

# RETENTION OF AMYLASE IN THE SECRETORY GRANULES OF PAROTID GLAND AFTER EXTENSIVE RELEASE OF $Ca^{++}$ BY IONOPHORE A-23187

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## ABSTRACT

The effect of the ionophore A-23187 was tested on isolated secretory granules of rat parotid gland. The ionophore caused extensive release of calcium from the granules without effecting release of amylase or other secretory proteins. It is therefore concluded that the role of calcium in the granules is probably not that of a stabilizing agent.

**KEY WORDS**  $Ca^{++}$ -ionophore ·  $Ca^{++}$  release · secretory granule · parotid gland · amylase release

A high content of calcium was reported for the secretory granules of rat parotid gland (7, 17), for the submaxillary gland (5), for the membranes of zymogen granules of guinea pig pancreas (4), and for the chromaffin granules of the adrenal medulla (3). It has been suggested that calcium might serve to stabilize the highly concentrated protein in the secretory granules (17). In apparent support of this idea, it was found that slices and isolated secretory granules of rat parotid gland release amylase and calcium to the same relative extent (17).

The ionophore A-23187, which has recently come into use (12, 14), presented another approach to test whether or not release of calcium by the isolated, membrane-bounded granules can be dissociated from release of amylase. This ionophore, which is relatively specific for divalent cations, has been used to facilitate the movement of calcium through the membranes of various biological systems in order to determine the role of the metal (6, 8, 13, 15).

The present communication demonstrates that the ionophore can cause the release of most of the

calcium content of the parotid secretory granule while the amylase is retained inside.

## MATERIALS AND METHODS

### *Incorporation of $^{45}Ca$ into Rat Parotid Glands In Vivo*

Male albino rats were used.  $^{45}Ca$  (15  $\mu Ci$ ) in 0.5 ml of saline, containing also 2 mM  $CaCl_2$ , was injected intraperitoneally during light ether anaesthesia. The injections were given at 7 a.m., and the dissection of the glands was performed 24 h later. After injection, the rats were kept without food, receiving water ad lib.

### *Isolation of the Secretory Granules*

For each experiment, at least four animals were used. Collection of glands at 4°C in 0.3 M sucrose medium, homogenization, and isolation of the secretory granule fractions were carried out as described by Amsterdam et al. (1). Granules were not isolated on a urografin gradient (10) since subsequent resuspension in the absence of urografin causes lysis of the granules. Examination of electron micrographs showed that about 98% of the structural entities were secretory granules while the remainder were mostly mitochondria.

### *Standard Assay System*

The reaction mixture prepared in a volume of 1 ml at 0–4°C contained: secretory granules equivalent to 150–

3,000 amylase units (0.3–6.0 mg protein); sucrose, 0.3 M; glycylglycine buffer, pH 7.5, 20 mM. The ionophore A-23187 in absolute ethanol was added as the last component to give a final ethanol concentration of 1% (vol/vol). Control systems without ionophore contained 1% ethanol. After incubation for various times at 37°C, aliquots were taken from the reaction mixture to determine total amylase and total  $^{45}\text{Ca}$ . The reaction mixture was subsequently centrifuged at 12,000 *g* for 1 min, and aliquots for analysis were taken from the supernate. The amounts of amylase and  $^{45}\text{Ca}$  in the supernate were calculated as percent released from the granules into the medium.

### Analytical Methods

Amylase was determined according to Bernfeld (2). Protein content was estimated with the Lowry reagent, using bovine serum albumin as standard (11).  $^{45}\text{Ca}$  was measured in a toluene-Triton scintillation mixture.  $^{40}\text{Ca}$  was determined by atomic absorption (17). The following modification was introduced: Samples were deproteinized with 10% sulfuric acid (wt/vol). All samples and standards contained KCl, 50 mM; NaCl, 50 mM;  $\text{MgCl}_2$ , 10 mM, and  $\text{H}_2\text{SO}_4$ , 10% wt/vol. These additions were made to equalize the effect of various alkaline ions on the determination of calcium. Measurements were performed with a Varian atomic absorption spectrophotometer, model AA-5 (Varian Associates, Instrument Div., Palo Alto, Calif.). Electron microscopy was performed on thin sections of pellets of secretory granules prepared, fixed, and stained as described previously (1).

### Chemicals

$^{45}\text{CaCl}_2$  was purchased from New England Nuclear (Boston, Mass.). The ionophore A-23187 was a gift from Eli Lilly and Co. (Indianapolis, Ind.). The solid form was stored at room temperature, protected from light. Stock solutions (2 mg/ml) in absolute ethanol were stored at 4°C, protected from light.

### RESULTS

The release of  $^{45}\text{Ca}$  and amylase as a function of ionophore concentration is shown in Fig. 1. The ionophore, which is probably located in the hydrophobic granule membrane, caused extensive release of  $^{45}\text{Ca}$  almost without release of amylase. The trend of reduction in  $^{45}\text{Ca}$  release with increasing ionophore concentration might perhaps be due to binding of the metal to the ionophore in the granule membrane (see legend to Fig. 1). According to Fig. 1, a concentration of 2  $\mu\text{g}$  ionophore/ml was chosen for subsequent experiments.

Substituting Tris or *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid [HEPES] buffer at pH 7.5 instead of the standard glycylglycine did

not affect the results.  $^{45}\text{Ca}$  release by the ionophore was also not significantly changed by substituting 120 mM sodium acetate for 240 mM sucrose in order to test the effect of ionic strength. In a number of experiments, protein release from the granules was measured in parallel with amylase release. Good agreement was found between the two determinations, indicating that amylase release is representative of the proteins of the granules. The effect of the ionophore as a function of time is shown in Fig. 2. It is evident that 2 min of incubation at 37°C were sufficient for maximal release of  $^{45}\text{Ca}$  under the conditions of the experiment. It is also clear that the extensive  $^{45}\text{Ca}$  release did not lead to a subsequent increase in amylase release.

The finding that prolonged incubation did not release more than 65% of total  $^{45}\text{Ca}$  suggested that perhaps an equilibrium had been established. To test this possibility,  $^{45}\text{Ca}$  release was measured at various granule concentrations (Fig. 3). It is evident that the relative release of  $^{45}\text{Ca}$  decreased with increasing granule concentration. The findings indicate a certain affinity of the granule components for calcium. It should be noted that the percent of maximal and minimal release of calcium varied with the batch of granule preparation but always showed a dependence on granule con-

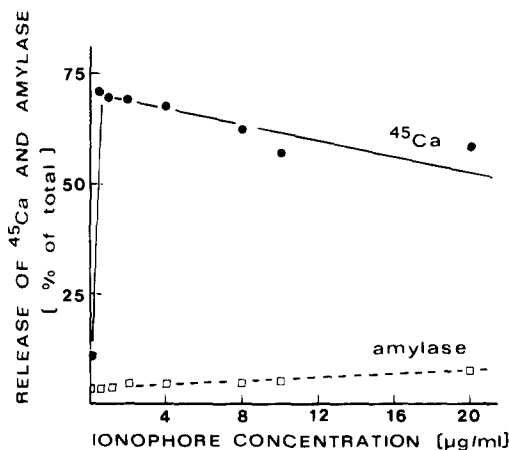


FIGURE 1 Release of  $^{45}\text{Ca}$  and amylase as a function of ionophore concentration. Granules, 500 amylase U/ml, were incubated at 37°C for 10 min. A constant volume of different concentrations of ionophore in ethanol was added to the systems. At 20  $\mu\text{g}$  ionophore/ml there is roughly 10% less  $^{45}\text{Ca}$  release than at 1  $\mu\text{g}$ /ml. It can be calculated that this decrease is equivalent to 6 nmol calcium (17) per 40 nmol ionophore added (12).

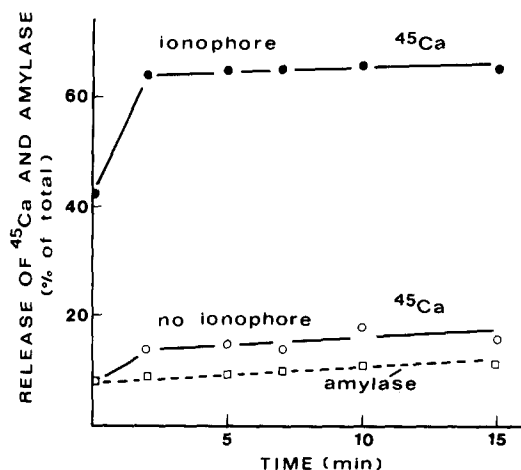


FIGURE 2 Release of  $^{45}\text{Ca}$  as a function of time. The ionophore,  $2\ \mu\text{g}/\text{ml}$ , was added to the granules,  $500$  amylase U/ml. The granules were subsequently incubated at  $37^\circ\text{C}$  for various times. Amylase release was the same in the presence and in the absence of ionophore.

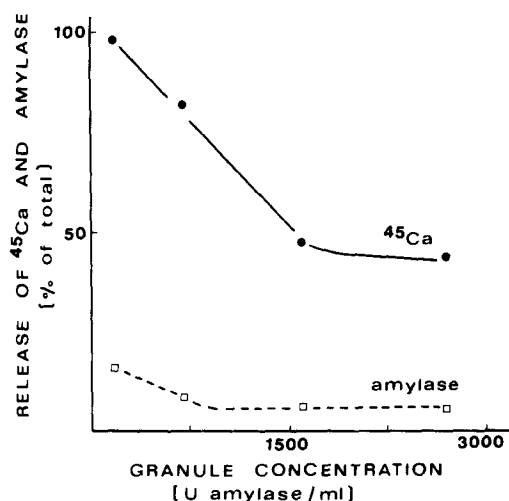


FIGURE 3 Effect of granule concentration on the release of  $^{45}\text{Ca}$ . Ionophore concentration was  $2\ \mu\text{g}/\text{ml}$ .

centration. The dependence of  $^{45}\text{Ca}$  release on granule concentration was tested also by diluting a concentrated solution of granules after insertion of the ionophore (not shown). It was found that release of  $^{45}\text{Ca}$  increased with dilution of the granule suspension. The increase of  $^{45}\text{Ca}$  release by dilution of a control without ionophore was only minor. If, indeed, the amount released through the ionophore reaches equilibrium with the amount still bound to the granules, it would be expected that a chelator in the medium would

increase the release of calcium. Fig. 4 shows that both ethylene glycol-bis( $\beta$ -aminoethyl ether)- $N,N,N',N'$ -tetraacetate (EGTA) and EDTA markedly increased the release of  $^{45}\text{Ca}$  from granules treated with the ionophore (note also figure legend). In the absence of ionophore, the extent of  $^{45}\text{Ca}$  release by the chelators is small and about equal to the amylase release. The somewhat more extensive  $^{45}\text{Ca}$  release with EDTA could be due to chelation of  $\text{Mg}^{++}$  (17) which, in turn, might increase the portion of granule calcium accessible to the ionophore. Essentially all the  $^{45}\text{Ca}$  was released also in the absence of chelators, when  $10\ \text{mM}$   $^{40}\text{Ca}^{++}$  or  $\text{Mg}^{++}$  was added in the presence of the ionophore. Presumably, the  $\text{Ca}^{++}$  and the  $\text{Mg}^{++}$  served to exchange through the ionophore for the  $^{45}\text{Ca}$  bound inside the granule.

The absolute amount of  $^{40}\text{Ca}$ , released from the granules through the ionophore, was also determined. When release of  $^{45}\text{Ca}$  was 92% in the presence of EDTA, release of  $^{40}\text{Ca}$ , measured by atomic absorption, was 77–82%. A similar amount of  $^{40}\text{Ca}$  was released in the absence of EDTA when 6–10 mM  $\text{MgCl}_2$  was present in the medium (Fig. 5). These latter experiments indi-

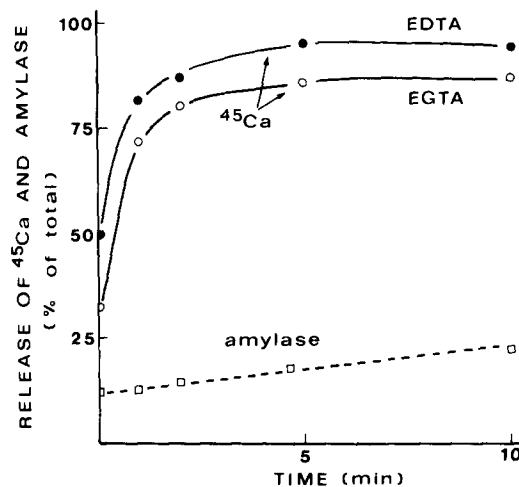


FIGURE 4 Effect of EDTA and EGTA on the release of  $^{45}\text{Ca}$  by the ionophore A-23187. Ionophore,  $2\ \mu\text{g}/\text{ml}$ , was added to test systems which contained granules,  $1,450$  amylase U/ml, EDTA,  $0.5\ \text{mM}$ , or EGTA,  $0.5\ \text{mM}$ . A system without the chelators but with ionophore released 41% of the  $^{45}\text{Ca}$  and 22% of the amylase during 10 min of incubation. Controls without ionophore, containing  $0.5\ \text{mM}$  EDTA or EGTA, released 25% of the  $^{45}\text{Ca}$  and 18% of the amylase in 10 min. Amylase release in the presence of ionophore plus EDTA or EGTA had the same values for both systems.

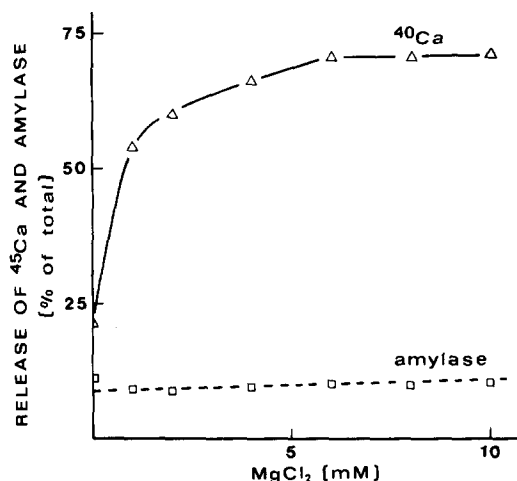


FIGURE 5 Release of  $^{40}\text{Ca}$  by the ionophore as a function of  $\text{Mg}^{++}$  concentration. Ionophore,  $2 \mu\text{g/ml}$ , was added to the granules,  $1,500 \text{ amylase U/ml}$ . A control without ionophore, containing  $10 \text{ mM MgCl}_2$ , released  $18\%$  of total  $^{40}\text{Ca}$  and  $9\%$  of total amylase.

cated a somewhat less release of  $^{40}\text{Ca}$  than observed for  $^{45}\text{Ca}$ . The negligible mitochondrial contamination (see Materials and Methods) cannot explain this difference. Various attempts to find the source of this apparent discrepancy have so far failed.

The granules after release of  $90\%$  of the  $^{45}\text{Ca}$  (see Fig. 4) were examined by electron microscopy. Thin sections of pellets prepared by centrifugation showed that the structure of membrane-bounded granules was preserved. Their staining and appearance was similar to that of control granules (1) which did not receive the ionophore.

## DISCUSSION

The present study suggests that the high amount of calcium in the secretory granules of rat parotid gland does not function as a stabilizer of these subcellular structures. With respect to leakage of amylase and other proteins, the isolated granules were just as stable after release of most of their calcium content. The possibility that in the cell the granules have different properties cannot, however, be excluded. Since the granules lost considerable amounts of calcium only after addition of the ionophore, it seems most likely that the mature granule membrane is impermeable to calcium (17). This deduction raises, of course, the question, at what stage of granule assembly did the calcium become concentrated within the organelle? The experiments indicate that neither the

content nor the membrane of the mature granule is capable of high affinity binding of the large amounts of calcium present. It is therefore not likely that calcium becomes concentrated in the granules simply by binding to the secretory proteins during granule assembly. It is, however, conceivable that the immature granule membrane functions as a calcium pump, later losing this property, becoming impermeable as the granule ripens. A calcium pump had previously been found in smooth membrane vesicles from parotid gland (16).

Several functions might be assigned to the calcium in the secretory granules: facilitation of assembly of the secretory proteins during early stages of granule formation, activation of the fusion of the mature granule membrane with the cell membrane in exocytosis, and building up of the teeth enamel when the calcium is secreted by the gland, together with the other exportable material (9). It will be of interest to see whether the calcium in the chromaffin granules (3) is also readily released by the ionophore without concomitant release of catecholamines, ATP, and the proteins of these organelles.

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