INVOLVEMENT OF MEMBRANE ASSOCIATED PROTEIN IN ADP-INDUCED LYSIS OF CHROMAFFIN GRANULES

Futoshi IZUMI, Takeshi KASHIMOTO, Takeshi MIYASHITA and Akihiko WADA
Department of Pharmacology II, Osaka University School of Medicine, 33 Joancho, Kitaku, Osaka 530

and

Motoo OKA
Department of Pharmacology, Tokushima University, School of Medicine, 3-18-15, Kuramotocho, Tokushimashi 770, Japan

Received 29 March 1977

1. Introduction

Recently, we reported that a fraction of cytoplasmic protein from bovine adrenal medulla stimulated release of catecholamine from isolated chromaffin granules, and that its stimulatory effect was dependent on ADP-Mg^{2+} and a low concentration of calcium [1]. The aggregation of blood platelets which is a typical fusion process, also depends on ADP and a protein factor [2].

Further studies on the protein factor showed that stimulation of catecholamine release was due to lysis of granules and that part of the protein factor was associated with the membranous fraction of chromaffin cells.

The final step of catecholamine release by exocytosis must be the interaction and fusion of granules with chromaffin cell membranes. This paper is on the involvement of the protein factor in the fusion process.

2. Materials and methods

Bovine adrenal glands were used throughout. Chromaffin granules were isolated with a millipore filter [3,4] and cytoplasmic releasing factor was prepared [1] as described previously.

Microsomes were prepared and solubilized as follows: The adrenal medulla was dissected out and placed in a cold solution of 150 mM KCl, 40 mM Tris–HCl (pH 7.4), 4 mM ATP, 2 mM $MgSO_4$, 5 mM EGTA and 0.05 mM 2-mercaptoethanol; it was then homogenized with a glass Potter homogenizer; the homogenate was centrifuged at 10 000 × *g* for 1 h to remove particulate material; the supernatant was centrifuged at 105 000 × *g* for 2 h, and the pellet was used as the microsome preparation. It was washed twice with 40 mM Tris–HCl (pH 7.4) by centrifugation (105 000 × *g*, 2 h), suspended in 150 mM KCl containing 40 mM Tris–HCl (pH 7.4) and homogenized vigorously with a tightly fitting teflon homogenizer. Then the mixture was centrifuged at 105 000 × *g*, 1 h. By these procedures part of the membrane associated protein was solubilized. The resulting microsomal pellet was again suspended in 150 mM KCl containing 40 mM Tris–HCl (pH 7.4) and cholic acid in the same solution was added dropwise to a final concentration of 0.25%. The mixture was stirred gently for 2 h in an ice-bath, then insoluble microsomes were removed by centrifugation. The supernatant was brought to 35% saturation ammonium sulfate and insoluble protein was collected, dissolved in 15 ml 150 mM KCl containing 40 mM Tris–HCl (pH 7.4) and dialysed against 1 liter of the same solution for 48 h, changing the outer fluid three times.

For analysis of the action of the catecholamine releasing factor, [^{14}C]adrenaline uptake was measured in granules that had been treated with catecholamine

releasing factor. Details of the procedure for measurement of uptake of [^{14}C]adrenaline are given in the legend to fig.2.

3. Results and discussion

When isolated chromaffin granules were incubated in KCl medium containing ADP-Mg^{2+}, 9.2 ± 1.5% (mean ± SD, n = 15) of their catecholamine was released into the medium in 5 min. Under similar conditions, but in the presence of catecholamine releasing factor (2 mg protein/ml of incubation medium), 45 ± 7.5% (mean ± SD, n = 5) of their catecholamine was released (fig.1).

This great increase in catecholamine release in the presence of catecholamine releasing factor could be due to release of about half the catecholamine from each granule or release of all the catecholamine from about half the granule. We were unable to differentiate between these two possibilities by sucrose density-gradient analysis or measurements of the optical densities of suspensions of granules.

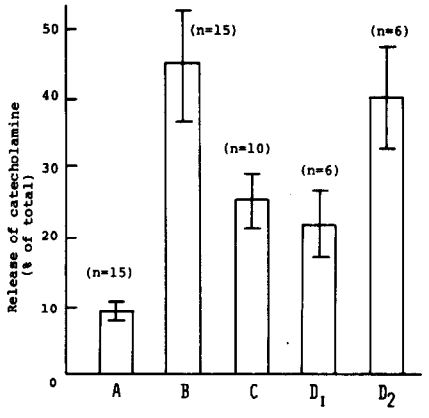

Fig.1. Stimulatory effects of cytoplasmic and microsomal proteins on the release of catecholamine from isolated bovine chromaffin granules. Granules (450 μg catecholamine) were incubated in a solution consisting of 150 mM KCl, 40 mM Tris–HCl (pH 7.4), 4 mM ADP and 2 mM $MgSO_4$. Incubations were carried out for 5 min at 37°C. The proteins added to cause catecholamine release were as follows: (A) None. (B) Cytoplasmic protein (0–35% fraction with ammonium sulfate, 2 mg/ml of incubation medium). (C) Protein extracted from microsomes with 150 mM KCl and 40 mM Tris–HCl (pH 7.4) (2 mg/ml). (D_1 and D_2) Protein solubilized from microsomes with 0.25% of cholic acid (D_1, 2 mg/ml : D_2, 10 mg/ml). Values are amounts of catecholamine released into the medium as percentages of the total catecholamine. Standard deviations are shown by vertical bars, and the number of experiments are shown at the top of each column.

So finally we used the catecholamine uptake capacity of granules as an index of the number of intact granules. In isotonic sucrose medium, granules take up catecholamines in the presence of ATP-Mg^{2+} and this uptake is completely blocked by reserpine [5,6]. Therefore, since ruptured granules do not take up catecholamine, the differences in the uptakes of catecholamine in the absence and presence of reserpine should be an estimate of the number of intact granules. The uptake of [^{14}C]adrenaline (2×10^{-5} M) was linearly proportional to the numbers of granules in the range that contained 50–500 μg catecholamines (data not shown).

Granules were treated with catecholamine releasing factor in the presence of ADP-Mg^{2+}, collected by centrifugation and suspended in isotonic sucrose medium and their [^{14}C]adrenaline uptake capacity was estimated in the presence of ATP-Mg^{2+}. As shown in fig.2, the amount of [^{14}C]adrenaline taken up by the granules during the second incubation was linearly proportional to the amount of catecholamine remaining in the granules after the first incubation. The amount of [^{14}C]adrenaline taken up is proportional to the number of intact granules; thus the amount of catecholamine remaining in the granules after the first incubation must also be proportional to the number of intact granules. Therefore, the abrupt release of catecholamines from the granules in the presence of the catecholamine releasing factor seems to be due to lysis of granules.

Granules incubated in KCl medium with ATP-Mg^{2+} released 15 ± 2.4% (mean ± SD, n = 5) of their catecholamine into the medium in 5 min. The catecholamine uptake capacities of these granules were as good as those of control granules. Under these conditions the granules seemed to become swollen.

According to the theory of exocytosis [7–9], the last step in catecholamine release is the fusion of granules with chromaffin cell membranes and lysis of the granules. However, it is not known how the membranous components are involved in this process. As an approach to this problem, we tried to demonstrate the catecholamine releasing factor in the microsomal fraction of chromaffin cells. We found that microsomes

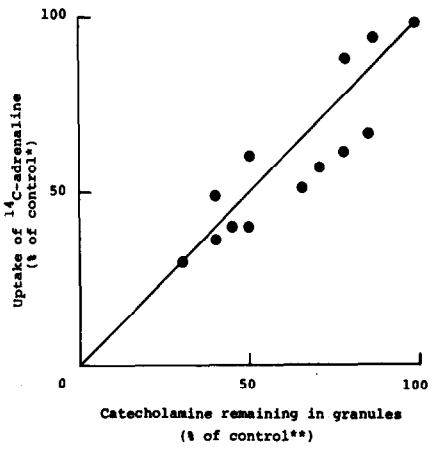

Fig.2. Correlation between [^{14}C]adrenaline uptake and amount of catecholamine in the granules. Granules (450 μg catecholamine) were first incubated with cytoplasmic protein (fraction precipitated with 0–35% saturation of ammonium sulfate, 2 mg/ml) in solution consisting of 150 mM KCl, 40 mM Tris–HCl (pH 7.4). 4 mM ADP and 2 mM $MgSO_4$. Incubation were carried out in 7 ml centrifuge tubes for 3–15 min at 37°C. After the first incubation, granules were precipitated by centrifugation (20 000 × *g*, 10 min), suspended in 1.0 ml 0.32 M sucrose containing 40 mM Tris–HCl (pH 7.4) and mixed with 2.0 ml 0.32 M sucrose containing 3 × 10^{-5} M DL-[7-^{14}C]adrenaline (270 × 10^4 cpm), 6 mM ATP, 3 mM $MgSO_4$ and 40 mM Tris–HCl (pH 7.4). Tubes were incubated for 5 min at 37°C, then the granules collected by centrifugation. Their [^{14}C]-adrenaline and non-radioactive catecholamines were extracted with 0.4 N perchloric acid. The radioactive adrenaline was counted in a liquid scintillator. Nonradioactive catecholamines was measured by the ethylenediamine condensation method. Values are expressed as percentages of those of the control.

Control: unincubated granules in reaction of catecholamine release:

** Catecholamine content: 420 ± 15 μg (mean ± SD., *n* = 4).

* [^{14}C]adrenaline uptake: 270 000 ± 21 000 cpm (mean ± SD, *n* = 4).

that had been washed twice with 40 mM Tris–HCl (pH 7.4) slightly inhibited release of catecholamines, possibly by trapping the low concentration of calcium. One increasing the ionic strength with 150 mM KCl some of the membrane associated protein was solubilized by vigorous homogenization and found to stimulate the release of catecholamine in the presence of ADP-Mg^{2+} (fig.1,C), like the catecholamine releasing factor in the cytoplasmic fraction. Further treatment of the microsomal fraction with cholic acid solubilized additional protein that enhanced catecholamine release (fig.1,D_1). Increase in the concentration of cholic acid to 0.5% caused progressive increase in the amount of protein solubilized, but higher concentrations caused protein denaturation. Therefore, we used 0.25% cholic acid in subsequent experiments. With this concentration the amount of protein solubilized was limited and its specific activity was less than that in the cytoplasmic fraction. Thus it seems that cholic acid caused release not only of catecholamine releasing protein, but also of other proteins nonspecifically. However, a high concentration of solubilized protein stimulated release of catecholamine to the same extent as the cytoplasmic factor (fig.1,D_2). The results show that part of the catecholamine releasing factor is associated with the microsomal fraction.

The lytic effect of the catecholamine releasing factor and the fact that it was associated with microsomes, strongly suggest that it is involved in the fusion of granules with the cell membrane. It seems that the catecholamine releasing factor is a so-called peripheral protein [10]. On activation with a low concentration of calcium in the presence of ADP-Mg^{2+}, it may become associated with chromaffin cell membranes and may be necessary for fusion of granules.

The fusion process cannot be studied adequately by biochemical procedures using whole cells or tissues, as pointed out by Davis and Lazarus [11]. However, it should be possible to obtain much information on the fusion process using the in vitro system for release of catecholamine from isolated granules.

Acknowledgements

We thank Mrs K. Tsuji for expert technical assistance. We also thank Dr Y. Miyake for valuable advice and discussion.

References

[1] Izumi, F., Oka, M., Morita, K. and Azuma, H. (1975) FEBS Lett. 56, 73–76.

[2] Nishimura, Y., Yamada, Y., Ithoh, M., Takenaka, O. and Inada, Y. (1975) FEBS Lett. 51, 171–173.

[3] Oka, M., Ohuchi, T., Yoshida, H. and Imaizumi, R. (1966) Life Sci. 5, 427–432.

[4] Oka, M., Izumi, F. and Kashimoto, T. (1972) Japan. J. Pharmacol. 22, 207–214.
[5] Kirshner, N. (1962) J. Biol. Chem. 237, 2311–2317.
[6] Hillarp, N. A. (1958) Acta Physiol. Scand. 43, 82–96.
[7] De Robertis, E. and Sabatini, D. D. (1960) Fed Proc. 19, 70–78.
[8] Smith, U. (1972) Science (NY) 179, 79–82.
[9] Poisner, A. N. (1973) in: Frontiers in Catecholamine Research, pp. 477–482, Pergamon Press, England.
[10] Singer, S. J. (1974) Ann. Rev. Biochem. 43, 805–833.
[11] Davis, B. and Lazarus, N. R. (1976) J. Physiol. 256, 709–729.