OCCURRENCE OF A Ca^{2+}- AND MODULATOR PROTEIN-ACTIVATABLE ATPase IN THE SYNAPTIC PLASMA MEMBRANES OF BRAIN

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1. Introduction

Ca^{2+}-dependent modulator protein was discovered as an activator of phosphodiesterase [1,2], or a protein factor required for the Ca^{2+}-dependent activation of phosphodiesterase [3,4]. Later, this protein was shown to be structurally similar to troponin-C [5,6] and to cause Ca^{2+}-dependent activation of several enzymes including phosphodiesterase, brain adenylate cyclase [7], myosin light chain kinases from skeletal muscles [8–10] and chicken gizzard muscle [11], and actomyosin ATPase [12,13]. Recently, two groups [14,15] have demonstrated that the activator protein [16,17] for the erythrocyte membrane ATPase is identical to this modulator protein.

(Ca^{2+} + Mg^{2+})-ATPase activity was also found in brain tissue [18,19]. However, requirement of brain enzyme for an activator has not been reported. In the present study, we are able to show the dependency of the activity of brain enzyme upon the modulator protein. Although brain (Ca^{2+} + Mg^{2+})-ATPase activity was detected in all particulate fractions upon subcellular fractionation, only enzyme in the synaptic plasma membrane fraction was responsive to modulator protein.

2. Materials and methods

2.1. *Preparation of synaptic membranes and other subcellular fractions*

Synaptic plasma membranes (P_{3-4}) and other subcellular fractions were prepared from cerebral cortices of male Sprague Dawley rats as in [20] with a slight modification [19]. Rat cerebral cortices were homogenized with 10 vol. 0.32 M sucrose and centrifuged at 900 × *g* for 10 min. The supernatant fluid was then centrifuged at 11 500 × *g* for 30 min. The resultant pellet (crude mitochondrial fraction) was further fractionated by sucrose density gradient centrifugations as shown in fig.1. Nerve-ending particles were disrupted by hypotonic treatment as follows: the particulate material in fraction B was collected by centrifugation (105 000 × *g* for 60 min) then homogenized with water (2.5 ml water/g original tissue). Fractions A, C, P_{1-2}, P_{3-4} and P_{5-6} obtained as shown in fig.1 were suspended in 10 mM Tris–HCl (pH 7.5) and 1 mM EGTA and then centrifuged at 105 000 × *g* for 60 min. This procedure was repeated once more: EGTA concentration in the second time centrifugation was 100 μM instead of 1 mM. The

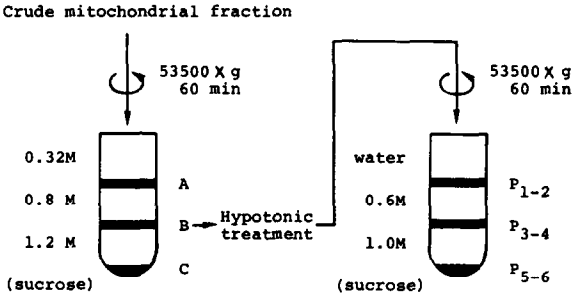

Fig.1. Fractionation of crude mitochondrial fraction by sucrose gradient centrifugations.

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resultant pellet, suspended in 10 mM Tris–HCl (pH 7.5), were used in the following study. All preparations were at 4°C.

2.2. *Analytical procedures*

$(Ca^{2+} + Mg^{2+})$-ATPase activity was determined as follows: 75 ~ 150 μg membrane protein was incubated at 37°C in a reaction mixture (0.8 ml) containing 50 mM Tris–HCl (pH 7.5), 2 mM $MgCl_2$, 100 mM KCl, 100 μM EGTA, 100 μM ouabain, 2 mM ATP and with (for total ATPase activity) or without (for Mg^{2+}-ATPase activity) the addition of 100 μM $CaCl_2$. Modulator protein (2.5 μg/tube) was added where indicated. After 30 min incubation, the reaction was terminated by the addition of 0.2 ml 32% trichloroacetic acid. The mixture was centrifuged and the supernatant was subjected to P_i determination as in [21]. $(Ca^{2+} + Mg^{2+})$-ATPase activity was estimated as the difference between the activities of total ATPase and Mg^{2+}-ATPase. $(Na^+ + K^+)$-ATPase and succinate dehydrogenase (SDH) activities were determined as in [22] and [23], respectively. Modulator protein was assayed as in [24]. Protein was determined as in [25].

3. Results and discussion

Figure 2 shows the dependence of $(Ca^{2+} + Mg^{2+})$-ATPase activity of synaptic plasma membranes (P_{3-4}) on additions of both Ca^{2+} and modulator protein. In the presence of 100 μM EGTA, activation of enzyme was observed with added Ca^{2+} at 50–500 μM, the maximum activation being attained at ~100 μM. With added Ca^{2+} at < 100 μM, say 90 μM, the free $[Ca^{2+}]$ in the reaction medium was calculated to be in the order of ⩽ μM, taking Ca^{2+}-EGTA K_d 10^7 M^{-1} [26]. Addition Ca^{2+} at > 500 μM was inhibitory. Addition of modulator protein increased enzyme activity by about 200%. The activation by modulator protein occurred over the entire $[Ca^{2+}]$ range where Ca^{2+} was stimulatory to enzyme. In the absence of added Ca^{2+}, addition of modulator protein produced no effect on enzyme activity, indicating that modulator protein does not affect Mg^{2+}-ATPase. Modulator protein activated $(Ca^{2+} + Mg^{2+})$-ATPase in a dose-dependent fashion (fig.3). Addition of 16 μg modulator protein/mg synaptic plasma membrane protein produced a maximum effect. This amount of modulator protein is well in the physiological concentration range, because 1 g brain tissue contains ~ 500 μg modulator protein [24], and 30 mg of the total particulate protein.

Fractions of P_{1-2}, P_{3-4} and P_{5-6} consist of mainly myelin plus ribosomes, synaptic plasma

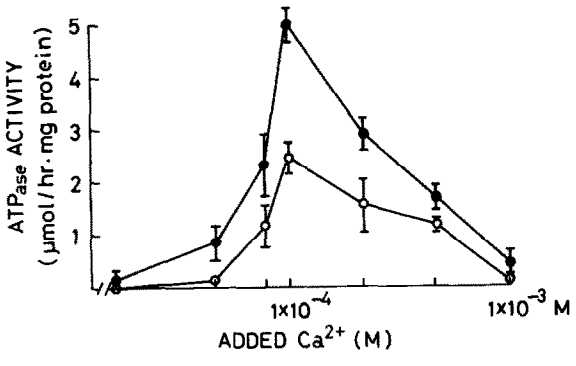

Fig.2. Effects of added Ca^{2+} and modulator protein on $(Ca^{2+} + Mg^{2+})$-ATPase activity of synaptic plasma membranes. Synaptic plasma membrane samples which had been treated with EGTA (see section 2) was assayed for $(Ca^{2+} + Mg^{2+})$-ATPase activity in the presence of 100 μM EGTA and added Ca^{2+} as indicated with (•) or without (○) the addition of modulator protein. Bars represent standard deviation (n=4).

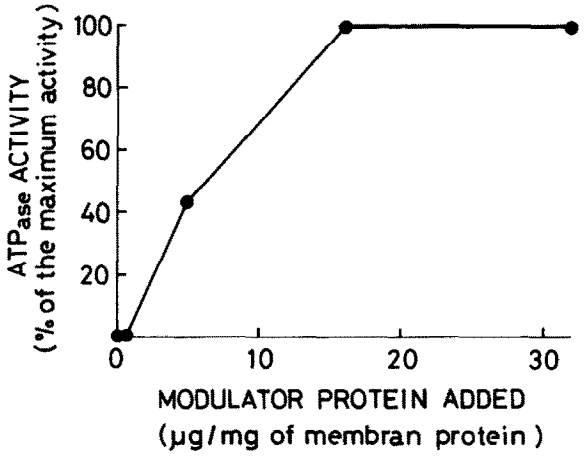

Fig.3. Dependence of $(Ca^{2+} + Mg^{2+})$-ATPase activity of synaptic plasma membranes on added modulator protein. The reaction mixture contained 100 μM EGTA and 100 μM $CaCl_2$.

Table 1
Enzyme activities in subcellular fractions

Fraction	$(Ca^{2+} + Mg^{2+})$-ATPase		$(Na^+ + K^+)$-ATPase	SDH
	Modulator(−) (μmol.mg prot.$^{-1}$.h^{-1})	Modulator(+)	(μmol.mg prot.$^{-1}$.h^{-1})	(A.mg prot.$^{-1}$. 30 min^{-1})
A	1.99 ± 0.62	2.27 ± 0.67	3.1 ± 1.5	0.03 ± 0.01
C	0.80 ± 0.23	0.84 ± 0.20	6.6 ± 0.4	0.45 ± 0.05
P_{1-2}	2.36 ± 0.56	2.82 ± 0.54	5.5 ± 0.4	0.02 ± 0.00
P_{3-4}	2.50 ± 0.36[a]	4.98 ± 0.42[a]	15 ± 0.9	0.08 ± 0.08
P_{5-6}	2.02 ± 0.66	2.53 ± 0.34	4.8 ± 0.6	0.35 ± 0.04

[a] $p < 0.01$, n=4

membranes and synaptosomal mitochondria, respectively [19,22]. In this study, fractions were monitored by measuring $(Na^+ + K^+)$-ATPase and succinate dehydrogenase (SDH) activities as markers for synaptic plasma membranes and mitochondria, respectively. The former was concentrated in P_{3-4} fraction (table 1), suggesting that the fraction P_{3-4} was high in synaptic plasma membrane content. The latter was concentrated in both fractions C and P_{5-6} (table 1). Although the activity of $(Ca^{2+} + Mg^{2+})$-ATPase was distributed in all particulate fractions examined, modulator protein dependency of enzyme activity was seen only in P_{3-4}.

Unless precautions were taken to remove endogenous modulator protein from the membrane fractions by repeated cycles of the suspension of the membranes in EGTA solution and the subsequent centrifugation, the modulator protein dependency of enzyme activity could never be observed. We found that the levels of modulator protein in synaptic plasma membrane samples before and after the EGTA treatment were 3.6 μg and 0.9 μg, respectively, per mg membrane protein; the difference is the amount removed from the membranes by such treatment. The result is consistent with [24] reporting the occurrence of two forms of membrane-bound modulator protein in the brain: one form was removed from the membranes by EGTA treatment while the other form was not. It is possible that, in vivo, the activity of $(Ca^{2+} + Mg^{2+})$-ATPase is regulated by the membrane-bound modulator protein. There was an activity of $(Ca^{2+} + Mg^{2+})$-ATPase that was independent of modulator protein addition (fig.2, table 1). Whether or not this is due to another species of enzyme is yet to be seen.

A functional linkage of erythrocyte membrane $(Ca^{2+} + Mg^{2+})$-ATPase with Ca^{2+}-pump activity of the membranes was well documented [27,28]. There is some ambiguity for the role of the brain $(Ca^{2+} + Mg^{2+})$-ATPase (19,29]: it may be related to the extrusion of Ca^{2+} to the extracellular space or the sequestration of intracellular Ca^{2+} by particulate components [30,31]. Moreover, it may be related to a system of actomyosin-like fibers which seem to be associated with synaptic structures [32,33]. Nevertheless, it is expected that the physiological function of Ca^{2+} in brain tissue will be elucidated to a large extent by further study on this enzyme in relation with modulator protein.

References

[1] Cheung, W. Y. (1970) Biochem. Biophys. Res. Commun. 38, 533–538.
[2] Cheung, W. Y. (1971) J. Biol. Chem. 246, 2859–2869.
[3] Kakiuchi, S., Yamazaki, R. and Nakajima, H. (1970) Proc. Jap. Acad. 46, 587–592.
[4] Kakiuchi, S., Yamazaki, R., Teshima, Y. and Uenishi, K. (1973) Proc. Natl. Acad. Sci. USA 70, 3526–3530.
[5] Stevens, F. C., Walsh, M., Ho, H. C., Teo, T. S. and Wang, J. H. (1976) J. Biol. Chem. 251, 4495–4500.
[6] Watterson, D. M., Harrelson, W. G., Keller, P. M., Sharief, F. and Vanaman, T. C. (1976) J. Biol. Chem. 251, 4501–4513.
[7] Brostrom, C. O., Huang, Y. C., Breckenridge, B. M. and Wolff, D. J. (1975) Proc. Natl. Acad. Sci. USA 72, 64–68.
[8] Yagi, K., Yazawa, M., Kakiuchi, S., Ohshima, M. and Uenishi, K. (1978) J. Biol. Chem. 253, 1338–1340.
[9] Waisman, D. M., Singh, T. J. and Wang, J. (1978) J. Biol. Chem. 253, 3387–3390.

[10] Barylko, B., Kuznicki, J. and Drabikowski, W. (1978) FEBS Lett. 90, 301–304.
[11] Dabrowska, R., Sherry, J. M. F., Aromatorio, D. K. and Hartshorne, D. J. (1978) Biochemistry, 17, 253–258.
[12] Amphlett, G. W., Vanaman, T. C. and Perry, S. V. (1976) FEBS Lett. 72, 163–168.
[13] Dedman, J. R., Potter, J. D. and Means, A. R. (1977) J. Biol. Chem. 252, 2437–2440.
[14] Gopinath, R. M. and Vincenzi, F. F. (1977) Biochem. Biophys. Res. Commun. 77, 1203–1209.
[15] Jarrett, H. W. and Penniston, J. T. (1977) Biochem. Biophys. Res. Commun. 77, 1210–1216.
[16] Bond, G. H. and Clough, D. L. (1973) Biochim. Biophys. Acta 323, 592–599.
[17] Luthra, M. G., Hildenbrandt, G. R. and Hanahan, D. J. (1976) Biochim. Biophys. Acta 419, 164–179.
[18] Nakamaru, Y. (1968) J. Biochem. 63, 626 -631.
[19] Ohashi, T., Uchida, S., Nagai, K. and Yoshida, H. (1970) J. Biochem. 67, 635–641.
[20] Whittaker, V. P., Michelson, I. A. and Kirkland, R. J. A. (1964) Biochem. J. 90, 293–301.
[21] Takahashi, Y. (1955) J. Jap. Biochem. Soc. 26, 690.
[22] Saito, K., Uchida, S. and Yoshida, H. (1972) Jap. J. Pharmacol. 22, 787–798.
[23] Kirshner, N., Krishner, A. G. and Kamin, D. L. (1966) Biochim. Biophys. Acta 113, 332–338.
[24] Teshima, Y. and Kakiuchi, S. (1978) J. Cyclic Nucl. Res. 4, 219–231.
[25] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[26] Nanninga, L. B. and Kempen, R. (1971) Biochemistry 10, 2449–2456.
[27] Schatzmann, H. J. and Vincenzi, F. F. (1969) J. Physiol. 201, 369–395.
[28] Lee, K. S. and Shin, B. C. (1969) J. Gen. Physiol. 54, 713–729.
[29] Nakamaru, Y. and Schwartz, A. (1971) Arch. Biochem. Biophys. 144, 16–29.
[30] Otsuka, M., Ohtsuki, I. and Ebashi, S. (1965) J. Biochem. 58, 188–190.
[31] Yoshida, H., Kadota, K. and Fujisawa, H. (1966) Nature 212, 291–292.
[32] Puszkin, S., Nicklas, W. J. and Berl, S. (1972) J. Neurochem. 19, 1319–13333.
[33] Blomberg, F., Cohen, R. S. and Siekevitz, P. (1977) J. Cell Biol. 74, 204–225.