to the release of amylase from them by Ca\(^{2+}\).

When a suspension of the granules was incubated with ATP-Mg\(^{2+}\), the turbidity decreased. However, addition of EGTA completely prevented this reduction in turbidity. Thus a low concentration of Ca\(^{2+}\), probably present in the reaction mixture as a contaminant, seems to act on the granules as a trigger in the presence of ATP-Mg\(^{2+}\) causing a change in their structure. When EGTA was added to granules which had been preincubated
with ATP-Mg\textsuperscript{2+} for a few minutes, it did not block the reduction in turbidity. So it seems impossible to stop structural change once this has started by removal of Ca\textsuperscript{2+}.

Addition of CaCl\textsubscript{2} to granules in medium containing ATP-Mg\textsuperscript{2+} and EGTA did not cause a conformational change in the granules. However, on addition of a protein-like substance from the supernatant to this medium, a structural change in the granules was observed on addition of CaCl\textsubscript{2}. Though it is uncertain what effect this substance has on the granules, we suggest that it may take part in the rearrangement of their membranes rich in phospholipid. We are now investigating this problem.

Experiments on the distribution of phospholipids in the granule suspension after various treatments, showed that the phospholipids in the supernatant did not differ qualitatively from those in the precipitate. Furthermore, ultramicroscopic studies showed that the structures in intact zymogen granules in resting acinar cells from the parotid glands disappeared on reduction in turbidity of the granule suspension, and many membrane-like structures and small dense, osmiophilic particles of about 100 Å diameter appeared. Thus it seems likely that the release of amylase from zymogen granules induced by Ca\textsuperscript{2+} is closely related to a structural change in the granules, that is, splitting of the granules into small particles of about 100 Å diameter and fragmentation of the limiting membrane enclosing the granules.

It has been suggested that Mg-ATPase in synaptic vesicles (14, 15) or chromaffin granules (3, 4, 16-19) participates in the release of the storage substance and structural change of the vesicles or granules. However, the Mg-ATPase activity in zymogen granules was not inhibited by EGTA at a concentration which blocked both the release of amylase from the granules and the structural change in the granules caused by ATP-Mg\textsuperscript{2+}. Furthermore, we observed that the release of amylase was not depressed by salyrganic acid at a concentration which inhibited the Mg-ATPase activity in the granules.\textsuperscript{*} Thus there does not seem to be a correlation between changes in Mg-ATPase activity in the granules and the release of amylase from them, namely, a structural change of the granules.

Accordingly, it seems that a structural change in the zymogen granules is one of processes involved in secretion of amylase from the parotid glands and that the change is regulated by a low concentration of Ca\textsuperscript{2+}.

**SUMMARY**

Addition of ATP reduced the turbidity of a suspension of zymogen granules isolated from rat parotid glands. This effect of ATP-Mg\textsuperscript{2+} was blocked by the further addition of EGTA. Addition of 4 × 10\textsuperscript{-4} M CaCl\textsubscript{2} did not reduce the turbidity of the suspension in medium containing 3 mM ATP-Mg\textsuperscript{2+} and 0.5 mM EGTA, but did reduce that of a suspension in the same medium with a protein-like factor at a concentration of 200 γ/ml protein from the cytoplasm of the parotid glands. The effective concentration of Ca\textsuperscript{2+} causing this reduction in turbidity was roughly estimated as lower than 10\textsuperscript{-5} M. The presence of both 3 mM ATP-Mg\textsuperscript{2+} and 200 γ/ml protein of the protein-like factor was necessary.

\textsuperscript{*}unpublished observation.
for this effect of Ca\textsuperscript{2+}. These results are closely related with the release of amylase from the granules reported by us previously.

Experiments on separation of zymogen granules on a density gradient and changes in the distribution of phospholipid in the granules in suspension after various treatments suggested that the conformation of the granules changed with the reduction in density. Furthermore, ultramicroscopic observations suggested that the zymogen granules were split into small and osmiophilic particles of about 100 Å diameter during this change in density.

The mode of action of a low concentration of Ca\textsuperscript{2+} on the structure of the granules was discussed on the basis of these results.

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