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NOVEL MICROSOMAL ANION-SENSITIVE Mg^{2+}-ATPase ACTIVITY IN RAT BRAIN*

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Abstract—Ethacrynic acid (EA) highly sensitive Mg^{2+}-ATPase activity was demonstrated in rat brain microsomes. Marker enzyme studies suggested that the EA highly sensitive Mg^{2+}-ATPase activity originated mainly from plasma membranes, and possibly from synaptic vesicles. Oligomycin did not affect the EA highly sensitive Mg^{2+}-ATPase activity. Sulfhydryl reagents, such as *N*-ethylmaleimide and 5,5′-dithiobis-(2-nitrobenzoic acid), and anion transport inhibitors, such as 4-acetamide-4′-isothiocyano-stilbene-2,2′-disulfonic acid, 4,4′-diisothiocyano-stilbene-2,2′-disulfonic acid and 2,4-dinitro-1-fluorobenzene, completely inhibited the EA highly sensitive Mg^{2+}-ATPase activity with apparent K_i values at 5, 5, 8, 8 and 10 μM respectively. Treatment of microsomes with ethylenediaminetetraacetic acid and ammonium sulfate increased the EA highly sensitive Mg^{2+} and Na^+,K^+-ATPase activities, but not EA less sensitive Mg^{2+}- or HCO_3^--ATPase activity, 2- to 3-fold that in crude microsomes. Relative substrate specificities of ATP ≫ GTP > ITP > UTP, CTP, a K_m for ATP at 0.77 mM, and an optimal pH at pH 7.4 were observed. Among the anions tested (Cl^-, Br^-, F^-, HCO_3^-, I^-, SCN^-, NO_3^-), EA highly sensitive Mg^{2+}-ATPase activity was stimulated significantly by Cl^- and reduced by NO_3^-. These data suggest that a novel, plasma membrane-located and anion-sensitive Mg^{2+}-ATPase activity exists in the brain.

Ethacrynic acid (EA‡) is a potent diuretic whose pharmacological activity is probably based on inhibition of active chloride transport [1]; it is also known to be active toward sulfhydryl groups [2]. As we described previously [3, 4], microsomes of rabbit brain possess Mg^{2+}-ATPase activity that is sensitively reduced by ethacrynic acid at concentrations below 0.3 mM. This ethacrynic acid sensitive (EA highly sensitive) Mg^{2+}-ATPase activity is affected differently by anions [3], and is reduced by sulfhydryl reagents [4]. Further, the activity appears to be of non-mitochondrial origin by its localization and sensitivity to sulfhydryl reagents [4].

Anion-sensitive Mg^{2+}-ATPase activities have been reported in several tissues such as gastric mucosa [5], trout gill [6], kidney [7] and pancreatic islets [8]. Since mitochondrial Mg^{2+}-ATPase also is sensitive to anions [9], some of the anion-sensitive Mg^{2+}-ATPase activity was suspected to be due to contaminated mitochondria [10]. However, evidence for the anion-sensitive Mg^{2+}-ATPase activities in erythrocyte ghosts [11], intestinal brush border [12], adrenal chromaffin granules [13] and pituitary secretory granules [14] demonstrated the presence of non-mitochondrial anion-sensitive Mg^{2+}-ATPases. Since such non-mitochondrial activity has not yet been reported in the brain, we examined the EA highly sensitive Mg^{2+}-ATPase activity, as a candidate for a novel anion-sensitive Mg^{2+}-ATPase activity.

EXPERIMENTAL PROCEDURES

Materials. Brains were obtained from Wistar rats of both sexes after exsanguination and maintained frozen at −20° until thawed at the time of homogenization. Ethacrynic acid, a gift of Merck, Sharp & Dohme Research Laboratories (West Point, PA), was alkalinized to pH 7.4 with Tris for dissolution. Reagents used were as follows: ouabain, ATP, AMP, EDTA, kynurenamine, oligomycin, DIDS (Sigma Chemical Co., St. Louis, MO), DNFB (Wako Pure Chemical Industries, Osaka) and SITS (ICN Nutritional Biochemical, Cleveland, OH). All other reagents were of the highest available purity.

Preparation methods. All procedures were performed at 0–4°. Tissues were homogenized in 8 vol. of ice-cold buffer solution containing 0.25 M sucrose, 1 mM EDTA and 12.5 mM Tris–acetate (pH 7.4) and centrifuged as described previously [3, 4] (1000 *g*, 15 min; 10,000 *g*, 15 min; 92,000 *g*, 30 min). Translucent layers of the final pellets were collected and washed with homogenization buffer by centrifugation (92,000 *g*, 30 min). The resulting pellets were suspended in homogenization buffer or 5 mM EDTA–Tris (pH 7.4) and used as microsomal fractions. Mitochondrial fractions were obtained from 10,000 *g* pellets as described previously [3, 4]. For

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‡ Abbreviations: EA, ethacrynic acid; Tris, tris (hydroxymethyl) aminomethane; SITS, 4-acetamido-4′-isothiocyano-stilbene-2,2′-disulfonic acid; DIDS, 4,4′-diisothiocyano-stilbene-2,2′-disulfonic acid; DNFB, 2,4-dinitro-1-fluorobenzene; TNBS, 2,4,6-trinitrobenzenesulfonic acid; DNCB, 2,4-dinitro-1-chlorobenzene; DNBS, 2,4-dinitrobenzenesulfonic acid; and DTNB, 5,5-dithiobis-(2-nitrobenzoic acid).

density gradient fractionation, 0.2 ml of either 10,000 *g* pellets or 92,000 *g* pellets suspended in homogenization buffer was layered on a 5-ml discontinuous gradient of 0.32 M, 0.6 M, 0.8 M, 1.0 M and 1.2 M sucrose (1 ml each) in 1 mM EDTA and 12.5 mM Tris–acetate (pH 7.4). After centrifugation in a swinging rotor (Hitachi RPS 65TA) at 100,000 *g* for 60 min, fractions were collected by suction from the top of the gradients using an automatic liquid charger (Toyo ALC-21), and diluted with 1 mM EDTA in 12.5 mM Tris–acetate (pH 7.4) to be 0.25 M in sucrose concentration using an Abbott refractometer.

Treatment of microsomes. All steps were carried out at 0–4°. Whole 92,000 *g* pellets (crude microsomes) or microsomes obtained as described above were suspended in 5 mM EDTA (pH 7.4 with Tris), stirred for 30 min, and then centrifuged at 10,000 *g* for 5 min. The supernatant fraction was brought to 30% saturation point by the dropwise addition of saturated ammonium sulfate solution (pH 7.4 with Tris), stirred for 20 min, and was centrifuged at 10,000 *g* for 15 min. The precipitate was suspended in 5 mM EDTA (pH 7.4 with Tris) and dialyzed overnight against the same solution. The resulting preparation was stored at −70° and used as the EDTA-treated microsomes.

Enzyme assay. ATPase activities were determined by spectrophotometric measurement of inorganic phosphate. Mg^{2+}-ATPase was assayed in 0.2 ml medium containing 100 mM Tris–acetate (pH 7.4), 1 mM EDTA, 6 mM magnesium acetate, 6 mM Na_2ATP, 1 mM ouabain and 10–40 μg of enzyme protein. Mg^{2+}-ATPase activity in the presence or absence of 0.3 mM ethacrynic acid was designated as EA less sensitive or total Mg^{2+}-ATPase activity respectively. The difference between the EA less sensitive and total Mg^{2+}-ATPase activities was denoted as the EA highly sensitive Mg^{2+}-ATPase activity. Na^+,K^+-ATPase activity was defined as the difference between paired tubes containing 100 mM Tris–acetate (pH 7.4), 1 mM EDTA, 6 mM magnesium acetate, 6 mM Na_2ATP, 100 mM NaCl, 10 mM KCl and 10–40 μg of enzyme protein with or without 1 mM ouabain. HCO_3-ATPase activity was measured as the difference of paired tubes for Mg^{2+}-ATPase with or without 20 mM $NaHCO_3$. After preincubation at 37° for 5 min, the reaction was started by the addition of ATP. The incubation was carried out at 37° for 10–20 min, and was stopped by adding 10% trichloroacetic acid and cooling the tubes in ice. The mixtures were centrifuged at 3000 *g* for 5 min, and a 0.3-ml sample of each supernatant fraction was used for the determination of inorganic phosphate liberated, as determined by the method of Chen *et al.* [15].

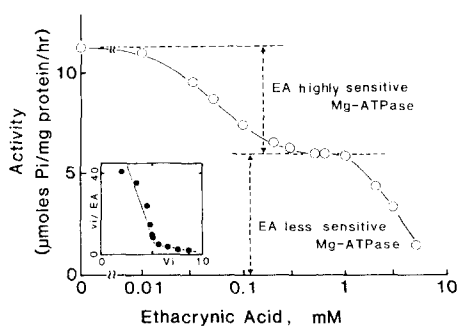

Fig. 1. Effect of ethacrynic acid on brain microsomal Mg^{2+}-ATPase activity. Activity was assayed by spectrophotometrically measuring inorganic phosphate liberated from ATP as described in Experimental Procedures. Microsomes were preincubated with ethacrynic acid for 5 min, and the ATP hydrolyzing reaction was started by addition of Na_2ATP (6 mM). The inset is an Eadie–Scatchard plot of the data in Fig. 1. Vi indicates ethacrynic acid-induced decrease in Mg^{2+}-ATPase activity, i.e. (total Mg^{2+}-ATPase activity in the absense of ethacrynic acid) − (Mg^{2+}-ATPase activity at a definite ethacrynic acid concentration).

5′-Nucleotidase was assayed according to the method of Avruch and Wallach [16]. Monoamine oxidase activity was determined [17] using kynurenamine as a substrate and measuring the rate of initial decrease at 360 nm absorbance. Protein concentration was determined by the method of Lowry *et al.* [18], using bovine serum albumin as a standard.

RESULTS

Effect of ethacrynic acid on microsomal Mg^{2+}-ATPase. As was observed in rabbit brain [3], microsomal Mg^{2+}-ATPase activity of rat brain was inhibited by ethacrynic acid biphasically (Fig. 1). Approximately 50% of the activity was inhibited by ethacrynic acid in the range of 10–300 μM, and the remaining activity was inhibited at concentrations over 1 mM. Eadie–Scatchard plots (Fig. 1, inset) of the data were resolved into two components with different slopes, apparent K_i values for them being 50 μM and 3 mM respectively. The former or the latter component was defined as ethacrynic acid (EA) highly sensitive or EA less sensitive Mg^{2+}-ATPase activity respectively.

Distribution. Distribution of EA highly sensitive Mg^{2+}-ATPase activity was compared with that of membrane marker enzymes after discontinuous sucrose density gradient fractionation of 10,000 *g* pellets or 92,000 *g* pellets (Fig. 2). Na^+,K^+-ATPase and 5′-nucleotidase were assayed as marker enzymes for plasma membranes and monoamine oxidase as a marker enzyme for mitochondria. HCO_3-ATPase was assayed for comparison. Among subfractions of 10,000 *g* pellets, EA highly sensitive Mg^{2+}-ATPase activity was highest in 0.8 M/1.0 M interface. The distribution pattern of EA highly sensitive Mg^{2+}-ATPase activity was distinguishably different from the patterns of total Mg^{2+}-ATPase, HCO_3-ATPase and monoamine oxidase activities, and was similar to the patterns of Na^+,K^+-ATPase and 5′-nucleotidase activities. After hypotonic shock treatment of 10,000 *g* pellets, EA highly sensitive Mg^{2+}-ATPase activity was much higher in the 0.32 M/0.6 M, 0.6 M/0.8 M and 1.0 M/1.2 M interfaces as compared with that in the 0.8 M/1.0 M interface. The distribution pattern was similar to that of 5′-nucleotidase, and again quite different from those of total Mg^{2+}-ATPase, HCO_3-ATPase and monoamine oxidase. Na^+,K^+-ATPase in these fractions did not parallel the EA highly sensitive Mg^{2+}-ATPase activity. With subfractions of 92,000 *g* pellets, EA highly sensitive Mg^{2+}-ATPase activity was high in all fractions and

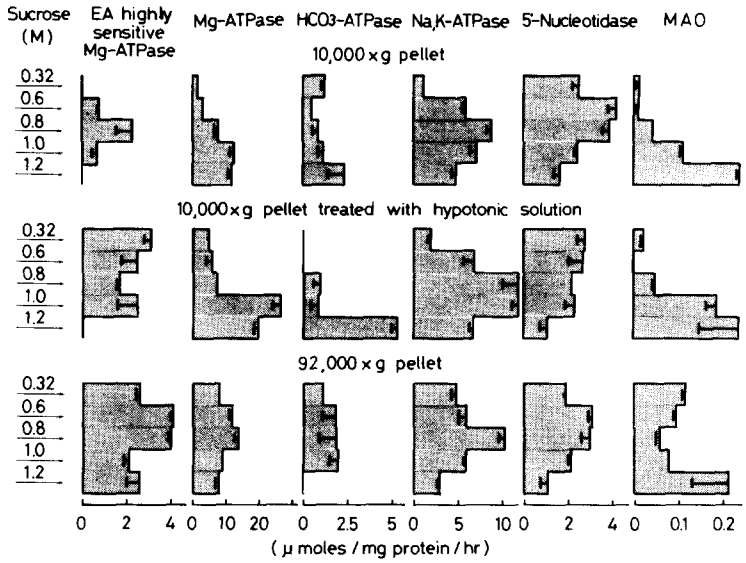

Fig. 2. Activities of ethacrynic acid highly sensitive Mg^{2+}-ATPase and marker enzymes in subfractions after discontinuous sucrose density gradient fractionation. Original fractions were 10,000 *g* pellets suspended in homogenization buffer (top), 10,000 *g* pellets treated with 1 mM EDTA (pH 7.4 with Tris) (middle) and 92,000 *g* pellets suspended in homogenization buffer (bottom). All subfractions were diluted to 0.25 M in sucrose concentration with 1 mM EDTA in 12.5 mM Tris–acetate (pH 7.4). MAO: monoamine oxidase. Values (μmoles product/mg protein/hr) are expressed as the mean of three determinations with S.E. indicated by a bar.

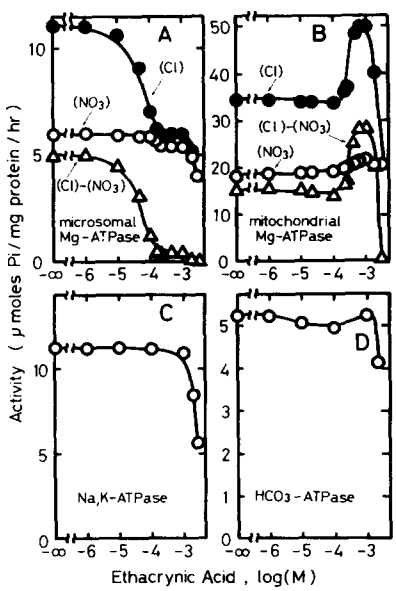

Fig. 3. Effects of ethacrynic acid on ATPase activities. (A) Microsomal Mg^{2+}-ATPase activity in the presence of 100 mM NaCl (●—●) or $NaNO_3$ (○—○). The difference between the former and the latter was calculated (△—△). (B) Mitochondrial Mg^{2+}-ATPase activity in the presence of 100 mM NaCl (●—●) or $NaNO_3$ (○—○). The difference between them was calculated (△—△). (C) Microsomal Na^+,K^+-ATPase activity. (D) HCO_3-ATPase activity in subfractions under 1.2 M sucrose from 10,000 *g* pellets treated with 1 mM EDTA. All membrane vesicles used as enzyme sources were suspended in homogenization buffer.

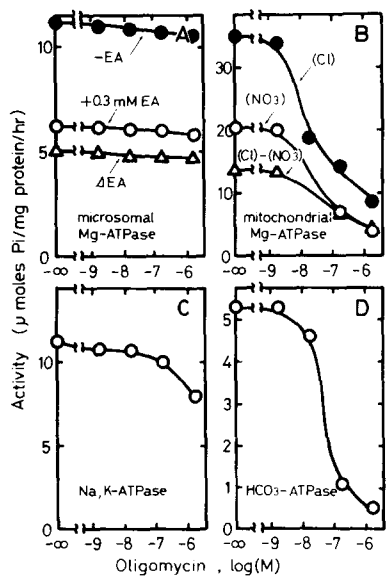

Fig. 4. Effects of oligomycin on ATPase activities. (A) Microsomal Mg^{2+}-ATPase activity in the presence of 100 mM NaCl with (○—○) or without (●—●) 0.3 mM ethacrynic acid. The difference between them was calculated (△—△). (B) Mitochondrial Mg^{2+}-ATPase activity in the presence of 100 mM NaCl (●—●) or $NaNO_3$ (○—○). The difference between them was calculated (△—△). (C) Microsomal Na^+,K^+-ATPase activity. (D) HCO_3-ATPase activity in the heaviest subfractions of 10,000 *g* pellets treated with 1 mM EDTA (pH 7.4 with Tris). Oligomycin was dissolved in acetic acid, and the vehicle for oligomycin up to 2 μM had no effect on any ATPase activity examined.

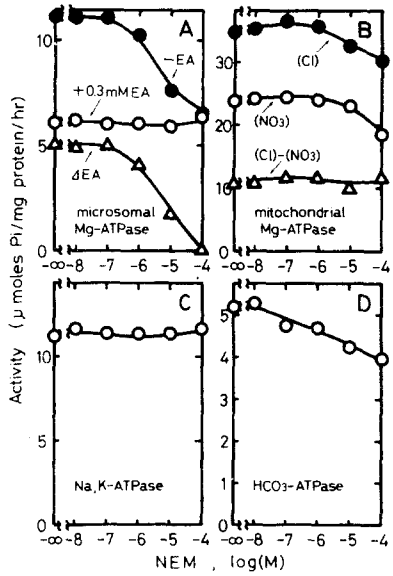

Fig. 5. Effects of *N*-ethylmaleimide (NEM) on ATPase activities. (A) Microsomal Mg^{2+}-ATPase activity in the presence of 100 mM NaCl with (○—○) or without (●—●) 0.3 mM ethacrynic acid. The difference between them was calculated (△—△). (B) Mitochondrial Mg^{2+}-ATPase activity in the presence of 100 mM NaCl (●—●) or $NaNO_3$ (○—○). The difference between them was calculated (△—△). (C) Microsomal Na^+,K^+-ATPase activity. (D) HCO_3-ATPase activity in the heaviest subfractions of 10,000 *g* pellets treated with 1 mM EDTA (pH 7.4 with Tris).

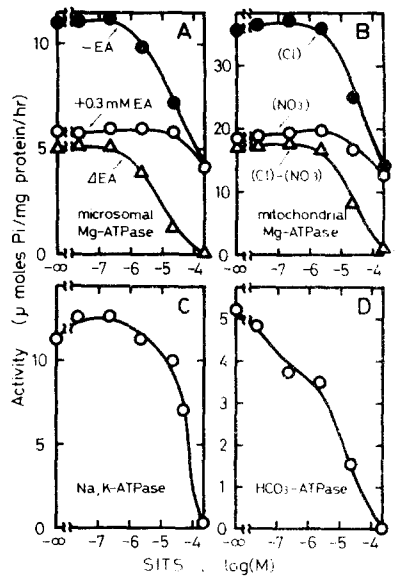

Fig. 6. Effects of SITS on ATPase activities. (A) Microsomal Mg^{2+}-ATPase activity in the presence of 100 mM NaCl with (○—○) or without (●—●) 0.3 mM ethacrynic acid. The difference between them was calculated (△—△). (B) Mitochondrial Mg^{2+}-ATPase activity in the presence of 100 mM NaCl (●—●) or $NaNO_3$ (○—○). The difference between them was calculated (△—△). (C) Microsomal Na^+,K^+-ATPase activity. (D) HCO_3-ATPase activity in the heaviest subfractions of 10,000 *g* pellets treated with 1 mM EDTA (pH 7.4 with Tris).

especially in 0.6 M/0.8 M and 0.8 M/1.0 M interfaces in a pattern similar to those of total microsomal Mg^{2+}-ATPase, 5′-nucleotidase and Na^+,K^+-ATPase. Activities of HCO_3-ATPase and monoamine oxidase, which were probably due to contaminated mitochondria and their disrupted membranes, were distributed differently from that of EA highly sensitive Mg^{2+}-ATPase activity. The data suggest, as discussed later in more detail, that microsomal EA highly sensitive Mg^{2+}-ATPase activity originates mainly from plasma membranes, but not from mitochondria.

Inhibitors. As shown in Fig. 3A, microsomal EA highly sensitive Mg^{2+}-ATPase activity was observed in the presence of 100 mM NaCl. However, when NaCl was replaced by $NaNO_3$, EA highly sensitive activity was selectively reduced. The difference between the activity in Cl^- medium and that in NO_3^- medium was completely inhibited by 0.3 mM ethacrynic acid. On the other hand, mitochondrial Mg^{2+}-ATPase activity was not reduced by ethacrynic acid below 0.1 mM, but rather stimulated by this acid over 0.5 mM (Fig. 3B). The control activity was low in NO_3^- medium as compared with that in Cl^- medium. Stimulation by ethacrynic acid was obvious only in Cl^- medium. Ethacrynic acid in concentrations as high as 2 mM or more reduced the mitochondrial Mg^{2+}-ATPase activity. Both Na^+,K^+-ATPase and HCO_3-ATPase activities were inhibited by ethacrynic acid over 2 mM (Fig. 3, C and D).

As shown in Fig. 4A, neither microsomal EA highly sensitive nor less sensitive Mg^{2+}-ATPase activity was affected by oligomycin up to 2 μM.

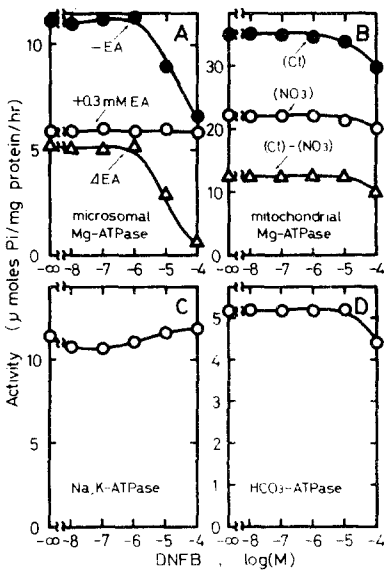

Fig. 7. Effects of DNFB on ATPase activities. (A) Microsomal Mg^{2+}-ATPase activity in the presence of 100 mM NaCl with (○—○) or without (●—●) 0.3 mM ethacrynic acid. The difference between them was calculated (△—△). (B) Mitochondrial Mg^{2+}-ATPase activity in the presence of 100 mM NaCl (●—●) or $NaNO_3$ (○—○). The difference between them was calculated (△—△). (C) Microsomal Na^+,K^+-ATPase activity. (D) HCO_3-ATPase activity in the heaviest subfractions of 10,000 *g* pellets treated with 1 mM EDTA (pH 7.4 with Tris). DNFB was dissolved in diethylether and diluted in distilled water. The solvent for DNFB up to 0.1 mM did not affect any of the ATPase activities examined.

whereas mitochondrial Mg^{2+}-ATPase activity was sensitively inhibited by oligomycin with an apparent K_i of 0.1 μM (Fig. 4B). Mitochondrial Mg^{2+}-ATPase activity in the presence of Cl^- or NO_3^- was similarly affected by oligomycin. Na^+,K^+-ATPase activity was reduced slightly by 2 μM oligomycin (Fig. 4C). HCO_3-ATPase activity was inhibited markedly by oligomycin with an apparent K_i of 0.1 μM (Fig. 4D).

EA highly sensitive Mg^{2+}-ATPase activity was reduced by *N*-ethylmaleimide with an apparent K_i of 5 μM (Fig. 5A). EA less sensitive Mg^{2+}-ATPase activity was not affected at all. Mitochondrial Mg^{2+}-ATPase activity or HCO_3-ATPase activity was slightly inhibited to approximately 80% of the control (Fig. 5, B and D). Na^+,K^+-ATPase activity was not affected by *N*-ethylmaleimide up to 0.1 mM (Fig. 5C).

SITS, which is known to inhibit erythrocyte anion transport, inhibited EA highly sensitive Mg^{2+}-ATPase activity (apparent $K_i = 8$ μM) with only a minor effect on EA less sensitive Mg^{2+}-ATPase activity (Fig. 6A). However, SITS also inhibited mitochondrial Mg^{2+}-ATPase in the presence of Cl^- (Fig. 6B). Replacement of Cl^- with NO_3^- minimized further inhibition of the activity caused by SITS. Both Na^+,K^+- and HCO_3-ATPases were inhibited by SITS with apparent K_i values of 70 and 5 μM respectively (Fig. 6, C and D). Another erythrocyte anion transport inhibitor, DNFB, selectively inhibited EA highly sensitive Mg^{2+}-ATPase activity (apparent $K_i = 10$ μM) without any significant effect on other ATPase activities examined (Fig. 7).

EDTA. EDTA (1–10 mM) added in the incubation medium stimulated EA highly sensitive Mg^{2+}-ATPase activity with the maximal effect at 5 mM, while, with EA less sensitive Mg^{2+}-ATPase activity, EDTA at 5 mM or more markedly inhibited the activity (Fig. 8A).

Treatment of microsomes. When microsomal pellets were suspended in 1–10 mM EDTA solution (pH 7.4 with Tris), stirred for 30 min at 4°, and centrifuged at 10,000 *g* for 5 min, supernatant fractions contained higher EA highly sensitive Mg^{2+}-ATPase activities with the increase in EDTA concentration (Fig. 8B). In the precipitates, as compared with those in the supernatant fractions, lower EA highly sensitive and higher EA less sensitive Mg^{2+}-ATPase activities were observed (data not shown). The supernatant fraction of microsomal suspension in 5 mM EDTA was then fractionated by 30% saturation with ammonium sulfate. The precipitates suspended in and dialyzed against 5 mM EDTA contained EA highly sensitive Mg^{2+}-ATPase activity 2- to 3-fold higher than that in crude microsomes (Table 1). In this final preparation, Na^+,K^+-ATPase activity was concentrated, but HCO_3-ATPase and monoamine oxidase activities were not detectable. Mg^{2+}-ATPase activity in the EDTA-treated microsomes was affected by ethacrynic acid in a pattern similar to that in original microsomes with a plateau in a concentration range of ethacrynic acid from 0.1 to 1 mM (data not shown).

Substrate specificity. ATP and some other nucleoside phosphates were assayed at 6 mM and pH 7.4 (Table 2). EA highly sensitive Mg^{2+}-ATPase activity was highest when ATP was used as a substrate. GTP and ITP were hydrolyzed at 30–50% of the ATP rate. However, pyrimidine nucleotides examined were hydrolyzed at less than one-sixth the ATP rate.

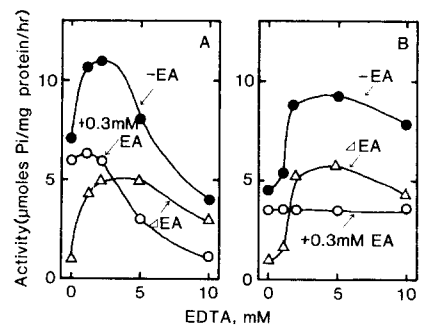

Fig. 8. Effects of EDTA on microsomal Mg^{2+}-ATPase activities. (A) Effects of EDTA added in the incubation medium. Microsomes suspended in homogenization buffer were used. (B) Mg^{2+}-ATPase activities of microsomes treated with different concentrations of EDTA. Microsomes were suspended in 1–10 mM EDTA solution, stirred for 30 min at 4° and centrifuged at 10,000 *g* for 5 min. Sixty to seventy-eight percent of microsomal protein was recovered in the supernatant fractions and assayed for Mg^{2+}-ATPase. Mg^{2+}-ATPase activities were measured in the presence (○—○) or absence (●—●) of 0.3 mM ethacrynic acid. The difference between them was calculated (△—△).

Table 1. Enzyme activities in crude, washed or EDTA-treated microsomal preparations*

Microsomes	Enzyme activity (μmoles product/mg protein/hr)				
	EA highly sensitive Mg^{2+}-ATPase	EA less sensitive Mg^{2+}-ATPase	Na^+,K^+-ATPase	HCO_3-ATPase	Monoamine oxidase
Crude	4.4 ± 0.5	11.3 ± 0.7	8.3 ± 1.0	1.1 ± 0.2	0.09 ± 0.01
Washed	4.8	5.8	11.7	0.5	0.05
EDTA-treated	10.7 ± 2.0	4.0 ± 1.4	22.9 ± 3.8	ND†	ND

* In crude or EDTA-treated microsomal preparations, values are expressed as mean ± S.E. (N = 3). Washed preparations were collected and then assayed for enzymes. Values in the collected washed preparation are shown. Assays were performed as described under Experimental Procedures. Crude: 92,000 *g* pellets were suspended in 5 mM EDTA. Washed: translucent 92,000 *g* pellets were washed with homogenization buffer and suspended in 5 mM EDTA. EDTA-treated: crude or washed microsomal pellets were suspended in 5 mM EDTA, stirred for 30 min, and centrifuged at 10,000 *g* for 5 min. The supernatant fractions were fractionated by 30% saturation with ammonium sulfate. The precipitates were suspended in and dialyzed against 5 mM EDTA and then assayed for enzymes.

† Not detectable.

Table 2. Substrate specificity of microsomal Mg^{2+}-ATPases*

Substrate	Mg^{2+}-ATPase activity (%)	
	EA highly sensitive	EA less sensitive
ATP	100	100
GTP	42	110
ITP	31	71
UTP	14	115
CTP	10	74
ADP	4	8
AMP	ND†	19
Cyclic AMP	ND	ND

* All substrates were tested at 6 mM and pH 7.4. The reactions were started by addition of substrates. Other assay conditions were as described under Experimental Procedures. All nucleotide solutions were adjusted to pH 7.4 with Tris prior to assay. EA highly sensitive or less sensitive Mg^{2+}-ATPase activity for ATP was 8.2 to 13.7 or 3.2 to 5.0 μmoles P_i/mg protein/hr respectively. Values are expressed as means of two or three determinations.

† Not detectable.

In contrast, EA less sensitive Mg^{2+}-ATPase activities for purine and pyrimidine nucleoside triphosphates were 70–120% of that for ATP. ADP, AMP or cyclic AMP was not hydrolyzed at the rate comparable to any nucleoside triphosphate examined. With EA highly sensitive and less sensitive Mg^{2+}-ATPase activity, the apparent K_m for ATP was 0.77 and 1.67 mM respectively.

pH Optimum. The maximal EA highly sensitive Mg^{2+}-ATPase activity was observed at pH 7.4, and the activity was reduced in the pH below 6.6 or over 7.8, when assayed in the presence of 100 mM imidazole–HCl (pH 6.2 to 7.8) or Tris–acetate (pH 7.4 to 9.0). EA less sensitive Mg^{2+}-ATPase activity did not change over the pH range from 6.2 to 9.0.

K_i *for inhibitors.* Using EDTA-treated microsomes, K_i values for inhibitors and related compounds were examined (Table 3). K_i values for *N*-ethylmaleimide and 5,5′-dithiobis-(2-nitrobenzoic acid) were both 5 μM. The K_i value for SITS also was comparable to that for a related stilbene, DIDS. A 10 μM concentration of DNFB inhibited 50% of EA highly sensitive Mg^{2+}-ATPase activity in the EDTA-treated microsomes. Related compounds with lower potencies in amino group labeling yielded apparent larger K_i values. Apparent K_i values for *N*-ethylmaleimide, SITS and DNFB were not different from those observed in the original microsomes.

Table 3. Apparent K_i values for inhibitors in EA highly sensitive Mg^{2+}-ATPase activity*

Inhibitors	K_i (μM)
N-Ethylmaleimide	5
5,5′-Dithiobis-(2-nitrobenzoic acid)	5
SITS	8
DIDS	8
DNFB	10
TNBS	90
DNCB	400
DNBS	~1000

* Inhibitors and treated microsomes were incubated at 37° for 5 min in the presence or absence of 0.3 mM ethacrynic acid, and reactions were started by the addition of ATP. The difference of the activities was calculated as EA highly sensitive Mg^{2+}-ATPase activity. EA less sensitive Mg^{2+}-ATPase activity was not affected by any inhibitor in the concentration range examined. Abbreviations: SITS, 4-acetamido-4′-isothiocyano-stilbene-2,2′-disulfonic acid; DIDS, 4,4′-diisothiocyano-stilbene-2,2′-disulfonic acid; DNFB, 2,4-dinitro-1-fluorobenzene; TNBS, 2,4,6-trinitrobenzenesulfonic acid; DNCB, 2,4-dinitro-1-chlorobenzene; and DNBS, 2,4-dinitrobenzenesulfonic acid.

Anions. Effects of sodium salts with different anions were tested (Table 4). Among the anions examined, Cl^- significantly stimulated, and NO_3^- markedly reduced EA highly sensitive Mg^{2+}-ATPase activity. Stimulation by Cl^- of the brain Mg^{2+}-

Table 4. Effects of anions on microsomal Mg^{2+}-ATPase activities*

Anions	Mg^{2+}-ATPase activity			
	EA highly sensitive		EA less sensitive	
	μmoles P_i/mg protein/hr	ratio	μmoles P_i/mg protein/hr	ratio
Cl	11.0 ± 0.1*†	1.19	5.5 ± 0.4	1.02
Br^-	9.9 ± 0.4	1.08	5.4 ± 0.4	1.01
F	9.9 ± 0.9	1.08	4.0 ± 0.1†	0.75
HCO_3^-	9.4 ± 0.2	1.02	5.7 ± 0.5	1.06
No addition	9.2 ± 0.1	1.00	5.4 ± 0.4	1.00
I^-	8.9 ± 0.5	0.97	5.4 ± 0.3	1.01
SCN^-	8.0 ± 0.5	0.87	4.5 ± 0.5	0.83
NO_3^-	4.2 ± 0.4†	0.45	4.8 ± 0.6	0.90

* ATPase activity in the presence or absence of 0.3 mM ethacrynic acid was designated as EA less sensitive Mg^{2+}-ATPase activity or total Mg^{2+}-ATPase activity respectively. The difference between these activities was denoted as EA highly sensitive Mg^{2+}-ATPase activity. A 25 mM concentration of sodium salts of each anion was added in the incubation medium. Other assay conditions were as described under Experimental Procedures. Values are expressed as means ± S.E. ($N = 3$). Ratios represent relative activities (no addition ≡ 1.00)

† $P < 0.05$ (anion tested: no addition) using Student's *t*-test.

ATPase activity was first observed in the EDTA-treated microsomes. On the other hand, EA less sensitive Mg^{2+}-ATPase activity was reduced by F^- and was not significantly affected by any other anions. Cl^- and NO_3^- over the range of 0–100 mM affected EA highly sensitive Mg^{2+}-ATPase activity differently in the EDTA-treated microsomes without any significant effect on EA less sensitive Mg^{2+}-ATPase activity. The maximal stimulation of EA highly sensitive Mg^{2+}-ATPase activity was observed in the 12.5 to 25 mM range of Cl^-, and reduction of the activity by NO_3^- was augmented with the increase in NO_3^- concentration. Potassium salts of the anions yielded quite similar results (data not shown).

DISCUSSION

Since ethacrynic acid, as previously observed with the rabbit brain [3], inhibited biphasically the rat brain microsomal Mg^{2+}-ATPase activity (Fig. 1), and the activity highly sensitive to ethacrynic acid was selectively affected by specific anions such as Cl^- or NO_3^- (Fig. 3 and Table 4), this EA highly sensitive Mg^{2+}-ATPase activity appears to differ from the remaining activity, i.e. EA less sensitive Mg^{2+}-ATPase activity.

Mitochondrial Mg^{2+}-ATPase also is known to be anion-sensitive [9], and HCO_3-ATPase activity observed in microsomal fractions of several tissues has been reported to be of mitochondrial origin [10]. Therefore, microsomal EA highly sensitive Mg^{2+}-ATPase activity of the rat brain was analyzed with respect to its origin. Subfractions obtained by sucrose density gradient centrifugation from 92,000 *g* pellets possessed higher EA highly sensitive Mg^{2+}-ATPase activities than those from 10,000 *g* pellets (Fig. 2). This suggests that EA highly sensitive Mg^{2+}-ATPase activity is associated with membrane particles with lower density as compared to mitochondria. From the distribution patterns of marker enzymes such as Na^+,K^+-ATPase, 5′-nucleotidase and monoamine oxidase, definite cell organelles appeared to localize at corresponding interfaces as described by Marchbanks [19]. Among the subfractions from 92,000 *g* pellets (crude microsomes), EA highly sensitive Mg^{2+}-ATPase activity was high in plasma membrane fractions with higher activities of marker enzymes. Further, when the microsomes were treated with EDTA and ammonium sulfate, both EA highly sensitive Mg^{2+}-ATPase and Na^+,K^+-ATPase activities were raised 2.2 to 2.7 times higher than those in crude microsomes, and neither HCO_3-ATPase nor monoamine oxidase activity was detectable (Table 1). These data suggest that EA highly sensitive Mg^{2+}-ATPase activity in 92,000 *g* pellets is mainly of plasma membrane origin. Among the subfractions from 10,000 *g* pellets, EA highly sensitive Mg^{2+}-ATPase activity was high in synaptosomal fraction (0.8 M/1.0 M interface) in parallel with the activities of plasma membrane marker enzymes. After hypotonic shock treatment, EA highly sensitive Mg^{2+}-ATPase activity was also observed in fractions of particles with lower density such as synaptic vesicles and disrupted synaptosomal membranes (0.32 M/0.6 M interface) as well as in fractions of particles with high density such as shrunken synaptosomes (1.0 M/1.2 M interface). This raises a possibility that, besides the synaptosomal membranes, intracellular organelles such as synaptic vesicles may be the origin of this enzyme activity. All these data suggest that EA highly sensitive Mg^{2+}-ATPase activity is mainly of plasma membrane origin, and is possibly present in synaptic vesicles. In contrast, HCO_3-ATPase activity in subfractions of 10,000 *g* pellets paralleled monoamine oxidase activity. Thus, HCO_3-ATPase in brain appeared, as reported previously [10], to originate from mitochondria. Dissociation of HCO_3-ATPase and monoamine oxidase activities in the heaviest fractions from 92,000 *g* pellets (Fig. 2) implies the possible localization of HCO_3-ATPase in an intramitochondrial compartment different from the outer membranes where monoamine oxidase exists.

Several reagents acted in different manners on the ATPases examined. In contrast to the finding that 50% inhibition of EA highly sensitive Mg^{2+}-ATPase activity was observed at 50 μM ethacrynic acid, this acid in concentrations up to 0.3 mM affected none of the activities of mitochondrial Mg^{2+}-, Na^+,K^+- and HCO_3-ATPase (Fig. 3). On mitochondrial Mg^{2+}-ATPase, ethacrynic acid in higher concentrations was rather stimulatory. The mechanisms involved are not known. However, this stimulatory effect of ethacrynic acid also appeared to be anion-dependent, because mitochondrial Mg^{2+}-ATPase activity measured in the presence of Cl^-, but not that in the NO_3^- medium, was accelerated by this acid. Ethacrynic acid reportedly inhibits Na^+,K^+-ATPase [20], HCO_3-ATPase [21] and pituitary secretory granule ATPase [14]. However, half inhibition of these enzymes is reached by ethacrynic acid in a concentration range of 2–5 mM. Thus, previous reports [3] on selective inhibition of microsomal Mg^{2+}-ATPase activity by a lower concentration of ethacrynic acid were confirmed with the rat brain microsomes. Oligomycin, a known inhibitor of mitochondrial Mg^{2+}-ATPase, inhibited both mitochondrial Mg^{2+}-ATPase and HCO_3-ATPase. Consistent with findings reported by Hobbs *et al.* [22], Na^+,K^+-ATPase activity was reduced by a higher concentration (>1 μM) of oligomycin. Thus, HCO_3-ATPase appeared to be similar to mitochondrial Mg^{2+}-ATPase in the sensitivity to oligomycin, and was found to be clearly different in this respect from EA highly sensitive Mg^{2+}-ATPase. As we reported previously with the rabbit brain microsomes [4], *N*-ethylmaleimide inhibited EA highly sensitive Mg^{2+}-ATPase activity and the inhibition appeared to be selective when the reagents were added at concentrations below 0.1 mM (Fig. 5 and Table 3). *N*-Ethylmaleimide reportedly inhibits Na^+,K^+-ATPase [23] and chromaffin granule Mg^{2+}-ATPase [24], at concentrations over 0.1 mM. Thus, EA highly sensitive Mg^{2+}-ATPase activity of rat brain microsomes also was found to be catalyzed by an enzyme system containing sulfhydryl groups with high affinity to sulfhydryl reagents. The so-called anion transport inhibitors, i.e. SITS, DIDS and DNFB, inhibited EA highly sensitive Mg^{2+}-ATPase activity (Figs. 6 and 7 and Table 3). Stilbene derivatives (SITS, DIDS), however, reduced other ATPase activities examined. Since these stilbene derivatives are known

Table 5. Summary of the effects of inhibitors on ATPase activities*

	Ethacrynic acid (0.3 mM)	Oligomycin (0.1 μM)	NEM (0.1 mM)	SITS (0.1 mM)	DNFB (0.1 mM)
EA highly sensitive Mg^{2+}-ATPase	↓	→	↓	↓	↓
EA less sensitive Mg^{2+}-ATPase	→	→	→	→	→
Na^+,K^+-ATPase	→	→	→	↓	→
HCO_3^--ATPase	→	↓	→	↓	→
Mitochondrial Mg^{2+}-ATPase	→	↓	→	↓	→

* The data in Figs. 3–7 are summarized. Key: (↓) inhibited, and (→) not changed.

to act specifically on amino groups [25] and the anion-sensitive components of the ATPase activities were sensitively reduced by the stilbenes (Fig. 6), the possible presence of an anion-sensitive and amino group containing site common to these ATPases is suggested. In contrast, DNFB selectively inhibited EA highly sensitive Mg^{2+}-ATPase (Fig. 7). DNFB and related compounds such as TNBS, DNCB and DNBS also are known reagents for amino groups, but DNFB further acts on sulfhydryl groups [25]. Inhibition by these reagents paralleled their potency in labeling amino groups (Table 3). It remains, however, to be determined whether DNFB (and related compounds) reduce EA highly sensitive Mg^{2+}-ATPase activity through acting on amino groups or on sulfhydryl groups in the enzyme protein. As summarized in Table 5, EA highly sensitive Mg^{2+}-ATPase activity is characterized by its high susceptibility to ethacrynic acid, *N*-ethylmaleimide and DNFB.

Among the anions tested, Cl^- stimulated and NO_3^- reduced the EA highly sensitive Mg^{2+}-ATPase activity, and HCO_3^- scarcely affected the activity. Non-mitochondrial anion-sensitive Mg^{2+}-ATPase activities reported are characteristic in their anion-sensitive nature. The activities in erythrocyte ghosts [11] and pituitary secretory granules [14] are markedly stimulated by HCO_3^- and to a lesser extent or not at all by Cl^-. Intestinal brush border Mg^{2+}-ATPase activity increases equally with HCO_3^- and Cl^- [12], and chromaffin granule Mg^{2+}-ATPase activity increases with Cl^- [13]. There may be some similarity in the anion-sensitive sites of the latter two enzymes and EA highly sensitive Mg^{2+}-ATPase. The effect of EDTA, however, clearly differentiates EA highly sensitive Mg^{2+}-ATPase activity from the other two, since EDTA (2–3 mM) markedly stimulates EA highly sensitive Mg^{2+}-ATPase activity, but completely inhibits Mg^{2+}-ATPase activities in intestinal brush border membranes and chromaffin granules.

To our knowledge, the anion-sensitive Mg^{2+}-ATPase activity reported by our laboratories appears to be a novel one in terms of its localization and enzymic characteristics. The existence of a Cl^--stimulated Mg^{2+}-ATPase activity in the plasma membranes raises a possibility that it operates as an anion-pump in the formation of Cl^- concentration gradient, which has been suggested by ion flux studies [26].

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