Biochemical Pharmacology, Vol. 31, No. 24, pp. 4077–4079, 1982.
Printed in Great Britain.

0006-2952/82/244077-03 $03.00/0

Non-mitochondrial origin of ethacrynic acid high-sensitive Mg^{2+}-ATPase activity in microsomal fractions from rabbit cortical gray matter

(*Received* 20 *March* 1982; *accepted* 20 *July* 1982)

In a previous report [1], we described Mg^{2+}-ATPase activity that was highly sensitive to ethacrynic acid (EA), in microsomal fractions from rabbit cortical gray matter. The enzyme activity was diminished significantly by several anions with the order of potencies being $NO_3^- > SCN^- > SO_4^{2-} > I^-$, CH_3COO^-, and it was inhibited by sulfhydryl reagents. So-called anion-sensitive ATPase, which is stimulated by HCO_3^- and inhibited by SCN^-, was found in the microsomal fractions of several tissues, and its role in anion transport was discussed [2–4]. Van Amelsvoort *et al.* [5–7], however, argued that this anion-sensitive ATPase was not located in plasma membranes but was of mitochondrial origin. To elucidate the origin of EA highly sensitive Mg^{2+}-ATPase, we examined the subcellular distribution of the EA highly sensitive Mg^{2+}-ATPase activity and the effects of various reagents on it.

Rabbits weighing 2–3 kg were anesthetized with pentobarbital sodium (30 mg/kg, i.v.) and perfused with cold physiological saline. The brain was removed and cerebral cortical gray matter was homogenized in 8 vol. (v/w) of ice-cold buffer solution containing 0.25 M sucrose, 1 mM EDTA and 12.5 mM Tris–acetate (pH 7.4). For differential centrifugation, initial homogenates were centrifuged at 100 *g* for 10 min to remove the cell debris and the supernatant fractions were regarded as starting homogenates, which were fractionated into P_1 (750 *g*, 15 min), P_2 (10,000 *g*, 15 min) and P_3 (92,000 *g*, 60 min) pellets and remaining supernatant fractions (S). The pellets were suspended in homogenization buffer. For mitochondrial preparation, P_2 pellets were suspended in 1.2 M sucrose solution containing 1 mM EDTA and 12.5 mM Tris–acetate (pH 7.4) and were centrifuged at 92,000 *g* for 60 min. The resulting mitochondrial pellets were suspended in homogenization buffer. For density gradient fractionation, 0.2 ml of P_3 fraction was layered on a 5-ml linear gradient of 0.4 to 1.6 M sucrose in 12.5 mM Tris–acetate (pH 7.4) and 1 mM EDTA. After centrifugation in a swinging rotor (Hitachi RPS65TA) at 100,000 *g* for 60 min, ten fractions were collected by suction from the top of the gradients using an automatic liquid charger (Toyo ALC-21).

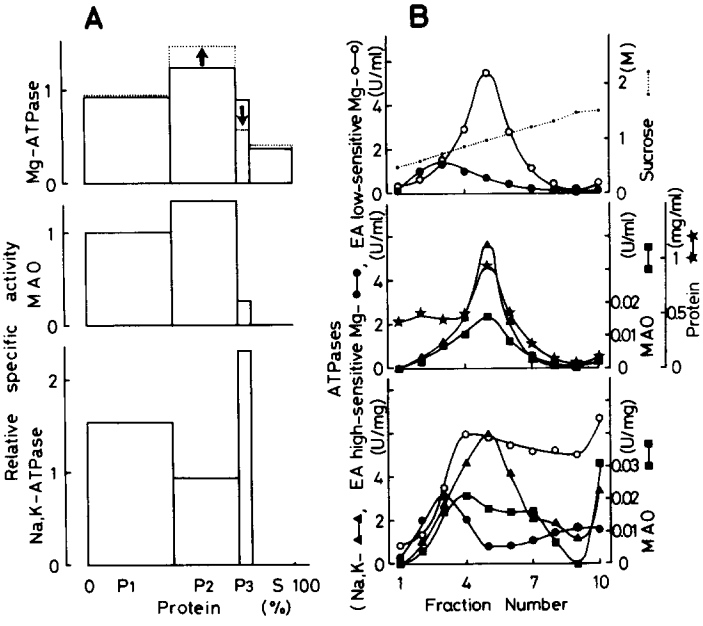

Fig. 1. Subcellular distribution of ethacrynic acid (EA) high-sensitive Mg^{2+}-ATPase activity in rabbit cerebral cortical gray matter. Key: (A) Differential centrifugation. (Top) Mg^{2+}-ATPase activity. ↓ or ↑ represents inhibition or stimulation of Mg^{2+}-ATPase activity by 0.3 mM EA. (Middle) Monoamine oxidase (MAO) activity. (Bottom) Na^+,K^+-ATPase activity. The standards for protein contents and enzyme specific activities were those of the starting homogenates. The standard specific activities of Mg^{2+}-ATPase, MAO and Na^+,K^+-ATPase were 9.5, 0.050 and 3.6 μmoles per mg protein per hr respectively. (B) Sucrose density gradient centrifugation of P_3 fraction. (Top) EA high- (●——●) and low- (○——○) sensitive Mg^{2+}-ATPase activities. (Middle) Na^+,K^+-ATPase (▲——▲) and MAO (■——■) activities and protein concentration (★——★). (Bottom) Specific activities. Symbols are the same as described above. Enzyme activities are given as μmoles per ml of fraction per hr (top and middle) or μmoles per mg of protein per hr (bottom).

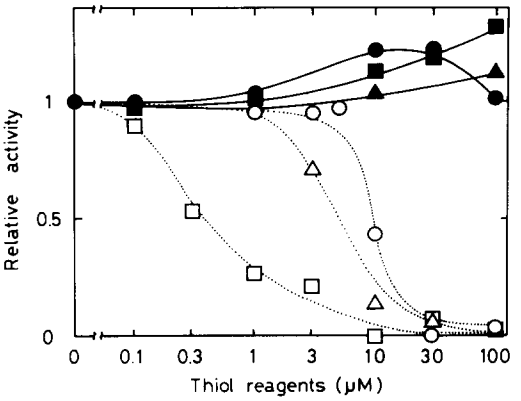

Fig. 2. Effects of sulfhydryl reagents on ethacrynic acid (EA) high-sensitive Mg^{2+}-ATPase and mitochondrial Mg^{2+}-ATPase activities. Key: (○, ●) *p*-chloromercuribenzoic acid (PCMB), (□, ■) 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and (△, ▲) *N*-ethylmaleimide (NEM). Dotted and solid lines represent EA high-sensitive Mg^{2+}-ATPase and mitochondrial Mg^{2+}-ATPase activities respectively.

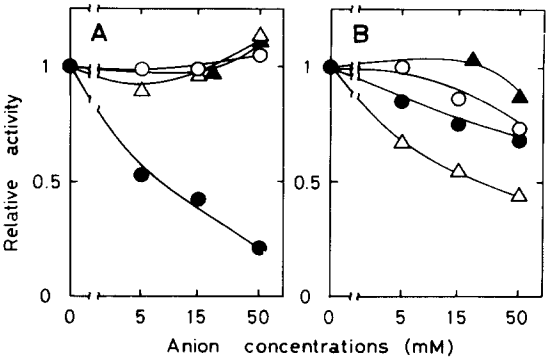

Fig. 3. Effects of HCO_3^-, Cl^-, NO_3^- and F^- on ethacrynic acid (EA) high- and low-sensitive Mg^{2+}-ATPase activities. Key: (A) microsomal EA high- or (B) low-sensitive Mg^{2+}-ATPase activity; (▲) $NaHCO_3$, (○) NaCl, (●) $NaNO_3$ and (△) NaF.

ATPase activities were measured as the rate of release of inorganic phosphate, as described previously [1]. The reaction was carried out at 37° for 10 min in 0.5 ml of a medium that contained 50 mM Tris–acetate (pH 7.4), 1 mM EDTA, 3 mM magnesium acetate, 3 mM ATP–Tris and 25–50 μg of enzyme protein with or without 0.3 mM EA. Mg^{2+}-ATPase activity in the presence or absence of 0.3 mM EA was described as total, or EA low-sensitive, activity respectively. The difference between total and EA low-sensitive activities was defined as the EA high-sensitive Mg^{2+}-ATPase activity. Na^+,K^+-ATPase activity denoted the difference between paired tubes that contained 50 mM Tris–acetate (pH 7.4), 1 mM EDTA, 3 mM magnesium acetate, 100 mM NaCl, 10 mM KCl and 3 mM ATP–Tris with or without 1 mM ouabain.

Monoamine oxidase (MAO) activity was measured by the method of Weissbach *et al.* [8]. Protein concentration was estimated by the method of Lowry *et al.* [9]. EA (free acid), provided by Merck, Sharp & Dohme Research Laboratories (West Point, PA, U.S.A.), was alkalinized to pH 7.4 with Tris for dissolution. *N*-Ethylmaleimide (NEM) and *p*-chloromercuribenzoic acid (PCMB) (Wako Pure Chemical, Osaka, Japan), and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (Nakarai Chemical, Kyoto, Japan) were used. Other chemicals used were of reagent grade.

Figure 1A shows the distribution of Mg^{2+}-ATPase, MAO and Na^+,K^+-ATPase activities after differential centrifugation. Relative specific activities were calculated taking the specific activities of the starting homogenates as the standards. The specific activities of Mg^{2+}-ATPase, MAO and Na^+,K^+-ATPase in the homogenate were 9.5, 0.050 and 3.6 μmoles per mg protein per hr respectively. The specific activity of Na^+,K^+-ATPase as marker enzyme of plasma membranes or of MAO as a marker enzyme of mitochondria was highest in the P_3 or P_2 fraction respectively. The inhibition of Mg^{2+}-ATPase activity by 0.3 mM EA, which denotes EA high-sensitive Mg^{2+}-ATPase activity as reported previously [1], was seen exclusively in the P_3 fraction; moreover, moderate stimulation by EA at 0.3 mM was observed in other fractions. Figure 1B shows the enzyme distribution pattern in the P_3 subfractions after sucrose density gradient centrifugation. Protein and the activities of Na^+,K^+-ATPase, MAO and EA low-sensitive Mg^{2+}-ATPase were most concentrated in fraction 5, where the sucrose concentration was 0.97 M (Fig. 1B, top and middle). In contrast, EA high-sensitive Mg^{2+}-ATPase activity had a peak in fraction 3, where the sucrose concentration was 0.72 M (Fig. 1B, top). The specific activity (Fig. 1b, bottom) of EA high-sensitive Mg^{2+}-ATPase or Na^+,K^+-ATPase was highest in fraction 3 or 5, respectively, but that of MAO or EA low-sensitive Mg^{2+}-ATPase was widely distributed in fractions 3–7 and 10, or fractions 4–10 respectively.

As shown in Fig. 2, EA high-sensitive Mg^{2+}-ATPase activity in microsomal fractions was sensitively inhibited by thiol reagents such as PCMB, DTNB or NEM, as reported previously. In contrast, Mg^{2+}-ATPase in mitochondrial fractions was not inhibited by any one of these reagents.

Figure 3 shows the effect of the HCO_3^- on EA high- or low-sensitive Mg^{2+}-ATPase activity in comparison with those of Cl^-, NO_3^- and F^-. EA high-sensitive activity was inhibited selectively by NO_3^- with a K_i of 7 mM, and EA low-sensitive activity was reduced preferably by F^- with a K_i of 20 mM. HCO_3^-, which is known to stimulate mitochondrial Mg^{2+}-ATPase activity [10], did not affect either microsomal EA high- or low-sensitive Mg^{2+}-ATPase activity.

Various organelles of brain can be differentiated and collected by means of their specific densities [11]. After sucrose density gradient fractionation, EA high-sensitive Mg^{2+}-ATPase was in a different membrane population as compared with that for Na^+,K^+-ATPase, the former being much less dense than the latter. This suggests that the EA high-sensitive Mg^{2+}-ATPase may be associated with plasma membranes and/or endoplasmic reticulum. MAO activity, and Na^+,K^+- and EA low-sensitive Mg^{2+}-ATPase activities concentrated in the 0.97 M sucrose fraction were presumably due to synaptosomes.

The present data suggest that EA high-sensitive Mg^{2+}-ATPase activity is distinguishable from mitochondrial Mg^{2+}-ATPase activity by its localization and sensitivity to sulfhydryl reagents and HCO_3^-.

Addendum—A mitochondrial Mg^{2+}-ATPase inhibitor, sodium azide, at 1 and 10 mM did not affect the EA high-sensitive Mg^{2+}-ATPase activity, while the EA low-sensitive Mg^{2+}-ATPase activity was reduced to 80 or 50% of the control by 1 or 10 mM sodium azide respectively. The data also suggest that the EA high-sensitive Mg^{2+}-ATPase activity in microsomes is unlikely to be due to the contaminated mitochondrial Mg^{2+}-ATPase. The EA low-sensitive Mg^{2+}-ATPase activity may be, in part, of mitochondrial origin.

Acknowledgements—We thank Professor S. Takaori for pertinent discussion. This study was funded, in part, from the Ministry of Education, Science and Culture, Japan.

Department of Pharmacology
Faculty of Medicine
Kyoto University
Kyoto 606, Japan

MITSUYOSHI HARA
MOTOKAZU FUJIWARA
CHIYOKO INAGAKI*

REFERENCES

1. M. Hara, S. Miwa, M. Fujiwara and C. Inagaki, *Biochem. Pharmac.* **31**, 877 (1982).
2. G. Sachs, A. Strych, G. Cline and B. I. Hirshwitz, *Biochim. biophys. Acta* **266**, 625 (1972).
3. B. Simon, R. Kinne and G. Sachs, *Biochim. biophys. Acta* **282**, 293 (1972).
4. C. T. Liang and B. Sacktor, *Archs Biochem. Biophys.* **176**, 285 (1976).
5. J. M. M. Van Amelsvoort, J. J. H. H. M. De Pont, A. L. H. Stoles and S. L. Bonting, *Biochim. biophys. Acta* **471**, 78 (1977).
6. J. M. M. Van Amelsvoort, J. J. H. H. M. De Pont and S. L. Bonting, *Biochim. biophys. Acta* **466**, 283 (1977).
7. J. M. M. Van Amelsvoort, J. W. C. M. Jansen, J. J. H. H. M. De Pont and S. L. Bonting, *Biochim. biophys. Acta* **512**, 296 (1978).
8. H. Weissbach, T. E. Smith, J. W. Daly, B. Witkop and S. Udenfriend, *J. biol. Chem.* **235**, 1160 (1960).
9. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
10. R. E. Ebel and H. Lardy, *J. biol. Chem.* **250**, 191 (1975).
11. R. M. Marchbanks, in *Research Methods in Neurochemistry* (Eds. N. Marks and R. Rodnight), Vol. 2, p. 79. Plenum Press, New York (1974).

* Author to whom all correspondence should be addressed.