ROLE OF Ca\textsuperscript{2+} IN THE SECRETION OF AMYLASE FROM THE PAROTID GLAND

Hajime ISHIDA,* Naomasa MIKI and Hiroshi YOSHIDA

Department of Pharmacology, Faculty of Medicine, Osaka University, Kita-ku, Osaka

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The secretion of amines and proteins from glands has been investigated by many workers and it has been suggested that Ca\textsuperscript{2+} may be important in the secretory process (1-4), but it is uncertain whether Ca\textsuperscript{2+} actually participates in the secretory mechanism inside the cell.

The parotid glands are known to be innervated by the sympathetic and parasympathetic nervous systems and acetylcholine and noradrenaline are physiological stimuli that cause secretion of amylase from the gland (5, 6). Amylase is synthesized and stored mainly in zymogen granules, in the parotid gland (7-10). Ultramicroscopically, zymogen granules are dense, round, osmiophilic bodies of about 1 \mu diameter, enclosed in a limiting membrane. They are easily isolated from the parotid gland by cell fractionation (8), and are well known to be important in secretion of amylase.

We study the mechanism of secretion by the parotid glands and the role of Ca\textsuperscript{2+} in the secretion process, especially in the release of amylase from zymogen granules.

A preliminary note on some of this work has already appeared (11).

MATERIALS AND METHODS

Preparation of parotid gland slices

Male Wistar rats were killed by a blow on the neck and the parotid glands were removed and rapidly transferred to ice-cold Ca\textsuperscript{2+} free Krebs Tris Ringer medium. Parotid gland slices were prepared from the glands in a total of about 100 mg wet weight.

Incubation of slices

The slices were pre-incubated for 15 minutes at 37 \degree C in a centrifugal tube (volume 30 ml) containing 10 ml of Ca\textsuperscript{2+} free Krebs Tris Ringer consisting of 121.3 mm NaCl, 4.8 mm KCl, 1.2 mm KH\textsubscript{2}PO\textsubscript{4}, 1.2 mm MgSO\textsubscript{4}, 16.5 mm Tris-HCl buffer (pH 7.4) and 10 mm glucose. Incubation was carried out for 5 minutes at 37 \degree C in tubes containing 10 ml of Krebs Tris Ringer with or without 3 mm CaCl\textsubscript{2} and the other constituents.

In medium with excess K\textsuperscript{+}, the final concentration of KCl was increased to 60 mm and that of NaCl was decreased to 55.1 mm.

The total amylase activity was not changed during incubation of slices under these conditions.

*Present address: Department of Pharmacology, Osaka University Dental School, Kita-ku, Osaka.
conditions.

After incubation the medium was adequately diluted with distilled water and used for estimation of amylase activity.

Preparation of zymogen granules

Parotid glands were removed from rats and homogenized in ice-cold 0.25 M sucrose and the zymogen granules were isolated by the method of Schramm and Dannon (8). The isolated granules were suspended in 0.25 M sucrose at a concentration of about 3 mg protein per 1.0 ml for use in experiments.

Incubation of zymogen granules

About 300 µg protein of granules in 0.1 ml were added to prewarmed medium containing 111.1 mM KCl, 3.33 mM MgCl₂, 11.1 mM Tris-HCl buffer (pH 7.4) and other additions, in 0.9 ml.

They were incubated for 5 minutes at 37°C and then 4 ml of ice-cold 0.25 M sucrose was added to stop the reaction. The mixture was centrifuged at 4,500 × g for 10 minutes and the supernatant was adequately diluted with distilled water and used for estimation of amylase and protein.

The total amylase activity and protein did not change during incubation of granules under these conditions.

Treatment of zymogen granules with EGTA

Zymogen granules were obtained by centrifugation, suspended in ice-cold 0.25 M sucrose containing 1 mM EGTA and kept for 40 minutes at 0°C. They were then separated by centrifugation, washed twice with 0.25 M sucrose, and suspended in 0.25 M sucrose at a concentration of about 3 mg protein per ml for the experiments.

Supernatant fraction

Parotid glands were homogenized with 5 volumes of cold 0.25 M sucrose and the supernatant (Sup.) was obtained by centrifugation of the homogenate at 100,000 × g for 30 minutes. It was diluted in 0.25 M sucrose at a concentration of about 5 mg protein per ml for use in experiments. The supernatant further fractionated, by the standard technique with neutralized (NH₄)₂SO₄, into two parts, precipitating at 0-40% and 40-80% saturation, respectively. Each precipitate was dissolved in 5 mM Tris-HCl buffer (pH 7.4) and dialyzed in a cellulose tube against the same buffer for 15 hours at 4°C. It was diluted in the same buffer at a concentration of about 5 mg protein per ml for use in experiments.

Estimation of amylase and protein

Amylase activity (EC 3.2.1.1.) in the medium was estimated by the method of Fuwa (12). Activity is expressed as units of amylase, one unit being the amount of amylase in mg of amylase required for 10% reduction of the blue value in 10 minutes at 30°C, as defined by Fuwa.

Protein in the medium was estimated by the method of Lowry et al. (13).

Chemicals used

The following chemicals were used: acetylcholine chloride (ACh), L-arterenol-D-bitartrate (Noradrenaline), eserine sulfate (Eserine), atropine sulfate (Atropine), cocaine
chloride (Cocaine), adenosine triphosphate disodium (ATP-2Na), adenosine triphosphate tris (ATP-Tris) and glycoletherdiamine tetraacetic acid (EGTA). The concentrations used are given in the tables and figures.

RESULTS

Effect of acetylcholine on the secretion of amylase from parotid gland slices

Amylase was secreted from slices of parotid gland on addition of $10^{-5}$ M acetylcholine (ACh) to incubation medium containing 3 mM CaCl$_2$ (Fig. 1). The action of ACh on

![Fig. 1](image_url)

FIG. 1. Time course of amylase secretion from parotid gland slices caused by acetylcholine.

Porotid gland slices in a total of about 100 mg wet weight were incubated for 5 minutes at 37°C in tubes containing 10 ml of incubation medium. The medium was Krebs Tris Ringer (121.3 mM NaCl, 4.8 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 3 mM CaCl$_2$, 16.5 mM Tris-HCl buffer (pH 7.4) and 10 mM glucose) containing $5 \times 10^{-4}$ M eserine and $10^{-5}$ M acetylcholine.

Each point represents the mean of 4 determinations.
The open circles : $10^{-4}$ M acetylcholine.
The solid circles : no acetylcholine.

TABLE 1. Effect of atropine on amylase secretion from parotid gland slices caused by acetylcholine.

| Additions | Amylase in medium (units/100 mg) |
|-----------|---------------------------------|
| Eserine ($5 \times 10^{-4}$ M) | 1887 ± 186 |
| Eserine ($5 \times 10^{-4}$ M) | 3428 ± 255 |
| ACh ($10^{-2}$ M) |  |
| Eserine ($5 \times 10^{-4}$ M) | 2072 ± 258 |
| ACh ($10^{-3}$ M) |  |
| Atropine ($10^{-4}$ M) |  |

Parotid gland slices were incubated under conditions described in Fig. 1. Each value represents the mean of 4 determinations ± standard deviation.
amylase secretion was blocked by the addition of $10^{-4}$ M atropine (Table 1). This shows that the ACh 'receptors' of the parotid glands are muscarinic in type. In Ca$^{2+}$ free medium, no amylase was secreted from the slices on addition of ACh (Table 2).

To confirm that amylase secretion caused by ACh depends on the presence of Ca$^{2+}$ in the incubation medium, we studied the effect of different concentrations of Ca$^{2+}$ on amylase secretion caused by ACh. In medium containing less than 3 mm CaCl$_2$, amylase secretion caused by $10^{-5}$ M ACh increased with increase in CaCl$_2$ concentration. However, at concentrations of CaCl$_2$ above 3 mm, amylase secretion from the slice caused by $10^{-5}$ M ACh decreased with increase in CaCl$_2$ concentration. This decrease might be due to the membrane stabilizing effect of Ca$^{2+}$. The optimal concentration of Ca$^{2+}$ for amylase secretion under our experimental conditions was 3 mm (Fig. 2).

**Table 2. Effect of CaCl$_2$ on amylase secretion from parotid gland slices caused by acetylcholine.**

| Additions | Amylase in medium (units/100 mg) |
|-----------|---------------------------------|
| CaCl$_2$  |                                 |
| (-) Esarine ($5 \times 10^{-4}$ M) | 1920 ± 169                     |
| (-) ACh ($10^{-5}$ M) | 1855 ± 177                     |
| CaCl$_2$  |                                 |
| (3 mm) Esarine ($5 \times 10^{-4}$ M) | 1670 ± 212                     |
| (3 mm) ACh ($10^{-5}$ M) | 3025 ± 235                     |

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**Effect of excess K$^+$ on the secretion of amylase from parotid gland slices**

Amylase secretion from the slices also occurred when the incubation medium contained excess K$^+$. However, in Ca$^{2+}$ free medium excess K$^+$ did not cause amylase secretion (Table 3).

Thus the CaCl$_2$ presented in the medium was necessary for amylase secretion

![Fig. 2. Effect of CaCl$_2$ concentration on amylase secretion from parotid gland slices caused by acetylcholine.](image)
caused by excess K', as well as for amylase secretion caused by ACh. No amylase was released from slices with excess K', when 1 mm cocaine, a membrane stabilizer, was present in the medium (Table 4).

Effect of noradrenaline on amylase secretion from parotid gland slices

Amylase was secreted from the slices on addition of $10^{-5}$ M noradrenaline to the in-

| Additions          | Amylase in medium (units/100 mg) |
|--------------------|----------------------------------|
| CaCl$_2$ (mm)     | KCl (mm)                        |
| 0                  | 6                               | 2530±245                   |
|                    | 60                              | 2397±236                   |
| 3                  | 6                               | 2763±242                   |
|                    | 60                              | 3727±218                   |

Parotid gland slices were incubated under conditions described in Fig. 1 and Table 2. In medium with excess K', the final concentration of KCl was increased to 60 mm and that of NaCl was decreased to 55.1 mm. Each value represents the mean of 4 determinations ± standard deviation.

Effect of noradrenaline on amylase secretion from parotid gland slices caused excess K'.

| Additions          | Amylase in medium (units/100 mg) |
|--------------------|----------------------------------|
| CaCl$_2$ (mm)     | KCl (mm) | Cocaine (mm) |                        |
| 6                  | 0       | 1            | 1830±278               |
| 3                  | 60      | 1            | 3280±182               |
|                    |         | 1            | 1950±212               |

Parotid gland slices were incubated under conditions described in Fig. 1 and Table 3. Each value represents the mean of 4 determinations ± standard deviation.

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Effect of noradrenaline on amylase secretion from parotid gland slices

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| Additions          | Amylase in medium (units/100 mg) |
|--------------------|----------------------------------|
| CaCl$_2$ (-)      | None                             | 900**                       |
|                   | Noradrenaline ($10^{-5}$ M)      | 1391**                      |
| CaCl$_2$ (3 mm)   | None                             | 989±32*                     |
|                   | Noradrenaline ($10^{-5}$ M)      | 3097±262*                   |

Parotid gland slices were incubated under conditions described in Fig. 1 and Table 2. * Mean of 4 determinations ± standard deviation. ** Mean of 2 determinations.
cubation medium and 3 mm CaCl₂ was also necessary for this release, because, on omission of CaCl₂ from the medium, the secretion of amylase was suppressed on addition of noradrenaline (Table 5).

On decreasing the time taken for preparation of slices leakage of amylase from the slices decreased. This is probably why the control level of amylase in this experiment was lower than in previous experiments.

Effect of ATP on the release of amylase from isolated zymogen granules

The above results show clearly that Ca²⁺ in the incubation medium is necessary for amylase secretion from the parotid gland caused by stimulants.

Next we studied the role of Ca²⁺ in the release of amylase from isolated zymogen granules.

Amylase was released from isolated zymogen granules on addition of 3 mm ATP to the reaction mixture (Fig. 3). The presence of Mg²⁺ is necessary for this effect of ATP. Studies on the effect of different concentrations of ATP on the release of amylase from zymogen granules, showed that the optimal molar ratio of ATP to Mg²⁺ was about one (Fig. 4). The effect of ATP on the release of amylase was dependent on the temperature of the reaction.

![Fig. 3. Effect of ATP on the release of amylase from isolated zymogen granules.](image)

About 300 µg protein of zymogen granules in 0.1 ml were added to the medium containing 111.1 mm KCl, 3.33 mm MgCl₂, 11.1 mm Tris-HCl buffer (pH 7.4) and 3.33 mm ATP, in 0.9 ml and incubated for 5 minutes at 37°C. Each point represents the mean of 4 determinations.

The open circles : 3 mm ATP.
The solid circles : no ATP.

![Fig. 4. Effect of ATP concentration of release of amylase from isolated zymogen granules.](image)

Zymogen granules were incubated under conditions described in Fig. 3. The ATP concentration was varied from zero to 6 mm. Each point represents the mean of 3 determinations.
Effect of EGTA on the release of amylase from isolated zymogen granules by ATP

On incubation of granules with 3 mM ATP, 3 mM MgCl₂ and 1 mM EGTA, there was no release of amylase from zymogen granules due to ATP (Table 6).

The amount of protein released into the medium tended to vary like with that of the amylase into the medium.

It seems likely that Ca²⁺, probably present in the reaction mixture as a contaminant, is concerned in the release of amylase from zymogen granules by ATP.

| Additions          | Amylase in medium (units/tube) |
|--------------------|-------------------------------|
| ATP (mM) | MgCl₂ (mM) | EGTA (mM) |
| 0     | 3           | 0          | 2949 ± 340  |
| 3     | 3           | 0          | 5083 ± 560  |
| 3     | 3           | 1          | 3166 ± 326  |

Zymogen granules were incubated under conditions described in Fig. 3. Each value represents the mean of 4 determinations ± standard deviation.

Effect of Ca²⁺ on the release of amylase from isolated zymogen granules

The concentration of Ca³⁺ necessary to regulate various phenomena inside the cell is known to be lower than 10⁻⁵ M, and so we investigated the effect of low concentrations of Ca³⁺ on the release of amylase from zymogen granules.

Zymogen granules were pretreated with 1 mM EGTA to reduce their Ca²⁺ content. Addition of 10⁻⁵ M CaCl₂ to reaction mixture containing 3 mM ATP and 3 mM MgCl₂ did not give any influence on the release of amylase from these granules. However, on incubation in the presence of 1 mg protein per ml of the supernatant with ATP and Mg²⁺, amylase release from the granules was observed on addition of 10⁻⁶ M or 5 x 10⁻⁶ M CaCl₂.

| Additions          | Amylase in medium (units/tube) |
|--------------------|-------------------------------|
| ATP (mM) | MgCl₂ (mM) | Sup. | CaCl₂ (M) | |
|       |        |     |           | 3755 ± 451 |
| 3     | 3      | 0   | 0         | 3875 ± 419 |
| 3     | 3      | 0   | 10⁻⁵      | 3308 ± 386 |
| 3     | 3      | 0   | 5 × 10⁻⁶  | 6250 ± 421 |
| 3     | 3      | 0   | 5 × 10⁻⁶  | 5115 ± 372 |

Zymogen granules treated with 1 mM EGTA were incubated under conditions described in Fig. 5.

One mg protein per ml of the supernatant fraction (Sup.) was used. The CaCl₂ concentration was 10⁻⁵, 5 × 10⁻⁶ M. Each value represents the mean of determinations.
TABLE 8. Effect of CaCl$_2$ and protein fraction on the release of amylase from isolated zymogen granules.

| Additions | ATP (mM) | MgCl$_2$ (mM) | Protein fr. (NH$_4$)$_2$SO$_4$ | CaCl$_2$ (M) | Amylase in medium (units/tube) |
|-----------|----------|---------------|-----------------------------|-------------|-------------------------------|
|           | 0 40     | 0             | 10$^{-6}$                   | 3 3         | 3250 ± 147 3500 ± 370         |
|           | 3 3      | 0             | 10$^{-6}$                   | 40-80%      | 3000 ± 357 5000 ± 418         |

Zymogen granules treated with 1 mM EGTA were incubated under conditions described in Fig. 5. The protein fraction precipitated at 0-40% or 40-80% saturation with (NH$_4$)$_2$SO$_4$ was used at the concentration of 2 mg protein per ml.

The CaCl$_2$ concentration was 10$^{-6}$ M.

Each value represents the mean of 8 determinations ± standard deviation.

Thus both the supernatant and ATP-Mg$^{2+}$ seemed to be required for release of amylase from the granules by CaCl$_2$.

Nature of factor in the supernatant fraction required for Ca$^{2+}$-induced release of amylase from isolated zymogen granules

We treated the supernatant with neutralized (NH$_4$)$_2$SO$_4$ and collected the fractions, precipitating at 0-40% and 40-80% saturation.

No release of amylase from the granules by CaCl$_2$ was observed on incubation in reaction mixture containing 2 mg protein per ml of the fraction precipitated at 0-40% saturation (Table 8). But on incubation with 2 mg protein per ml of the fraction precipitated at 40-80% saturation, amylase was released from the granules on addition of 10$^{-6}$ M CaCl$_2$ (Table 8). Furthermore we observed that the 40-80% fraction became inert by heat treatment, so the active factor required for the stimulated release by Ca$^{2+}$ seemed to be protein. The effective concentration of CaCl$_2$ was 10$^{-6}$ M under this experimental

![Fig. 5. Effect of CaCl$_2$ concentration on release of amylase from isolated zymogen granules.](image-url)
ATP was also necessary for the release of amylase from zymogen granules by CaCl₂ and the 40–80% fraction, and on its omission, no release of amylase was observed (Table 9).

**TABLE 9. Effect of ATP on the release of amylase from isolated zymogen granules by CaCl₂ and the protein fraction.**

| Additions | Protein fr. (NH₄)₂SO₄ (mM) | MgCl₂ (mM) | ATP (mM) | CaCl₂ (M) | Amylase in medium (units/tube) |
|-----------|-----------------------------|------------|---------|-----------|-----------------------------|
| 0         | 3                           | 10⁻⁶       | 0       | 3225 ± 382 |
| 40–80%    | 3                           | 10⁻⁶       | 0       | 5488 ± 347 |
|           | 0                           | 10⁻⁶       | 0       | 3250 ± 352 |
|           | 0                           | 10⁻⁶       | 0       | 2850 ± 416 |

Zymogen granules treated with 1 mM EGTA were incubated under conditions described in Fig. 5. Protein fraction precipitated at 40–80% saturation with (NH₄)₂SO₄ was used at the concentration of 2 mg protein per ml.

Each value represents the mean of 6 determinations ± standard deviation.

**DISCUSSION**

It was reported by Bdolah et al. (14) that Ca²⁺ and Mg²⁺ of the Krebs Ringer bicarbonate medium did not appear essential for protein secretion from slices of rat parotid gland. But, in this experiment, amylase was secreted from slices of parotid gland by stimulants on incubation in Krebs Tris Ringer medium containing Ca²⁺, as in the cases of release of protein from the submaxillary glands (3, 4), catecholamine from the adrenal glands (15–17) and vasopressin from the neurohypophysis (17–19). Thus Ca²⁺ may generally be important in the secretory mechanisms of glands. It has been reported that the membrane potential of the acinar cells of the parotid gland, recorded with an intracellular microelectrode, is below 35 mV, namely about 20 mV on the average, the inside being negative (20). It is unknown whether the potential of stimulated glands differs significantly from that of unstimulated glands. However, it seems likely that the parotid gland cells would be depolarized by stimulants, because cocaine and atropine, inhibit the depolarization of cell membranes, and block the secretory responses to excess K⁺ and ACh, respectively. But depolarization alone does not seem to be a sufficient stimulant for secretion, and it may be only one of the conditions required. Thus depolarization may merely be a change in the electrical sign of a membrane in response to a stimulant, because it has been reported that addition of amethocaine (21) or omission of Ca²⁺ (22) depresses the secretion of catecholamine from chromaffin cells, but does not block the depolarization of the cell.

Noradrenaline caused the secretion of amylase from slices of parotid glands as reported by Schramm et al. (23), but the presence of CaCl₂ was also necessary for this.
On the other hand, it has been reported that uptake of labelled calcium into squid giant exons (24), adrenal glands (25, 26) and the neurohypophysis (19) was caused by ACh or excess K⁺.

Thus secretory mechanisms of glands may have some intracellular process in common which is sensitive to Ca²⁺, induced to enter the cell from the extracellular fluid by a stimulant.

In the present work, we also investigated the effect of Ca²⁺ on the release of amylase from isolated granules.

A requirement for Ca²⁺ in the release of amylase from the granules was clearly demonstrated. The release of amylase from the granules was caused by ATP and the optimal molar ratio of ATP to Mg²⁺ was found to be about one, as in the cases of release of catecholamine from chromaffin granules (27, 28) and of ACh from synaptic vesicles (29).

EGTA, a specific chelator of Ca²⁺, blocked the release from the granules by ATP. It seems likely that Ca²⁺, probably present in the reaction mixture as a contaminant, may be involved in the release of amylase by ATP. Accordingly we decreased the Ca²⁺ content of the granules by treatment with EGTA. A release of amylase from these granules by Ca²⁺ was clearly demonstrated on addition of CaCl₂ to reaction mixture containing ATP, Mg²⁺ and the supernatant fraction of the gland. The effective concentration of Ca²⁺ for this release seemed comparable with that inside the cell.

The factor in the supernatant necessary for this release of amylase by Ca²⁺, ATP and Mg²⁺ from zymogen granules pretreated with EGTA seems to have protein-like nature, because it was precipitated at 40–80% saturation of (NH₄)₂SO₄ and its effect disappeared on heating the supernatant at 60°C for 60 minutes or in a boiling water bath for 30 minutes. The role of this protein-like substance in the release of amylase from the granules is unknown.

ATP was also necessary for release of amylase from the granules by Ca²⁺ and on its omission, no release was observed.

Ultramicroscopic examination and estimation of succinic dehydrogenase activity in the preparation of zymogen granules showed that our preparation was slightly contaminated with mitochondria. When the mitochondria had been removed by density gradient centrifugation, the preparation of granules had Mg-ATPase activity but not Na-K-ATPase activity,* as in the case of chromaffin granules and synaptic vesicles. Our results suggest that an increased intracellular concentration of Ca²⁺ caused by a stimulant may release amylase from zymogen granules in the presence of ATP, Mg²⁺ and a protein-like factor. This release may be closely connected with a change in structure of the zymogen granules and this change in structure may be related to Mg-ATPase in the granule.

Furthermore, we found that Ca²⁺ changes the structure of the granules (30) and is closely related with the release of amylase from them, as reported in this study.

* unpublished observation
ROLE OF Ca²⁺ IN AMYLASE SECRETION

SUMMARY

Secretion of amylase from slices of parotid gland was caused by acetylcholine (10^{-5} M), excess K⁺ (60 mm) or noradrenaline (10^{-3} M) when incubation was carried out in the presence of 3 mm CaCl₂, but not in Ca²⁺ free medium. The release of amylase from isolated zymogen granules was stimulated by addition of 3 mm ATP and 3 mm MgCl₂. However, this release was blocked by the further addition of 1 mm EGTA. In the presence of ATP-Mg²⁺, the release of amylase from EGTA-treated zymogen granules was stimulated by addition of 10^{-6} or 5 × 10^{-6} M CaCl₂ and a protein-like factor in the cytoplasm of the parotid gland at a concentration of 2 mg protein per ml. The effective concentration of Ca²⁺ for evoking release from zymogen granules was about 10^{-6} M.

The role of Ca²⁺ in the secretion of amylase from zymogen granules in the parotid gland was discussed on the basis of these findings.

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