SUBCELLULAR DISTRIBUTION OF MYOSIN (K^+, EDTA)-ATPase IN BOVINE ADRENAL MEDULLA

John E. HESKETH, Dominique AUNIS*, Michel PESCHELOCHE and Paul MANDEL
Centre de Neurochimie du CNRS, INSERM, 67085-Strasbourg Cedex, France

Received 10 June 1977

1. Introduction

The presence of myosin-like proteins has been reported in several nervous tissues (for review, see ref. [1]). Such a protein from the adrenal medulla has recently been characterized [2,3]. Knowledge of the localization of these proteins is poor but is of potential interest since it would provide information basic to evaluation of hypotheses concerning the function of actomyosin-like proteins in neurosecretion [4], ion transport [5], axoplasmic transport [6] and neuronal growth [7]. Myosin from both muscle and non-muscle sources is thought to be unique in possessing an ATPase (ATP phosphohydrolase, EC 3.6.1.3) activity which is exhibited in the presence of 0.6 M KCl and 2 mM EDTA [8]. Purification of myosin from various non-muscle tissues including the adrenal medulla [2,3] is associated with an increase in the specific activity of this (K^+, EDTA)-ATPase. In the present work, such ATPase activity has been used as a biochemical marker for myosin with which to follow its distribution in subcellular fractions. The work was carried out using bovine adrenal medulla, a system well proven as a model for neurosecretion [9].

Correspondence: Dr D. Aunis, Centre de Neurochimie, CNRS, 11, rue Humann, F-67085 Strasbourg Cedex, France

*Chargé de Recherches, INSERM

Abbreviations: ATP, adenosine triphosphate; ATPase: ATP phosphohydrolase (EC 3.6.1.3); EDTA: ethylene-diamine tetracetate

2. Materials and methods

2.1. *Subcellular fractionation of the adrenal medulla*

Bovine adrenal glands, obtained from the slaughterhouse, were kept on ice and medullae were dissected from cortices. Medullae from 4–5 glands (average weight 5–10 g) were homogenized in 0.32 M sucrose using a weight-to-volume ratio of 1 : 10, and a motor-driven homogenizer (2500 rev./min), with a glass tube and teflon pestle (Elvehjem-type Potter-homogenizer). The subsequent fractionation is adapted from the classical procedure of Smith and Winkler [10], as we have described in detail elsewhere [11].

2.2. *Fractionation of the crude chromaffin granule fraction on continuous sucrose density gradients*

This experimental procedure followed that reported recently by Wilson and Kirshner [12]. The crude chromaffin granule fraction was obtained by centrifuging the low-speed 800 × *g* supernatant at 26 000 × *g* for 20 min. The resulting pellet was suspended in 0.5 M sucrose buffered with 10 mM Tris–HCl at pH 7.4 (2.5 ml/g of starting medullae) and containing 300 units of catalase per ml. (EC 1.11.1.6, from Boehringer, Mannheim, Germany). Five ml of the suspension was layered on to 30 ml of a linear gradient formed from 15 ml of 1.0 M sucrose and 2.25 M sucrose each buffered with 10 mM Tris–HCl (pH 7.4), and containing 300 units of catalase per ml. Gradients were centrifuged at 90 000 × *g* for 5 h at +4°C in a Beckman SW-27 Rotor. Fractions of 1.7 ml were collected from the bottom of the tube, analyzed

North-Holland Publishing Company – Amsterdam

for sucrose density by refractometry, pooled and diluted with chilled water. Fractions were centrifuged at 100 000 × *g* for 60 min in a R-40 Rotor. Washings of membranes were repeated; membranes were then suspended in 1.5 ml of the 10 mM Tris–HCl buffer.

2.3. *Measurement of enzyme activities*

Enzyme activities were assayed using the following methods: phenylethanolamine *N*-methyl transferase (EC 2.1.1.x), Saavedra et al. [13]; dopamine-β-hydroxylase (EC 1.14.17.1), Pisano et al. [14]; monoamine oxidase (EC 1.4.3.4), Goridis, Neff [15]; acetylcholinesterase (EC 3.1.1.7), Ellman et al. [16]; guanylate cyclase (EC 4.6.1.2), Goridis and Reutter [17]; adenylate cyclase (EC 4.6.1.1), Rachamandran and Lee [18]; 5′-nucleotidase (EC 3.1.3.5), Hortnagl [19]; acid phosphatase (EC 3.1.3.2), Lowry [20]; (K^+, EDTA)-ATPase was measured by the liberation of inorganic phosphate (P_i) following incubation with 2 mM Na_2ATP in 0.6 M KCl, 2 mM EDTA, 10 mM imidazole (pH 7.0). Inorganic phosphate was measured by the method of Steward as described by Naylor et al. [21].

2.4. *Measurement of protein*

Protein was assayed using the Folin method of Lowry et al. [22], with bovine serum albumin as standard.

3. Results

As shown in fig.1, initial subcellular fractionation studies showed that over 60% of the recovered (K^+, EDTA)-ATPase activity was found in the low-speed 800 × *g* pellet constituted of nuclei, plasma membranes and cell debris. The distribution of the ATPase activity was paralleled by that of acetylcholinesterase, 5′-nucleotidase and adenylate cyclase. The double localization of acetylcholinesterase in plasma membranes and in cytosol, of 5′-nucleotidase in plasma membranes and in Golgi apparatus has been reported [12,23]. In contrast, adenylate cyclase has been shown to exclusively be associated with plasma membranes [12,24]. Concerning the (K^+-EDTA)-ATPase activity, 61%, 17%, 14%, 6.5% and 1.5% of total recovered activity were recovered in respectively the low-speed 800 × *g* pellet, the soluble

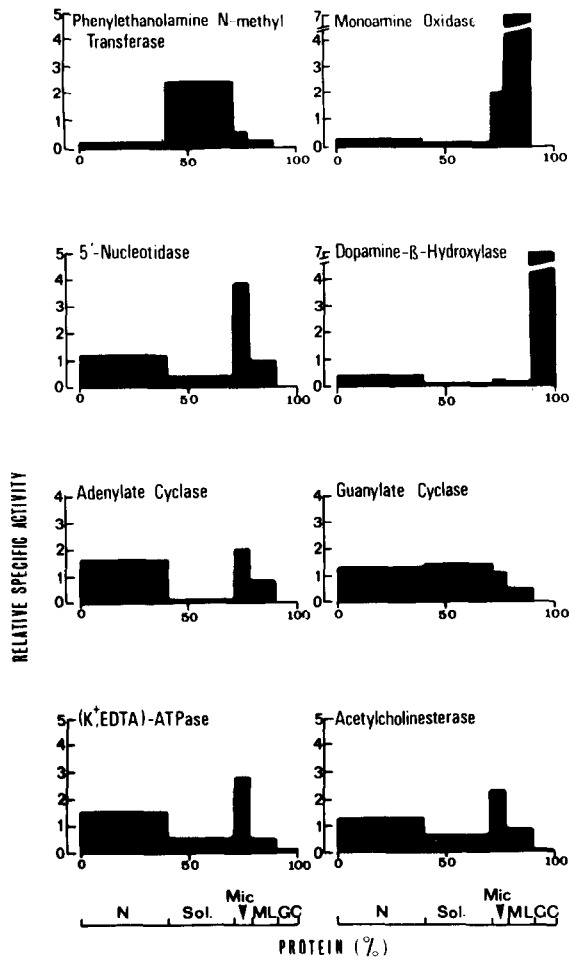

Fig.1. Distribution pattern of marker enzymes in subcellular fractions of bovine adrenal medulla. Relative specific activity is the ratio of percentage of total activity in the considered fraction over the percentage of total recovered protein in that fraction. Phenylethanolamine *N*-methyl transferase was considered as a marker of cytosol [30], adenylate cyclase of plasma membranes [12,24], guanylate cyclase of plasma membranes and of cytosol [24], monoamine oxidase of mitochondria [30], acetylcholinesterase of plasma membranes and cytosol [12,23], 5′-nucleotidase of Golgi apparatus and plasma membranes [12,19,24], dopamine-β-hydroxylase of chromaffin granule [30]. N = low-speed 800 × *g* pellet (Nuclei, plasma membranes, cell debris); Sol = soluble fraction (cytosol); Mic = microsomal fraction (small chromaffin granules, Golgi apparatus, rough endoplasmic reticulum); ML = mitochondria and lysosomal fraction (mitochondria, lysosomes, chromaffin granule membranes, Golgi apparatus); GC = chromaffin granules. Results given are the means from 3 or more preparations; variations were less than ± 10%. Recovery of enzyme activities was between 85–110%.

fraction, the microsomal fraction, the mitochondria-lysosomal fraction and the chromaffin granule fraction. However, the impure nature of the low-speed 800 × *g* pellet made the assumption that (K^+, EDTA)-ATPase recovered in that fraction could be in plasma membranes equivocable. Moreover, the possible precipitation of myosin because of the low ionic strength of the homogenizing buffer made further examination of membrane fractions necessary. Thus, experiments were carried out in which the activity of (K^+, EDTA)-ATPase and certain known membrane marker enzymes were studied following separation of a crude chromaffin granule fraction on continuous sucrose density gradients [12,24]. The results of our experiments (fig.2) showed the distribution of (K^+, EDTA)-ATPase activity to follow closely those of membrane-bound acetylcholinesterase, guanylate cyclase and adenylate cyclase. Some activity trailed throughout the gradients fractions. Plasma membrane marker enzymes [12,24] showed the highest specific activity in fraction 4: the specific activity of acetylcholinesterase was 8.1 μmol of acetylthiocholine hydrolyzed/h/mg of protein, an enrichment of 5.75-fold over the activity in the crude chromaffin granule fraction. The specific activity of adenylate cyclase in fraction 4 was enriched 4.4-fold from 30.2 pmol of cyclic AMP formed/min/mg protein to 135 and guanylate cyclase specific activity was enriched 6.2-fold from 10.0 to 62.0 pmol of cyclic GMP formed/min/mg protein. (K^+, EDTA)-ATPase specific activity was increased from 0.26 to 1.05 μmol of P_i liberated/h/mg protein, i.e., an enrichment of 4.4-fold.

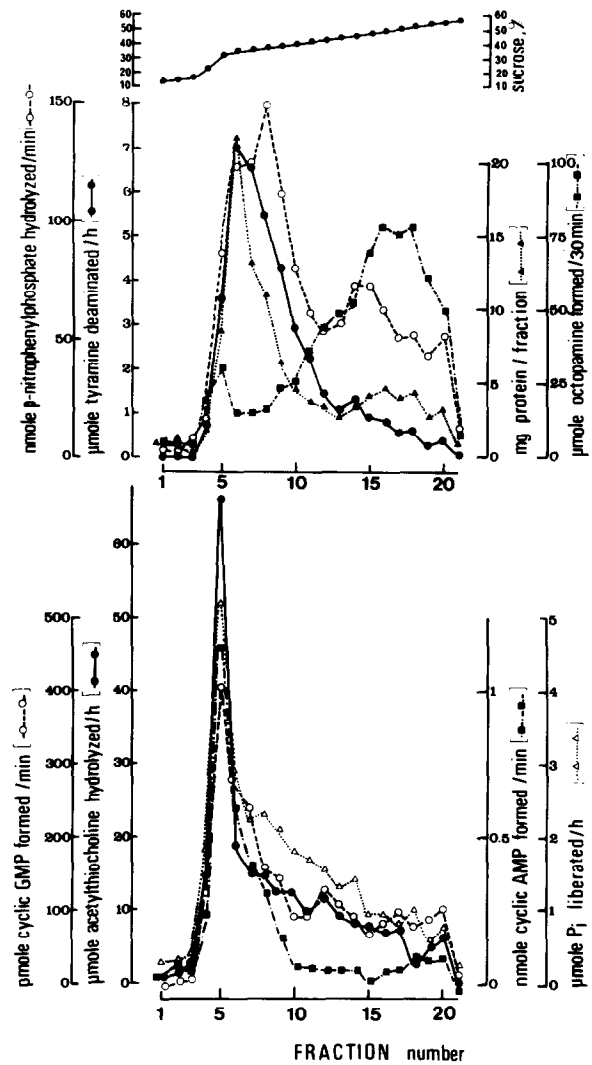

Fig.2. Distribution of marker enzymes in a typical sucrose density gradient after centrifugation of crude chromaffin granule fraction. (Top): Monoamine oxidase (•); dopamine-β-hydroxylase (▪); acid phosphatase (○); protein (▲). (Bottom): Acetylcholinesterase (•); adenylate cyclase (▪); guanylate cyclase (○); (K^+, EDTA)-ATPase (△). At the top of the panel, % sucrose in each fraction is given. Enzyme activities were checked for linearity towards time and protein concentration in assay medium. Experiment has been repeated twice with identical results. Recovery was 70.5% for acetylcholinesterase, 85% for guanylate cyclase, 75% for adenylate cyclase, 60% for membrane-bound dopamine-β-hydroxylase, 68% for acid phosphatase, 97% for monoamine oxidase and 65% for (K^+, EDTA)-ATPase, 225 mg of protein were deposited on the three parallel gradients and 107 mg (47.5%) recovered as membrane protein. The highest specific activity of monoamine oxidase was recovered in fraction 9, increasing from 0.3–0.67 μmol of tyramine deaminated/h/mg protein. Specific activity of acid phosphatase was increased from 9.4–28.5 nmol of *p*-nitrophenylphosphate hydrolyzed/min/mg protein in fraction 11 in which the specific activity was the highest. 5′-Nucleotidase was measured in all fractions, but not reported here because of the clarity of the figure: a plateau was observed (over fractions 5 to 11), and specific activity showed a bimodal distribution. Highest specific activities were recovered in fractions 4 and 11, suggesting the double localization of this enzyme in plasma membranes and Golgi apparatus [12,19,24].

Results shown in figs. 1 and 2 suggest that there is little ATPase activity associated with the chromaffin granule fraction: only 1.5% of the recovered activity was found in the purified granule fraction. Moreover, the pattern of sedimentation of dopamine-β-hydroxylase bound to chromaffin granule membranes appeared very different from that of the (K^+, EDTA)-ATPase activity (fig.2). Some soluble (K^+, EDTA)-ATPase activity could be detected, representing 17% of the total recovered activity.

4. Discussion

The association of (K^+, EDTA)-ATPase activity with myosin from muscle and non-muscle sources [8] suggests this enzyme activity to be a valid biochemical marker for myosin. Thus, the subcellular distribution of the (K^+, EDTA)-ATPase will reflect the distribution of myosin.

Our initial experiments (fig.1) showed 60% of the ATPase activity to be recovered in the crude plasma membranes, nuclei and cell debris pellet. Interpretation of this data was difficult for two reasons. Firstly, the impurity of the fraction and secondly the possibility that in vivo or under our in vitro conditions myosin may have been in some filamentous form and so the high recovery of the ATPase activity in the crude membrane and nuclear pellet could have been due to a sedimentation of myosin rather than due to association with subcellular structures. However, the parallel distribution of the (K^+, EDTA)-ATPase activity and of plasma membrane markers together with the high relative specific activity value for the ATPase and plasma membrane markers in the microsomal fraction suggested that some myosin may have been associated with plasma membranes. Further evidence for this view was obtained from gradient experiments (fig.2); the (K^+, EDTA)-ATPase activity and that of plasma membrane markers showed a parallel distribution and enrichment of specific activity. Overall, our results suggest there is some myosin associated with plasma membranes. The possible precipitation of myosin during fractionation could have led to an overestimation of the ATPase activity recovered in the crude nuclear and plasma membrane fraction, so that it is not possible to give an estimate of the percentage of activity associated with plasma membranes with confidence. It is unlikely that the assay for (K^+, EDTA)-ATPase measured any Na^+ plus K^+-stimulated ATPase activity (a known membrane enzyme) since the (K^+, EDTA)-ATPase activity was not found to be inhibited by ouabain and since Mg^{2+} necessary for the Na^+ plus K^+-stimulated activity was not present in the incubation medium.

Some (K^+, EDTA)-ATPase activity was recovered in the soluble fraction although this was somewhat variable. Such myosin in the soluble fraction might reflect an in vivo pool of soluble myosin or it could be an artifact of the homogenization and fractionation processes.

Both subcellular distribution and gradient experiments showed there to be little (K^+, EDTA)-ATPase activity associated with chromaffin granules. The little activity present was most likely due to contamination of the fractions. This paucity of activity in the granule fraction suggests that myosin is not a component of the chromaffin granule. This result is of interest since it has been suggested that there is myosin associated with synaptic vesicles [4], structures homologous to chromaffin granules. Such vesicular myosin was suggested to react with actin present in the synaptic membrane to bring about the release of neurotransmitter. The evidence for this hypothesis and for vesicular myosin is weak since the nature and purity of the preparations involved have been questioned [25,26]. Actin has been found to bind to chromaffin granules [27] but this was not shown to be due to myosin in the granules. The present results do not support the hypothesis of Berl et al. [4] for neurosecretion in that myosin does not seem to be associated with chromaffin granules.

In conclusion, our results suggest that in the bovine adrenal medulla myosin is associated not with chromaffin granules but partly with plasma membranes. There is immunohistochemical evidence from other tissues such as fibroblasts and chick embryonic cells in culture that there is myosin associated with the plasma membrane [28,29]. Membrane localization of myosin may also apply to other nervous tissues. The presence of myosin in the plasma membrane or in the soluble form does not exclude it playing a role in neurosecretion.

Acknowledgements

Miss D. Thierse is gratefully acknowledged for her

skillfull technical assistance. J. E. H. acknowledges the receipt of a European Exchange Fellowship from the Royal Society. M. P. is actually a recipient of a grant from French CNRS. This work was supported by grants from the French INSERM and CNRS.

References

[1] Bray, D. (1977) Biochimie 59, 1–6.
[2] Trifaro, J. M. and Ulpian, C. (1976) Neuroscience 1, 483–488.
[3] Johnson, D. H., McCubbin, W. D. and Kay, C. M. (1977) FEBS Lett. 77, 69–74.
[4] Berl, S., Puszkin, S. and Nicklas, W. J. (1973) Science 179, 441–446.
[5] Bowler, K. and Duncan, C. J. (1967) Nature 211, 642–643.
[6] Schmitt, F. O. (1968) Proc. Natl. Acad. Sci. USA 60, 1092–1101.
[7] Bray, D. (1973) Nature 244, 93–96.
[8] Pollard, T. D. and Weihring, R. R. (1974) CRC Critical Rev. Biochem. 2, 1–65.
[9] Helle, K. B. and Serck-Hanssen, G. (1975) Molec. Cell. Biochem. 6, 127–146.
[10] Smith, A. D. and Winkler, H. (1967) Biochem. J. 103, 480–482.
[11] Aunis, D., Bouclier, M., Pescheloche, M. and Mandel, P. (1977) J. Neurochem. in press.
[12] Wilson, S. P. and Kirshner, N. (1977) Molec. Pharmacol. 13, 382–385.
[13] Saavedra, J. M., Palkovits, M., Brownstein, M. and Axelrod, J. (1974) Nature 248, 695–696.
[14] Pisano, J. J., Creveling, C. R. and Udenfriend, S. (1960) Biochim. Biophys. Acta 43, 566–568.
[15] Goridis, C. and Neff, N. H. (1971) J. Neurochem. 18, 1673–1682.
[16] Ellman, G. L., Courtney, K. D., Andres, V. and Featherstone, R. M. (1961) Biochem. Pharmacol. 7, 88–95.
[17] Goridis, C. and Reutter, W. (1975) Nature 257, 698–700.
[18] Rachamandran, J. and Lee, W. (1970) Biochem. Biophys. Res. Comm. 41, 358–366.
[19] Hörtnagl, H. (1976) Neuroscience 1, 9–18.
[20] Lowry, O. H. (1957) in: Methods in Enzymology (Colowick, S. P. and Kaplan, N. O., eds) Vol. IV, pp. 371–372, Academic Press, New York.
[21] Naylor, G. J., Dick, D. A. T., Dick, E. G., Le Poidevin, D. and Whyte, S. F. (1973) Psychol. Med. 3, 502–508.
[22] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[23] Chubb, I. W. and Smith, A. D. (1975) Proc. R. Soc. Lond. Ser. B, 191, 245–261.
[24] Aunis, D., Pescheloche, M. and Zwiller, J., to be published.
[25] Wellington, B. S., Livett, B. G., Jeffrey, P. L. and Austin, L. (1976) Neuroscience 1, 23–34.
[26] Fine, R. E., Blitz, A. L., Hitchcock, S. E. and Kaminer, B. (1973) Nature 245, 182–186.
[27] Burridge, K. and Phillips, J. H. (1975) Nature 254, 526–529.
[28] Willingham, M. C., Ostlund, R. E. and Pastan, I. (1974) Proc. Natl. Acad. Sci. USA 71, 4144–4148.
[29] ap Gwynn, I., Kemp, R. B. and Jones, B. M. (1976) Cell Tiss. Res. 171, 351–358.
[30] Laduron, P. and Belpaire, F. (1968) Biochem. Pharmacol. 17, 1127–1140.