EFFECTS OF CYTOPLASMIC AND MICROSOMAL FRACTIONS ON ATP-Mg⁺⁺ STIMULATED CATECHOLAMINE RELEASE FROM ISOLATED ADRENOMEDULLARY GRANULES

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There is evidence that Ca⁺⁺ is required for the release of catecholamine from the adrenal medulla evoked by acetylcholine or a high K⁺ concentration (1, 2) and that an increased influx of Ca⁺⁺ into the cell occurs during this process (3). It has therefore been proposed that it is the entry of Ca⁺⁺ into the cell which somehow triggers the secretory process (4, 5).

On the other hand, the release of catecholamine from isolated catecholamine storage granules is shown to be stimulated by ATP and Mg⁺⁺ (6-8) and this ATP-Mg⁺⁺ stimulated release of catecholamine also requires a low concentration of Ca⁺⁺ (<10⁻⁵M), present as a contaminant in the reaction mixture (9). The authors suggested that ATP-Mg⁺⁺ stimulated release of catecholamine from the granules could be a step in the secretory response of the adrenal medulla.

To elucidate the exact intracellular mechanism of adrenal medullary secretion, it is necessary to determine whether or not intracellular elements other than catecholamine storage granules are involved in the regulation of catecholamine release from the granules. We have investigated the effects of the cytoplasmic and microsomal fractions on ATP-Mg⁺⁺ stimulated catecholamine release from the granules. Results suggest that these two fractions are important in regulation of catecholamine release from the adrenal medulla.

MATERIALS AND METHODS

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

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

2) *Preparation of cytoplasmic and microsomal fractions from adrenal medulla*

Bovine adrenal medulla was homogenized in ten volumes of isotonic KCl solution (pH

7.3). The coarse debris and nuclei were removed by centrifugation at $1,000\times g$, for 10 min. The granular fraction was then removed by centrifugation at $8,000\times g$, for 20 min. The supernatant was centrifuged at $105,000\times g$, for 2 hr in a Spinco model L ultracentrifuge for separation into the microsomal fraction and cytoplasmic fraction. The cytoplasmic fraction was treated with ammonium sulfate and the fraction precipitating at 40-80% saturation was collected. The precipitate was dissolved in 40 mM Tris-HCl buffer (pH 7.3) and dialysed against the same buffer for 24 hr at 4°C. The insoluble precipitate was removed by centrifugation and the supernatant used as the preparation of the cytoplasmic fraction. The microsomal fraction was suspended in 40 mM Tris-HCl buffer (pH 7.3) and passed through a Sephadex G-25 column (1.8 cm diameter, 20 cm height) to remove catecholamine, calcium ions and other lower molecular weight substances present as contaminants. The microsomal fraction was then suspended with isotonic KCl solution for use in experiments.

3) *Release of catecholamine from catecholamine storage granules*

The reaction was carried out at 37°C in plastic centrifuge tubes without shaking. The standard incubation medium contained 150 mM KCl, 40 mM Tris-HCl buffer (pH 7.3), 4 mM ATP and 2 mM MgSO_4 in a final volume of 3 ml. The reaction was started by adding the granules (about 2 mg of protein, containing 400 to 500 μg of catecholamine) and stopped by adding 4 ml of ice cold isotonic KCl solution. The tubes were then chilled in ice and centrifuged at $20,000\times g$, for 10 min. Catecholamine in the precipitate and supernatant were extracted with 0.4 N perchloric acid and determined fluorimetrically by the ethylenediamine condensation method (11).

RESULTS

1) *Effect of the cytoplasmic fraction on release of catecholamine from the granules*

Catecholamine storage granules isolated from bovine adrenal medulla were incubated in isotonic KCl solution for 5 min at 37°C. The release of catecholamine was stimulated by the presence of ATP and Mg^{++} , as reported previously (6). This ATP- Mg^{++} stimulated catecholamine release was found to be greatly increased by addition of the cytoplasmic fraction prepared from the adrenal medulla (Fig. 1), however addition of the cytoplasmic fraction in the absence of ATP and Mg^{++} did not accelerate the release of catecholamine from the granules.

2) *Effect of the concentration of cytoplasmic fraction on ATP- Mg^{++} stimulated catecholamine release*

As shown in Fig. 2, the presence of 1 mg of protein of the cytoplasmic fraction per ml of incubation medium accelerated the release of catecholamine. The effect of the cytoplasmic fraction was maximal when this fraction was added at a concentration of 3 to 4 mg protein per ml of incubation medium.

3) *Time course of catecholamine release in the presence of the cytoplasmic fraction*

The effect of the cytoplasmic fraction on the time course of ATP- Mg^{++} stimulated catecholamine release was examined (Fig. 3). The release of catecholamine caused by

FIG. 1. Effect of cytoplasmic fraction on release of catecholamine from adrenal medullary granules.

Incubation medium

KCl 150 mM, Tris-HCl (pH 7.3) 40 mM,

ATP 4 mM, MgSO₄ 2 mM

Cytoplasmic fraction (2.0 mg/ml)

CA granules (450-500 μ g CA)

Incubated for 5 min at 37°C

FIG. 2. Effect of cytoplasmic fraction on release of catecholamine from adrenal medullary granules

Incubation medium

KCl 150 mM, Tris-HCl (pH 7.3) 40 mM,

ATP 4 mM, MgSO₄ 2 mM

Cytoplasmic protein (1.0-4.0 mg/ml)

CA granules (450-500 μ g CA)

Incubated for 5 min at 37°C, n=4

FIG. 3. Time course of catecholamine release from granules in the presence of the cytoplasmic fraction.

Incubation medium

KCl 150 mM, Tris-HCl (pH 7.3) 40 mM,

ATP 4 mM, MgSO₄ 2 mM

Cytoplasmic protein (2 mg/ml)

CA granules (450-500 μ g CA)

Incubated at 37°C, n=5

FIG. 4. Heat denaturation of cytoplasmic fraction

Incubation medium.

KCl 150 mM, Tris-HCl (pH 7.3) 40 mM,

ATP 4 mM, MgSO₄ 2 mM

Cytoplasmic protein (2 mg/ml)

CA granules (450-500 μ g CA)

Incubated for 5 min at 37°C

ATP-Mg⁺⁺ was observed to be linear during the first 10 min of the reaction. The presence of the cytoplasmic fraction (2 mg protein/ml of incubation medium) strongly stimulated the release of catecholamine and this effect was found to be remarkable during a short period of incubation, suggesting that the cytoplasmic fraction increases the initial rate of catecholamine release from the granules.

4) *Inactivation of the activity of the cytoplasmic fraction by heat treatment*

The effect of heat treatment of the cytoplasmic fraction on its effect and/or catecholamine release was examined.

The cytoplasmic fraction was incubated in 150 mM KCl medium containing 40 mM Tris-buffer (pH 7.3) at 80°C for 10 min. As shown in Fig. 4, this greatly reduced its effect in increasing ATP-Mg⁺⁺ stimulated catecholamine release from the granules.

5) *Effect of cytoplasmic fractions from various organs on the release of catecholamine from the granules*

The effects of cytoplasmic fractions from other tissues (heart, spleen, liver) and serum albumin on ATP-Mg⁺⁺ stimulated catecholamine release from the granules were tested. The cytoplasmic fractions of these organs were prepared by the same procedure used with bovine adrenal medulla. The cytoplasmic fractions were added to the incubation medium at a concentration of 2 mg protein per ml. As shown in Table 1, the cytoplasmic fractions from bovine heart and spleen were found to accelerate the release of catecholamine from the granules, as in the case of the adrenal medulla, however, the cytoplasmic fraction from bovine liver and serum albumin did not have any affect.

6) *Effect of EGTA on the release of catecholamine in the presence of the cytoplasmic fraction*

Previously, it was reported that the release of catecholamine stimulated by ATP-Mg⁺⁺ was inhibited by EGTA, which chelates calcium, and that this inhibition was reversed by a low concentration of calcium added as Ca-EGTA buffer (9). This suggested that a low concentration of Ca⁺⁺ contaminating the medium could be important in ATP-Mg⁺⁺ stimulated catecholamine release, therefore, the effect of EGTA on the release of catecholamine

TABLE 1. Effects of cytoplasmic fractions from various organs on release of catecholamine. Incubtion medium,

KCl 150 mM, Tris-HCl (pH 7.3) 40 mM, ATP 4 mM, MgSO₄ 2 mM

Cytoplasmic fraction (2.0 mg/ml)

CA granules (450-500 µg CA)

Incubated for 5 min at 37°C

Organ	CA release (%)
None	100*
Adrenal medulla	214
Heart	177
Spleen	179
Liver	105
Serum albumin	98

* 100% = 80 µg of CA released/5 min

FIG. 5. Effect of EGTA on release of catecholamine from adrenal medullary granules.

Incubation medium

KCl 50 mM, Tris-HCl (pH 7.3) 40 mM,

ATP 4 mM, MgSO_4 2 mM

Cytoplasmic protein (2 mg/ml)

CA granules (450–500 μg CA)

Release of catecholamine after addition of EGTA is shown by the broken lines.

FIG. 6. Effect of microsome fraction on release of catecholamine from adrenal medullary granules.

Incubation medium.

KCl 150 mM, Tris-HCl (pH 7.3) 40 mM,

ATP 4 mM, MgSO_4 2 mM

Cytoplasmic protein (2 mg/ml)

CA granules (450–500 μg CA)

Release of catecholamine after addition of microsome fraction is shown by broken lines.

in the presence of the cytoplasmic fraction was examined. As shown in Fig. 5, the ATP-Mg^{++} stimulated release of catecholamine from catecholamine storage granules decreased immediately on addition of EGTA. EGTA also inhibited the release of catecholamine when this was accelerated by the presence of the cytoplasmic fraction.

7) Effect of the microsomal fraction on release of catecholamine from the granules

The effect of the microsomal fraction from bovine adrenal medulla on ATP-Mg^{++} stimulated release of catecholamine from the granules was examined (Fig. 6). The microsomal fraction, suspended with isotonic KCl solution, was added 2 min after the start of the reaction. The release of catecholamine stimulated by ATP-Mg^{++} was immediately depressed by the addition of the microsomal fraction (2.5 mg of protein). This inhibitory effect of the microsomal fraction was also observed when the release of catecholamine was greatly accelerated by the presence of cytoplasmic fraction (2 mg of protein per ml of incubation medium).

DISCUSSION

It has been suggested that acetylcholine or excess K^+ evokes catecholamine secretion from the adrenal medulla by promoting the influx of Ca^{++} into the cells (3). Therefore, Ca^{++} is thought to initiate the release of catecholamine by an intracellular action (4, 5).

On the other hand, studies on isolated catecholamine storage granules have shown that release of catecholamine is stimulated by ATP and Mg^{++} (6–8) and that their effect also depends on the presence of a low concentration of Ca^{++} ($<10^{-5}M$) which is present as a contaminant in the reaction mixture (9). Thus, it appears that ATP- Mg^{++} stimulated release of catecholamine may be a step in the secretory process of the adrenal medulla.

It was uncertain whether these findings observed with isolated granules actually represented the mechanism of catecholamine release occurring in intact cells. Therefore, the role of cytoplasmic and microsomal fractions in the release of catecholamine from the granules was examined in order to elucidate the intracellular mechanism regulating catecholamine release.

From experiments on the effects of the cytoplasmic fraction on ATP- Mg^{++} stimulated release of catecholamine from the granules, the following results were obtained:

- 1) ATP- Mg^{++} stimulated catecholamine release was accelerated significantly by addition of the cytoplasmic fraction and this effect was marked during the initial period of incubation.
- 2) The effect of the cytoplasmic fraction in accelerating catecholamine release stimulated by ATP and Mg^{++} was lost on heating the cytoplasmic fraction at $80^{\circ}C$ for 10 min.
- 3) The release of catecholamine stimulated by ATP and Mg^{++} in the presence of the cytoplasmic fraction was inhibited by the addition of EGTA, a specific chelator of Ca^{++} .

It is of interest that the initial rate of catecholamine release in the presence of ATP and Mg^{++} was accelerated by addition of the cytoplasmic fraction. This suggests that in intact cells where components of the cytoplasmic fraction are present at high concentration, the release of catecholamine from the granules may be much more rapid. This phenomenon may represent the rapid release of catecholamine from cells stimulated by acetylcholine or excess K^{+} .

The effective component in the cytoplasmic fraction appears to be a protein-like substance as this effect was observed with part of the cytoplasmic fraction precipitated by 40–80% saturation of ammonium sulfate. The fact that the effect of the cytoplasmic fraction was lost on heat treatment also supports this suggestion.

As reported previously (9), the release of catecholamine caused by ATP- Mg^{++} was inhibited by EGTA and this inhibition by EGTA was reversed by addition of Ca-EGTA buffer. From these findings, it was suggested that a low concentration of Ca^{++} ($<10^{-5}M$), probably present in the incubation mixture as a contaminant, could be important in the release of catecholamine caused by ATP and Mg^{++} . A similar inhibitory effect of EGTA was also observed under conditions where release of catecholamine from the granules was greatly accelerated by the presence of the cytoplasmic fraction. This suggests that the cytoplasmic fraction may accelerate ATP- Mg^{++} stimulated catecholamine release by potentiating the action of a low concentration of Ca^{++} on the granules.

In connection with this finding, it is of interest that serum protein is necessary for the release of serotonin from blood platelets caused by Ca^{++} (12) and that the cytoplasmic protein of the parotid gland accelerated the release of amylase from isolated zymogen granules in the presence of Ca^{++} , ATP and Mg^{++} (13–14).

Further studies are required on the mechanism by which the cytoplasmic protein-like substances potentiates the releasing action of a low concentration of Ca^{++} . A possible explanation is that when Ca^{++} has entered the cell due to stimulation by acetylcholine or excess K^{++} its action in releasing catecholamine from the granule may be potentiated by the protein-like component of the cytoplasmic fraction.

The cytoplasmic fractions from bovine heart and spleen also accelerated the release of catecholamine from the granules, but those from liver or serum albumin did not. These findings suggest that the cytoplasmic fraction from tissues which contain relatively high concentrations of catecholamine accelerate the release of catecholamine from the granules. The release of catecholamine from the heart or spleen is also known to depend on the presence of Ca^{++} (5). Therefore, catecholamine release from these tissues innervated with sympathetic nerves may also be regulated in part by the same type of intracellular components.

The effect of the microsomal fraction on ATP- Mg^{++} stimulated catecholamine release from the granules was examined to elucidate the role of microsomal elements in regulation of the secretory process in the adrenal medulla. It was found that the ATP- Mg^{++} stimulated catecholamine release from the granules was inhibited by addition of the microsomal fraction from adrenal medulla. This inhibitory effect of the microsomal fraction was also observed when the rate of catecholamine release was greatly accelerated by the presence of the cytoplasmic fraction.

Recently, Poisner and Hava reported that the microsomal fraction can bind Ca^{++} very rapidly in the presence of ATP and Mg^{++} (15). We also investigated the binding of Ca^{++} by the microsomal fraction under our experimental conditions and obtained similar results, showing that binding of radioactive Ca^{++} by the microsomal fraction is dependent on ATP and Mg^{++} (16). Therefore, it seems likely that a microsomal fraction, like EGTA, may inhibit the release of catecholamine from the granules, presumably by removing the low concentration of Ca^{++} , which is necessary for catecholamine release, from the medium.

In muscle, the microsomal fraction (endoplasmic reticulum) is known to participate in contraction and relaxation by regulating the intracellular Ca^{++} concentration (17, 18). It could be possible that the microsomal fraction may be important in release of catecholamine from medullary granules by regulating the intracellular Ca^{++} concentration, as proposed by Poisner and Hava (15).

Further experiments are now in progress as to whether or not other elements, such as the mitochondrial fraction are important in regulation of catecholamine release by controlling the intracellular Ca^{++} level in the adrenal medulla.

From the above results, both the cytoplasmic and microsomal fractions appear important in intracellular regulation of the secretory process: the cytoplasmic fraction accelerates catecholamine release from the granules by potentiating the releasing effect of Ca^{++} introduced into cells by acetylcholine or excess K^{+} , while the microsomal fraction inhibits the release of catecholamine by decreasing the intracellular level of free Ca^{++} . These findings in isolated catecholamine storage granules may represent in part the secretory process occurring in intact cells where immediate, rapid release of catecholamine is evoked by

acetylcholine or excess K^+ , and release rapidly decreases with time.

SUMMARY

The cytoplasmic fraction from adrenal medulla was found to accelerate the catecholamine release from the granules caused by ATP and Mg^{++} , presumably by potentiating the releasing effect of the low concentration of Ca^{++} contaminating the reaction mixture. On the other hand, the microsomal fraction inhibited the ATP- Mg^{++} stimulated catecholamine release from the granules both in the presence and absence of the cytoplasmic fraction, presumably by removing Ca^{++} from the incubation medium.

Roles of the cytoplasmic and microsomal fractions in regulating the initiation and termination of the secretory process were discussed on the basis of these findings.

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