ROLE OF CALCIUM FOR MAGNESIUM-ACTIVATED ADENOSINETRIPHOSPHATASE ACTIVITY AND ADENOSINETRIPHOSPHATE-MAGNESIUM STIMULATED CATECHOLAMINE RELEASE FROM ADRENAL MEDULLARY GRANULES

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There have been several reports that Mg^{++} -activated ATPase is present in the catecholamine storage granules of the adrenal medulla (1-3) and that this ATPase may be linked in some way with the ATP- Mg^{++} stimulated uptake and release of catecholamine by isolated granules (4-9).

Recently, we found that the low concentration of Ca^{++} present in the incubation medium as a contaminant participated in the release of catecholamine from the granules stimulated by ATP and Mg^{++} (10). This finding is interesting in relation to the fact that acetylcholine or excess potassium causes the release of catecholamine from a perfused adrenal gland by promoting increased uptake of Ca^{++} into the cells (11-13).

This paper reports investigations on the effect of Ca^{++} on the Mg^{++} -activated ATPase activity in isolated catecholamine storage granules from the adrenal medulla. The possible relationship of these results to the release of catecholamine from the granules is discussed.

MATERIAL AND METHODS

1) *Preparation of catecholamine storage granules from adrenal medulla*

Catecholamine storage granules were prepared from bovine adrenal medulla as described previously (14). After removal of the cortex, the medullary tissue was cut into pieces and homogenized in a glass Potter homogenizer with 5-6 volumes of 0.32 M sucrose containing 40 mM of Tris-HCl buffer (pH 7.3). The homogenate was centrifuged at $1,000 \times g$ for 10 min to remove coarse debris and nuclei. The supernatant was passed through membrane filters as described previously (14). The filtrate passing through a 0.3μ pore filter was then centrifuged at $6,000 \times g$ for 20 minutes. The pellet was suspended with isotonic KCl solution (150 mM KCl, 40 mM Tris-HCl buffer, pH 7.3) and centrifuged again at $6,000 \times g$ for 20 minutes. The pellet was finally suspended in isotonic KCl solution and used as the preparation of catecholamine storage granules.

2) *Measurement of ATPase activity of granules*

The standard incubation medium contained 150 mM KCl, 40 mM Tris-HCl buffer (pH 7.3), 4 mM ATP and 2 mM $MgSO_4$. The reaction was started by adding the granules (about 2 mg of protein, containing 400-500 μg of catecholamine). The final volume of the incu-

bation mixture was 4 ml. The reaction was stopped by adding 1 ml of 50% (w/v) trichloroacetic acid. After deproteinization the inorganic phosphate in the supernatant was estimated following the method of Takahashi et al. (15). Protein was estimated according to Lowry et al. (16).

3) Release of catecholamine from the granules

The reaction was carried out in plastic centrifuge tubes without shaking. The standard incubation medium was the same as that used for measurement of the ATPase activity of the granules. The reaction was terminated by adding 4 ml of ice cold isotonic KCl solution. Then the tubes were immediately chilled in ice and centrifuged at $20,000 \times g$ for 10 minutes. Catecholamine in the precipitate and supernatant were extracted with 0.4 N perchloric acid and determined fluorimetrically by the ethylenediamine condensation method (17).

4) Reagents

ATP, disodium salt was obtained from Sigma Chemical Co., passed through a Dowex 50 (H^+ type, 200–400 mesh) column ($0.4 \text{ cm} \times 4 \text{ cm}$) to remove contaminating calcium ions and converted to the tris-form.

Ca-EGTA was prepared by mixing various ratios of $CaCl_2$ and *EGTA following the method of Portzehl et al. (18). The mixture was adjusted to pH 7.3 by addition of KOH.

RESULTS

1) Effects of EGTA on Mg^{++} -activated ATPase and ATP- Mg^{++} stimulated catecholamine release

The Mg^{++} -activated ATPase activity of the granules and the ATP- Mg^{++} stimulated catecholamine release in the presence of various concentrations of Mg^{++} were examined. Incubation was carried out at $37^\circ C$ for 5 minutes. The release of catecholamine from the granules was strongly stimulated by the addition of ATP- Mg^{++} in parallel with activation of Mg^{++} -activated ATPase. As shown in Fig. 1, Mg^{++} -activated ATPase activity was inhibited significantly by addition of 2.5 mM of EGTA, which chelates with calcium. This inhibitory effect of EGTA was not affected by the increase of Mg^{++} concentration in the incubation medium.

The ATP- Mg^{++} stimulated release of catecholamine was examined under

FIG. 1. Inhibitory effect of EGTA on Mg^{++} -activated ATPase of adrenal medullary granules and ATP- Mg^{++} stimulated catecholamine release.

The reaction was carried out in standard incubation medium for 5 minutes at $37^\circ C$. EGTA (2.5 mM) was present or absent. The values are the means of 5 experiments.

●—● Mg^{++} -activated ATPase activity
○—○ ATP- Mg^{++} stimulated catecholamine release

*EGTA: 1,2-bis (2-dicarboxymethylaminoethoxy)-ethane

the same experimental conditions. ATP- Mg^{++} stimulated release of catecholamine was reduced to 35% of the control value by addition of 2.5 mM EGTA. The inhibitory effect of EGTA on catecholamine release was much stronger than the effect of Mg^{++} -activated ATPase.

EGTA did not interfere with the assay of inorganic phosphate or catecholamine, since it had no effect when added to the sample after the reaction had stopped.

2) Reversal by Ca-EGTA of EGTA inhibition of Mg^{++} -activated ATPase and ATP- Mg^{++} stimulated catecholamine release

To confirm that the effect of EGTA was due to its chelation with contaminating Ca^{++} , the ratio of Ca^{++} to EGTA was varied keeping the EGTA concentration at 2.5 mM. In intact granules, the inhibition of Mg^{++} -activated ATPase by EGTA was progressively reversed on raising the Ca/EGTA ratio (Fig. 2). Addition of CaCl_2 at a concentration equivalent to that of EGTA almost completely prevented the inhibition by EGTA.

The inhibition of ATP- Mg^{++} stimulated release of catecholamine by EGTA was also reversed by raising the Ca/EGTA ratio. However, even in the presence of equimolar amounts of Ca^{++} and EGTA catecholamine release increased to about 70% of the control value.

3) Effects of EGTA and Ca-EGTA on Mg^{++} -activated ATPase in catecholamine storage granules in hypotonic KCl solution

Next, the effects of EGTA and Ca-EGTA were examined in hypotonic KCl (25 mM) solution in which the granules ruptured abruptly, to see whether the inhibitory effect of EGTA on Mg^{++} -activated ATPase was dependent on intact structure of the granules.

Mg^{++} -activated ATPase activity was estimated in hypotonic (25 mM) KCl solution, other conditions being as in the experiment in isotonic KCl solution. In hypotonic KCl solution Mg^{++} -activated ATPase was again inhibited about 50% by 2.5 mM EGTA. As shown in Fig. 4, maximum inhibition was observed with 1.0 mM EGTA. On raising the Ca/EGTA ratio, the Mg^{++} -activated ATPase activity was progressively restored and it was completely restored to the control level at a lower Ca/EGTA ratio than in isotonic solution. In medium containing equimolar amounts of Ca^{++} and EGTA, the Mg^{++} -activated ATPase activity was above the control value (Fig. 3). Similar results were obtained at a final EGTA concentration of 5 mM.

FIG. 2. Mg^{++} -activated ATPase activity of adrenal medullary granules and ATP- Mg^{++} stimulated catecholamine release at various Ca-EGTA ratios.

Reactions were carried out for 5 minutes at 37°C in standard incubation medium but with various Ca/EGTA ratios. The values represent the means of 5 experiments and standard deviation are indicated by vertical I-shaped bars.

* 100% activity = 0.43 ± 0.04 $\mu\text{moles Pi/mg prot/minutes}$

** 100% catecholamine release = 75 ± 9.0 $\mu\text{g/5 minutes}$

*** pCa ; Calculated value of free Ca^{++}

FIG. 3. Mg^{++} -activated ATPase activity of adrenal medullary granules in hypotonic KCl medium with various Ca/EGTA ratios.

The procedure was as for Fig. 2, except that the final concentration of KCl was 25 mM. The values represent the means of 4 experiments and the standard deviation are indicated by vertical I-shaped bars.

* 100% activity = 0.51 ± 0.06 $\mu\text{moles Pi/mg prot/minutes}$ ($n=7$)

** pCa: Calculated value of free Ca^{++}

4) Effect of ionic strength on Mg^{++} -activated ATPase

Since the effects of EGTA and Ca-EGTA on Mg^{++} -activated ATPase activity were greater in hypotonic KCl solution than in isotonic KCl solution, we examined the effect of the ionic strength of KCl on Mg^{++} -activated ATPase. The Ca^{++} -sensitivity of Mg^{++} -activated ATPase was measured as the difference between the activities in the presence of 2.5 mM of Ca-EGTA and of EGTA, that is the maximum and minimum activities, respectively. As shown in Fig. 5 the Ca^{++} -sensitive activity of Mg^{++} -activated ATPase decreased on increasing the KCl concentration in the incubation medium. Potassium was not specific for this effect of ionic strength on the action of Ca^{++} , for sodium or Tris ion could replace potassium.

FIG. 4. Effect of EGTA on Mg^{++} -activated ATPase in hypotonic KCl solution.

The incubation medium contained 4 mM ATP, 2 mM MgSO_4 , 40 mM Tris (pH 7.3), 25 mM KCl, about 2 mg protein of granules and various concentrations of EGTA. The reaction was carried out for 5 minutes at 37°C. The values represent the means of 3 experiment and standard deviation are indicated by vertical I-shaped bars.

* 100% activity = 0.51 ± 0.06 $\mu\text{moles Pi/mg prot/minutes}$ ($n=7$)

FIG. 5. Effect of ionic strength on Ca^{++} -sensitive activity of Mg^{++} -activated ATPase of adrenal medullary granules.

The incubation medium contained 40 mM Tris (pH 7.3), 4 mM ATP, 2 mM MgSO_4 , about 2 mg protein of granules and the various concentrations of KCl shown, with 2.5 mM EGTA or Ca-EGTA. The reaction was carried out for 5 minutes at 37°C. The values represent the means of 3 experiments and the standard deviation are indicated by vertical I-shaped bars.

* 100 % activity = 0.41 ± 0.05 $\mu\text{moles Pi/mg prot/minutes}$ ($n=6$)

5) Time course of the Mg^{++} -activated ATPase reaction in the presence of EGTA or Ca-EGTA

The time course of the Mg^{++} -activated ATPase reaction was examined in hypotonic KCl medium. In the presence of 2.5 mM EGTA, the reaction was linear during the first 20 min of incubation. With Ca-EGTA, Mg^{++} -activated hydrolysis of ATP was linear during the first 5 minutes and then gradually decreased (Fig. 6).

FIG. 6. Time course of Mg^{++} -activated ATPase reaction in the presence of Ca-EGTA or EGTA.

The incubation medium contained 25 mM KCl, 40 mM Tris (pH 7.3), 4 mM ATP, 2 mM MgSO_4 and about 2 mg protein of granules. Either Ca-EGTA or EGTA was added at the concentration of 2.5 mM. The final volume of the incubation medium was 4 ml. The reactions were carried out at 37°C. The values represent the means of 4 experiments and the standard deviation are indicated by vertical I-shaped bars.

6) Stability of Mg^{++} -activated ATPase

The granules were lysed with 25 mM KCl in 40 mM Tris-HCl buffer (pH 7.3) and then preincubated at 37°C for the various periods shown in Fig. 7. Mg^{++} -activated ATPase activity was estimated under standard conditions except that the final concentration of KCl was 25 mM. The reaction was carried out at 37°C for 5 minutes. The effect of Ca^{++} on Mg^{++} -activated ATPase activity was expressed as described above. As shown in Fig. 7, the effect of Ca^{++} on Mg^{++} -activated ATPase decreased gradually on preincubation without ATP, and after preincubation for 5 minutes at 37°C the effect of Ca^{++} was about half the control level.

FIG. 7. Heat denaturation of the Ca^{++} -sensitive activity of Mg^{++} -activated ATPase in adrenal medullary granules.

The incubation medium contained 4 mM ATP, 2 mM MgSO_4 , 40 mM Tris (pH 7.3), 25 mM KCl and about 2 mg protein of granules. EGTA or Ca-EGTA was presented. The reaction was carried out for 5 minutes at 37°C. The values represent the means of 3 experiments and the standard deviation are indicated by vertical I-shaped bars.

* 100% activity = 0.41 ± 0.05 $\mu\text{moles Pi/mg prot/minutes}$ ($n=6$).

DISCUSSION

The existence of Mg^{++} -activated ATPase in adrenal medullary storage granules and its possible role in the uptake and release of catecholamine by the granules have been re-

ported by several workers (4-9). Calcium ions are essential for the release of catecholamine from perfused adrenal (11, 12) or adrenal slices (19) by acetylcholine or a high potassium concentration. It is generally thought that free Ca^{++} penetrating into the chromaffine cells due to the addition of acetylcholine or a high potassium concentration may initiate the release of catecholamines from the granules in some way (13). But the intracellular mechanism of the action of Ca^{++} is unknown.

Accordingly, we examined the effect of a low concentration of Ca^{++} on the Mg^{++} -activated ATPase activity of the granules and on ATP- Mg^{++} stimulated catecholamine release from the granules.

As shown in Fig. 1, EGTA, which chelates with calcium was found to inhibit the Mg^{++} -activated ATPase activity of the granules. This inhibitory effect of EGTA on Mg^{++} -activated ATPase activity was not influenced by various concentrations of Mg^{++} , suggesting that the effect of EGTA was due to its removal of contaminating Ca^{++} from the incubation medium. EGTA also strongly inhibited the ATP- Mg^{++} stimulated release of catecholamine.

Next we used Ca-EGTA buffer to obtain a low concentration of calcium ions into the incubation medium. The ratio of Ca^{++} to EGTA was varied from zero to 1.0 keeping the total amounts of EGTA at 2.5 mM. On raising the Ca/EGTA ratio, the Mg^{++} -activated ATPase activity was progressively restored and with equimolar amounts of Ca^{++} and EGTA, the Mg^{++} -activated ATPase activity was restored to 96% of the control level. Inhibition of catecholamine release by EGTA was also reversed by Ca-EGTA, though to a lesser extent. The fact that the reversal of inhibition of catecholamine release was less than that of Mg^{++} -activated ATPase activity might be due to increase in the ionic strength of the medium, in the former case, an inevitable results of adjusting the pH of the preparation of Ca-EGTA buffer, since ATP- Mg^{++} stimulated release of catecholamine is known to be very sensitive to increase in ionic strength.

Therefore, the results suggest that a low concentration of calcium may be important for the activity of Mg^{++} -activated ATPase of catecholamine containing granules and for the release of catecholamine stimulated by ATP and Mg^{++} .

We also examined the effects of EGTA or Ca-EGTA on the Mg^{++} -activated ATPase in hypotonic KCl medium to see whether the intact structure of the granules was necessary for the effects of these agents on Mg^{++} -activated ATPase. The inhibitory effect of EGTA on Mg^{++} -activated ATPase and its reversal by Ca-EGTA were also observed in hypotonic KCl medium, suggesting that the intact structure of catecholamine storage granules is not necessary for the effects of these agents. In hypotonic solution the inhibitory effect of EGTA was restored to the control level at a lower ratio of Ca/EGTA and with equimolar amounts of Ca^{++} and EGTA, Mg^{++} -activated ATPase activity was above the control level. These findings suggest that Mg^{++} -activated ATPase in the granules may be more sensitive to Ca^{++} in hypotonic KCl solution or that the effect of a low concentration of Ca^{++} may be antagonized by potassium ion. To study these possibilities we examined the effect of ionic strength on the action of Ca^{++} on Mg^{++} -activated ATPase. The stimulatory effect of Ca^{++} on Mg^{++} -activated ATPase decreased on increasing the potassium concentration.

Sodium or Tris ion could replace potassium ion. Therefore, the fact that the effects of EGTA and Ca-EGTA are less in isotonic KCl solution than in hypotonic solution could be explained by the higher ionic strength of the former medium. Under isotonic conditions the effect of Ca^{++} on the Mg^{++} -activated ATPase seems to be small. The actual concentration of free calcium in the medium at various Ca/EGTA ratios was not measured but calculated according to Schwarzenbach (20). The concentration of free calcium which is associated with the Mg^{++} -activated ATPase and ATP- Mg^{++} stimulated catecholamine release seems to be about 10^{-7} to 10^{-5} M.

When the total EGTA concentration was 5 mM and the Ca/EGTA ratio was 0.8, the free concentration of EGTA seems to be 1 mM. As shown in Fig. 4, at this concentration EGTA exhibited maximum inhibition of Mg^{++} -activated ATPase. Therefore, the possibility that EGTA acts directly on the granules and that the reversal of inhibition by Ca-EGTA is due to decrease in the free EGTA concentration is excluded.

The effect of Ca^{++} on the Mg^{++} -activated ATPase was greater during a short incubation period (Fig. 6) and was not observed after heat denaturation, so that the sensitivity of Mg^{++} -activated ATPase to Ca^{++} is very unstable to heat.

The fact that the maximum inhibitory effect of EGTA on Mg^{++} -activated ATPase in intact granules was 40% of the total activity raises the question of the physiological role of EGTA resistant Mg^{++} -activated ATPase. Addition of EGTA to the incubation medium significantly increased the uptake of ^{14}C -adrenaline into the granules unpublished. Thus EGTA resistant Mg^{++} -activated ATPase may be related to the uptake of catecholamine.

From these data, it seems that a low concentration of calcium penetrating the chromaffine cells, due to the presence of acetylcholine or a high potassium concentration, may activate the Mg^{++} -activated ATPase of these granules and this activation, in turn, may cause catecholamine release from the granules.

SUMMARY

The effect of Ca^{++} on the Mg^{++} -activated ATPase (ATP Phosphohydrolase, EC, 3, 6, 1, 4) activity and on ATP- Mg^{++} stimulated catecholamine release from bovine adrenal medullary granules was studied.

1. The chelating agent, EGTA inhibited the Mg^{++} -activated ATPase activity of the granules. EGTA also strongly inhibited the ATP- Mg^{++} stimulated release of catecholamine from the granules.

2. Addition of Ca^{++} at a concentration equimolar to that of EGTA prevented the inhibition of Mg^{++} -activated ATPase by EGTA. Inhibition of catecholamine release by EGTA was also reversed by addition of Ca^{++} .

3. The inhibitory effect of EGTA on Mg^{++} -activated ATPase and its reversal by Ca-EGTA were also observed in the disrupted granules.

4. These results suggest that a low concentration of Ca^{++} may be important for the activity of Mg^{++} -activated ATPase of the granules and for the release of catecholamine stimulated by ATP and Mg^{++} .

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