CATECHOLAMINE RELEASING FACTOR IN BOVINE ADRENAL MEDULLA

Futoshi IZUMI, Motoo OKA*, Kyoji MORITA and Hitoshi AZUMA
Department of Pharmacology II, Osaka University School of Medicine, 33, Joancho, Kitaku, Osaka, Japan 530

Received 16 May 1975

1. Introduction

Recently, the importance of cytoplasmic proteins in the process of catecholamine secretion has been pointed out. These proteins include microtubule protein (tubulin) [1], microfilament [2], cyclic AMP-dependent protein kinase [3] and contractile-like protein [4].

To obtain direct biochemical evidence of exocytosis in catecholamine release from the adrenal medulla, we studied the cytoplasmic proteins of chromaffine cells, finding a protein factor which stimulated catecholamine release from isolated granules containing catecholamine. Results indicated that the stimulatory effect of this protein factor was dependent on the presence of adenine nucleotides such as ATP, ADP and AMP. This factor required magnesium and a low concentration of calcium for its action.

2. Materials and methods

Bovine adrenal glands were used throughout the experiments. Fresh adrenal glands were obtained from a local slaughter house. The adrenal medulla was dissected out and placed in a cold solution of 150 mM, KCl, 50 mM Tris–HCl (pH 7.4), 4 mM ATP, 2 mM $MgSO_4$, 5 mM EGTA and 0.05 mM 2- mercaptoethanol. Then it was homogenized with a glass Potter homogenizer. The homogenate was centrifuged successively at 10 000 *g* for 1 hr and at 105 000 *g* for 2 hr. The final supernatant was subjected to stepwise fractionation with 0–35, 35–50 and 50–100% saturation of ammonium sulfate. Precipitation with ammonium sulfate was repeated three times to prevent cross-contamination of the fractions. The precipitates obtained at each fractionation step by centrifugation at 10 000 *g* for 30 min were dialyzed against 150 mM KCl, in 50 mM Tris–HCl (pH 7.4) until the dialysis fluid gave a negative reaction with Nessler's reagent. Then these fractions of cytoplasmic protein were used in experiments on catecholamine release and colchicine binding. Granules containing catecholamine were obtained using a millipore filter. The details of the procedures used for isolation of granules and measurement of catecholamine were described in our previous report [5]. For detection of colchicine binding, colchicine was incubated with one of the cytoplasmic protein fractions in the presence of GTP and magnesium for 1 hr at 37°C in the dark. Then the mixture was applied to a Sephadex G-75 column (1.8 X 40 cm) and protein-bound colchicine was separated from free colchicine by elution with 150 mM KCl–50 mM Tris–HCl (pH 7.4). The fractions of eluate were deproteinized by adding perchloric acid to a final concentration of 0.4 N and colchicine in each fraction was measured from its absorption at 240 nm.

3. Results and discussion

The catecholamine releasing activity of the cytoplasmic protein detected in the presence of ATP and magnesium was mainly recovered in the fraction precipitated with 0–35% saturation of ammonium

* Present Address: Department of Pharmacology, Tokushima University School of Medicine, 3-18-15, Kuramotocho, Tokushimashi, Japan.

Table 1
Release of catecholamine from isolated adrenomedullary granules and colchicine binding to cytoplasmic protein fractions

Fraction added	Catecholamine release[a] (% of total)	Colchicine binding[b] (mμ mol/mg protein)	
None	17.6 ± 2.1		
0–35%	40.0 ± 5.3	0.14	1.17
35–50%	23.5 ± 1.8	0.41	0.65
50–100%	22.8 ± 3.2	1.02	1.58

[a] Values are the mean average ± standard deviation (n = 4).
[b] Values represented are from two experiments.
The incubation medium for catecholamine release consisted of 150 mM KCl, 40 mM Tris–HCl (pH 7.4), 4 mM ATP, 2 mM $MgSO_4$, 2 mg of cytoplasmic protein and granules containing catecholamine (450 μg of catecholamine). The mixture was incubated for 5 min at 37°C and then centrifuged at 20 000 *g* for 10 min. Catecholamine in the supernatant was measured by the ethylenediamine-condensation method.

Cytoplasmic protein fractions (50 mg each) were incubated with 0.7 μmol of colchicine in solution containing 150 mM KCl, 40 mM Tris–HCl (pH 7.4), 0.1 mM GTP and 10 mM $MgSO_4$. The final vol. of incubation medium was 7.0 ml.

sulfate. However, colchicine bound predominantly to the fractions precipitated by higher concentration of ammonium sulfate and only weakly to this fraction.

Assuming that colchicine binds to a certain site on the microtubule protein, the catecholamine releasing factor seems to be quite distinct from microtubule protein, which has been suggested to be involved in the intracellular movement of granules during the secretory process (table 1).

Table 2 shows the effects of various nucleotides on the release of catecholamine in the presence of the catecholamine releasing factor (0–35% fraction) and magnesium, which was essential for this process. In the presence of the releasing factor, the release of catecholamine was greatly stimulated not only by ATP but also by ADP and AMP. The effect of ADP was greater than those of other diphosphonucleotide analogues, such as UDP, CDP and GDP. Like the latter, GTP caused only slight stimulation while adenosine, adenine or the protein factor alone caused no stimulation. In the absence of this factor, only ATP stimulated catecholamine release.

The fact that ADP and AMP stimulated catechol-

Table 2
Effects of nucleotides on the release of catecholamine and P_i liberation

	Catecholamine release (%)		P_i Liberation (μmol)[a]	
35% Fraction	None	Added	None	Added
AMP	9.0	37.5	0.15	1.16 (0.53)
ADP	9.0	52.0	0.83	4.61 (2.62)
ATP	17.2	40.2	1.30	3.10 (1.01)
GTP	9.1	15.1		

Slight effects: UDP, CDP, GDP
No effects: Adenosine, Adenine
35% fraction alone
[a] μmoles of phosphate liberated in 5 min, measured by the method of Martin and Doty [9].
Granules (450 μg of catecholamine) were incubated in a solution consisting of 150 mM KCl, 40 mM Tris–HCl (pH 7.4), 4 mM nucleotide, 2 mM $MgSO_4$ and 2 mg/ml of protein when added. Incubation was carried out at 37°C for 5 min. Then the mixture was centrifuged and catecholamine and inorganic phosphate in the supernatant were estimated. Values in parentheses show phosphate liberation in the absence of granules.
Values represented are the mean average from three experiments.

amine release in the presence of the protein factor suggests that under more nearly in vivo conditions, these adenine nucleotides are also involved in the release of catecholamine as they are formed in successive steps following ATP hydrolysis by magnesium stimulated-ATPase of the granule membranes.

Determination of inorganic phosphate revealed that the granules hydrolyzed not only ATP but also ADP and AMP. The releasing factor itself also showed phosphate-liberating activity, as shown in parentheses in table 2, but when present with the granules its activity in phosphate liberation was more than additive. Parallel measurements of catecholamine release and phosphate liberation in relation to time (data not shown) and to concentration dependency (fig.1) showed that the release of catecholamine was not proportional to the liberation of phosphate. Therefore, it seems that the increased release of catecholamine by the protein factor was not the result of increased phosphate liberation.

It has been suggested that phosphorylation of the granules may be involved in the secretory process [6]. In support of this we observed time-dependent phosphorylation of the granules when they were incubated with [γ-^{32}P] ATP in the presence of magnesium. Further purification of the 0–35% fraction by DEAE-cellulose column chromatography revealed that the catecholamine releasing protein was eluted with 150 mM KCl while protein kinase was eluted with 500 mM KCl. The latter had no effect on catecholamine release indicating that the catecholamine releasing protein is quite different from protein kinase.

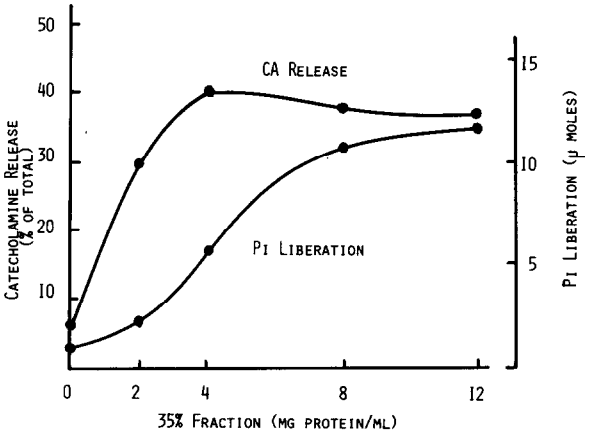

Fig.1. Release of catecholamine and P_i liberation in the presence of the 0–35% fraction. Granules (450 μg of catecholamine) were incubated in a solution consisting of 150 mM KCl, 40 mM Tris–HCl (pH 7.4), 4 mM ADP, 2 mM $MgSO_4$ and various amounts of the 0–35% fraction. Incubation was carried out at 37°C for 5 min. Data shown from one of the three experiments.

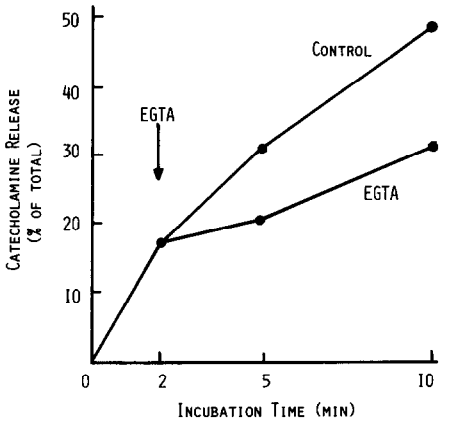

Fig.2. Effect of EGTA on the release of catecholamine in the presence of the 0–35% fraction. Granules (450 μg catecholamine) were incubated in a solution consisting of 150 mM KCl, 40 mM Tris–HCl (pH 7.4), 4 mM ADP, 2 mM $MgSO_4$ and 2 mg/ml of the 0–35% fraction. EGTA was added after 2 min of incubation period at a final concentration of 5 mM.

As shown in fig.2, the release of catecholamine in the presence of the 0–35% fraction was abolished on removal of contaminating calcium by addition of EGTA. Furthermore, although the stimulatory effect was observed in medium containing KCl and NaCl, it was not observed in sucrose medium which is thought to have a membrane-stabilizing effect. These findings suggest that the stimulatory effect of the protein factor is linked with the action of a low concentration calcium, which is thought to penetrate into chromaffine cells during stimulation by acetylcholine, the physiological secretogogue [7].

Decrease in the optical density of a suspension of granules can be used as an index of structural changes of the granules during catecholamine release. The decrease in optical density was found to be much greater in the presence of the catecholamine-releasing protein than in its absence, suggesting that the stimulatory effect of the protein factor is closely related with structural changes of the granules.

It is important to know on which steps of exocytosis the protein factor and adenine nucleotides act. They probably do not act on the step of intracellular movements of granules, because colchicine only combines weakly with the fraction containing the protein factor. However, they may well be involved in some parts of the process of fusion of granules to the surface membrane [8].

Acknowledgements

We thank Mrs K. Tsuji for expert technical assistance. We also thank Professor H. Wada for valuable advice.

References

[1] Redburn, D. A., Poisner, A. M. and Samson, E. Jr. (1972) Brain Res. 44, 615–624.
[2] Poisner, A. M. (1973) Proc. 4th International Congr. Endocrinol. (in press).
[3] Labrie, F., Lemaire, S., Poirier, G., Pelletier, G. and Boucher, R. (1971) J. Biol. Chem. 246, 7311–7317.
[4] Poisner, A. M. (1973) in: Frontiers in Neuroendocrinology (Gangong, W. F. and Martini, L., eds.) pp. 33–39, Oxford University Press, New York.
[5] Oka, M., Izumi, F. and Kashimoto, T. (1972) Japan. J. Pharmacol. 22, 207–214.
[6] Trifaró, J. M. and Dworkind, J. (1971) Molec. Pharmacol. 7, 52–65.
[7] Douglas, W. W. and Poisner, A. M. (1962) J. Physiol. 162, 385–392.
[8] Poste, G. and Allison, A. C. (1971) J. Theor. Biol. 32, 165–184.
[9] Martin, J. B. and Doty, D. M. (1949) Anal. Chem. 21, 965.