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DIFFERENTIAL EFFECT OF ETHACRYNIC ACID ON MICROSOMAL Mg^{2+}-ATPase

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Abstract—The effect of ethacrynic acid (EA) was studied on microsomal ATPase in various tissues of the rat and in kidneys of different species. Inhibition of Na,K-ATPase in microsomes of kidney medulla and cortex was observed only at 5×10^{-3} M EA. At concentrations of 10^{-5}–10^{-4} M, EA caused some enhancement of Na,K-ATPase activity. Microsomal Mg–ATPase of kidney medulla and cortex was inhibited by EA at concentrations of 10^{-5}–10^{-3} M in various rodents (rat, mouse, guinea-pig and rabbit) as well as in human and cat kidney. EA showed the same differential effect on kidney microsomal Mg-ATPase when microsomes were prepared without treatment with desoxycholate, when chloride was replaced by sulfate in the medium. EA inhibited preferentially Mg-ATPase vs Na, K-ATPase in microsomes of rat brain and rat submaxillary gland.

Ethacrynic acid (EA) is a well known diuretic drug [1]. However, the mechanism of action of this drug has been the subject of many studies and controversial interpretations. While it was initially assumed to act through inhibition of NaK-ATPase activity [2–4], this mechanism was later questioned [5, 6]. Recently it has been suggested that EA inhibits a "chloride pump" in the thick ascending limb of the rabbit kidney [7]. However, no specific chloride-activated ATPase has been demonstrated to date. EA has also been reported to inhibit various other enzymes related to energy supply [8] and could, thus, affect transport indirectly.

We have recently studied sodium extrusion from kidney slices *in vitro* and found that ouabain, an established inhibitor of Na,K-ATPase, depressed sodium extrusion in slices of kidney medulla by about 60 per cent but only 30 per cent of sodium extrusion was inhibited in the cortex [9]. This corroborates the higher Na,K-ATPase activity in kidney medulla compared to kidney cortex [10]. In contrast, EA had the opposite effect—only 23 per cent inhibition of sodium extrusion in medullary slices but 77 per cent inhibition in cortical slices [9]. Thus, the action of EA on sodium extrusion does not conform with the distribution of Na,K-ATPase in the kidney.

In a study of the effect of various diuretics on kidney microsomal ATPase, EA and Furosemide were the only diuretics which inhibited kidney medullary Mg-ATPase more effectively than Na,K-ATPase [11]. Therefore, it seemed of interest to study whether this preferential inhibition of Mg-ATPase was a specific effect of EA in rat kidney medulla or whether this was a general effect of EA. The present study reports the results of these experiments and extends the preferential effect of EA on microsomal Mg-ATPase to different species and various tissues.

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Na,K-ATPase activity is attributable to the plasma membrane. However, since in the method of preparation, the fraction obtained contains also endoplasmic reticulum, the more general term "microsomal" fraction is used so as to avoid the implication of a pure membrane preparation.

MATERIALS AND METHODS

Animals. Kidneys from several species of rodents were obtained: rats (of the Hebrew University strain) weighing 180–250 g; guinea pigs, weighing 350–450 g; mice, weighing 25–30 g; rabbits, weighing 1.5–2.0 kg. In all these species kidneys from males only were used. In addition to rodents this study also includes human kidneys (obtained at surgery) and kidneys of cats.

The kidneys were removed from rats, mice and guinea-pigs under ether anesthesia. Rabbits were injected with pentobarbitone (intracardiac) and cats were anesthetized with i.p. pentobarbitone sodium. Human kidneys were obtained from partial nephrecties performed for recurrent nephrolithiasis or from cases of accidental sudden death (we would like to acknowledge the kind help of Prof. A. Durst of the Dept. of Surgery, Hadassah University Hospital, Jerusalem in providing the human material).

Preparation of enzyme. In all cases the effect of EA was studied on the activity of microsomal (Na + K)-ATPase and Mg-ATPase from kidney cortex and medulla, separately. The kidneys were rapidly removed and cooled in ice. After removal of the capsule, the kidneys were cut sagittally and were separated into two parts: cortex and medulla, as described previously [12]. Each portion was weighed and homogenized 10:1 (v/w) in a solution containing 0.25 M sucrose, 2 mM EDTA, buffered with Tris (5 mM) to pH 7.4–7.5. Homogenization was carried out in a glass homogenizer immersed in ice. The procedure of preparation was essentially adopted from Jørgensen and Skou [13] as previously described [12]. The

homogenates were centrifuged at 7000 *g* for 15 min; the supernatant was separated and the pellet was resuspended and rehomogenized (same medium and same volume as the first homogenization) and centrifuged again at 7000 *g* for 15 min. The combined supernatants were centrifuged at 48,000 *g* for 40 min. The pellet was resuspended and rehomogenized in the buffered sucrose medium (1:1) and then 9 volumes of a hypotonic buffer (containing 0.67 mg/ml sodium desoxycholate (DOC), 2 mM EDTA and 25 mM Tris, pH 7.0) were added. The samples were incubated for 30 min at room temperature and then centrifuged at 25,000 *g* for 30 min. The pellet was washed with 0.25 M sucrose to eliminate any DOC. The pellet was then resuspended (1:1) in a medium of the same composition as the initial homogenization solution and was used as the enzymatic preparation.

The sediment obtained at 48,000 *g* as well as the pellets obtained after treatment with DOC were subjected to electron-microscopy (we would like to thank Professor E. Rosenmann of the Department of Pathology, Hebrew University School of Medicine for performing the E.M. study). No mitochondria were found and the material consisted of vesicles surrounded by typical membrane. Enzymatic assay showed only rudimentary activity of succinic dehydrogenase (less than 0.1 μmol succinate/mg prot. × min.) (succinic dehydrogenase is characteristic for mitochondria).

In the case of kidney, the fraction obtained by this method was also enriched in alkaline phosphatase activity (assayed in the presence of 0.5 mM Mg^{2+}) from 9 in the homogenate to 52 μmol Pi/mg protein × hr. This enzyme is characteristic for plasma membrane. Similarly glucose 6-phosphatase activity, characteristic for endoplasmic reticulum, was also enriched (from 6.7 in the homogenate to 20 μmol Pi/mg protein × hr). Contribution of red blood cell membranes was assumed to be negligible since the kidneys were perfused with ice-cold saline before cutting and homogenization.

Protein determination was carried out according to Lowry *et al.* [14].

Assay of enzymatic activity. 0.05 ml of the enzyme preparation, which contained 0.05–0.10 mg of protein, was used per assay. The total volume of incubation was 1.5 ml. The medium had the following composition: 100 mM NaCl, 10 mM KCl, 4 mM $MgCl_2$, 33 mM Tris buffer, pH 7.4 and 4.0 mM ATP (disodium salt, buffered to pH 7.4). The reaction mixture, except substrate, was preincubated for 10 min at 37° in a thermostatic bath with constant shaking. The reaction was started by the addition of ATP and was stopped by the addition of 1 ml of 10% trichloroacetic acid (TCA). The samples were centrifuged for 6 min at 4000 rev./min. in order to cause precipitation of the protein. Inorganic phosphate was determined in the supernatant by the method of Fiske and Subbarow [15]. The (Na + K)-ATPase activity was defined as the difference between the inorganic phosphate liberated during incubation in the complete medium and incubation in a K^+-free medium. Preliminary experiments have shown that addition of 10^{-3} M ouabain to the complete medium had the same effect as omission of K^+. Therefore, K^+ free medium was used routinely for differentiation of Na,K-ATPase. Correction was made for the spontaneous, non-enzymatic, breakdown of ATP, measured as the inorganic phosphate liberated under the same experimental conditions in the absence of enzyme, and for the inorganic phosphate present in the tissue, measured as the inorganic phosphate in a reaction mixture to which TCA was added prior to addition of the enzyme.

Ethacrymic acid was added to the incubation medium at final concentrations of $10^{-5} \times 10^{-3}$ M. The enzyme was preincubated with the electrolytes and EA for 10 min at 37° and then ATP was added and the reaction started. All reagents were prepared in bidistilled water. Ouabain was purchased from Sigma and Ethacrynic acid was obtained as a gift from Assia Pharmaceuticals, Ramat Gan, Israel and from Merck, Sharp & Dohme, West Point, Pa.

Cysteine—adduct of EA. The cysteine-adduct of EA was prepared according to a personal communication by Dr. E. J. Cragoe, Jr. from Merck, Sharp & Dohme, West Point, Pa. Two solutions were prepared. The first, by adding 1 mmol (303 mg) of EA to a solution of 1 mmol (84 mg) Na-bicarbonate in 3 ml of distilled water. The second solution was prepared by the addition of 1.1 mmol (194 mg) of pure L-cysteine hydrochloride monohydrate to a solution of 2.2 mmol (185 mg) of Na-bicarbonate in 3 ml of distilled water. The two solutions were united, allowed to stand for one hr and then diluted with distilled water to a final volume of 10 ml. The concentration of the solution was 0.1 M of EA as the salt of its cysteine adduct. The solution was used immediately. The distilled water used for dissolving was boiled, to expel the O_2, and cooled under a stream of N_2, to prevent oxidation of the cysteine.

RESULTS

Effect of EA on microsomal ATPase in cortex and medulla of rat kidney

Figure 1 shows the effect of increasing concentrations of EA (10^{-5} M-5 × 10^{-3} M) on the activity of microsomal (Na + K)ATPase and Mg-ATPase of rat kidney cortex and medulla. In kidney cortex inhibition of Mg-ATPase activity by EA was already evident at the lowest concentration studied (10^{-5} M) and in the medulla significant inhibition was observed from 10^{-4} M EA. In contrast, microsomal (Na + K)-ATPase activity was not inhibited by EA even at 10^{-3} M but in fact it was somewhat enhanced at 10^{-5} and 10^{-4} M EA. At 10^{-3} M EA the activity of (Na + K)-ATPase was not significantly inhibited while the activity of Mg-ATPase was already inhibited by more than 50 per cent. At 5×10^{-3} M both enzyme activities were very markedly depressed but Mg-ATPase was inhibited more than (Na + K)-ATPase (88 vs 74 per cent in the cortex and 90 vs 78 per cent in the medulla).

Effect of EA on microsomal ATPase in kidney cortex and medulla of various species

To find out whether the effect of EA on microsomal (Na + K)-ATPase and Mg-ATPase was specific for the rat or represented a general phenomenon, we studied kidneys from various species. Figures 2 and 3 show that, in general, in all the species studied the

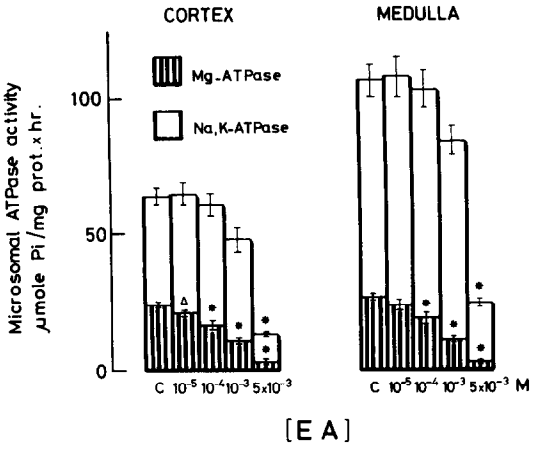

Fig. 1. Effect of ethacrynic acid on microsomal ATPase activity of rat kidney cortex and medulla. Each column is mean of five experiments. Vertical bars. S.E.

C—control—no ethacrynic acid added. 10^{-5} – 5×10^{-3} M: final concentration of ethacrynic acid in assay. Δ: $P < 0.05$ for difference between activity in presence of EA and control;*: $P < 0.005$ for difference between activity in presence of EA and activity in control (C). Note that for Na, K-ATPase activity (open columns) significant inhibition was observed only at the highest concentration of EA (5×10^{-3} M) in both cortex and medulla; at all EA concentrations below 5×10^{-3} M there was no significant inhibition. Mg-ATPase activity was significantly inhibited already at 10^{-5} M (cortex) and 10^{-4} M EA (medulla).

effect of EA was similar. Both in cortex and medulla of the kidney, inhibition of Mg-ATPase was found already at 10^{-5} M EA, while (Na + K)-ATPase was slightly enhanced at this concentration. A sizeable depression of (Na + K)-ATPase was evident only at $\geqslant 10^{-3}$ M EA. At 5×10^{-3} M EA both (Na + K)-ATPase and Mg-ATPase were markedly depressed but in most cases the inhibition was larger for Mg-ATPase than for (Na + K)-ATPase. There was no marked difference in the sensitivity to EA of the enzymes from kidney cortex and medulla of the same species but there were differences between species (Figs. 2 and 3). The enzyme obtained from cat kidney showed similar effects of EA. In human kidney, Mg-ATPase showed the highest sensitivity to EA. At 10^{-3} M EA human medullary Mg-ATPase was inhibited almost by 90 per cent while in the other species it was inhibited by 32 per cent–59 per cent. Human medullary (Na + K)-ATPase was inhibited at 10^{-3} M EA by 30 per cent while in the other species the inhibition was considerably smaller or absent altogether.

Effect of EA on microsomal ATPase of brain and submaxillary gland of the rat.

To study whether the effect of EA was tissue-specific, microsomal (Na + K)-ATPase and Mg-ATPase activity was studied in other tissues, too. Figure 4 shows the effect of EA on microsomal (Na + K)-ATPase of brain and submaxillary gland

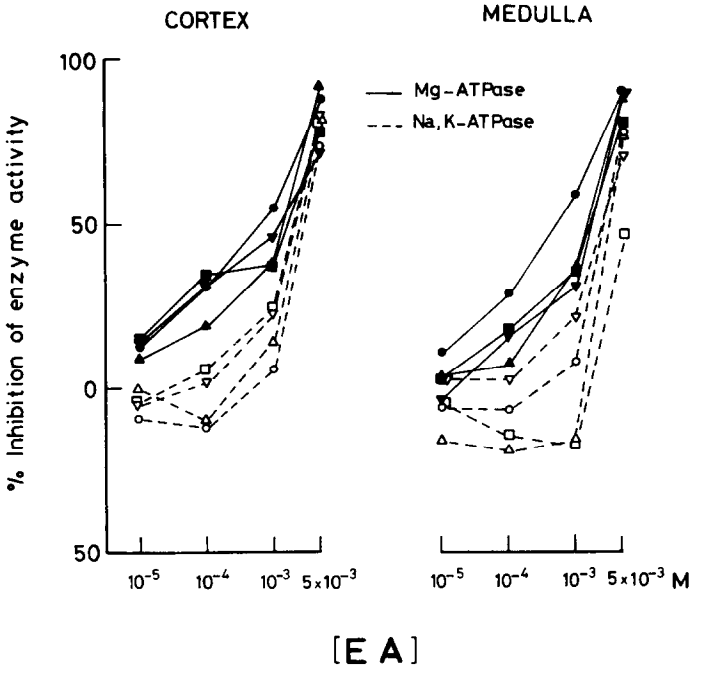

Fig. 2. Inhibition by EA of microsomal ATPase activity in kidney cortex and medulla of various rodents.

Ordinate: percent inhibition of ATPase activity, Continuous lines: Mg-ATPase; broken lines: Na, K-ATPase, Rat: ●, Mg-ATPase; ○, Na, K-ATPase. Rabbit: ▲, Mg-ATPase; △, Na, K-ATPase. Guinea pig: ▼, Mg-ATPase; ▽, Na, K-ATPase, Mouse: ■, Mg-ATPase; □, Na, K-ATPase.

Note that in each species at [EA] = 10^{-5}–10^{-3} M Mg-ATPase is inhibited more than Na, K-ATPase. In many cases Na, K-ATPase activity is *increased* at [EA] of 10^{-5}-10^{-4} M. Values above 0, inhibition; values below 0, increased enzyme activity.

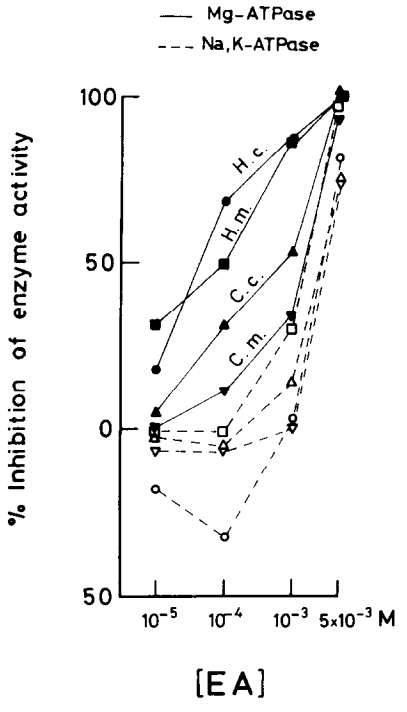

Fig. 3. Inhibition by EA of microsomal ATPase activity in human and cat kidney. Ordinate: percent inhibition of ATPase activity. Continuous lines: Mg-ATPase; broken lines: Na, K-ATPase. H.C.: Human kidney cortex; H.m.: human kidney medulla; C.C.: cat kidney cortex; c.m.: cat kidney medulla. Human kidney cortex: ●, Mg-ATPase; ○, Na,K-ATPase. Human kidney medulla: ■, Mg-ATPase; □, Na, K-ATPase. Cat kidney cortex: ▲, Mg-ATPase; △, Na,K-ATPase. Cat kidney medulla: ▼, Mg-ATPase; ▽, Na, K-ATPase. Values above 0, inhibition; values below 0, increased activity. Note that Mg-ATPase is more sensitive to inhibition than Na, K-ATPase at [EA] = 10^{-5} – 10^{-3} M. Some enhancement of Na,K-ATPase activity is seen at

$$[EA] = 10^{-5} - 10^{-4}\,M.$$

of the rat. Inhibition of brain microsomal Mg-ATPase started already at 10^{-5} M EA and reached 56 per cent inhibition at 10^{-3} M EA. (Na + K)-ATPase was not affected by EA even at 10^{-3} M EA. In the submaxillary gland, inhibition of Mg-ATPase was gradual, starting at 10^{-5} M EA and reaching 43 per cent inhibition at 10^{-3} M EA. Inhibition of submaxillary (Na + K)-ATPase started only at 10^{-3} M EA (23 per cent inhibition).

Action of some factors on the inhibition of microsomal ATPase by EA

Some other factors that could possibly affect inhibition of microsomal (Na + K)-ATPase and Mg-ATPase by EA were also studied.

Absence of K^+ *or presence of ouabain.* Since Na, K-ATPase activity was differentiated from Mg-ATPase activity routinely by omission of K^+ from the incubation medium, we checked the possible effect of absence of K^+ on the action of EA by adding ouabain instead of K^+-free medium. There was no difference in the effect of EA on Mg-ATPase, whether it was assayed in a K^+-free medium or in the complete medium with ouabain added.

Treatment with desoxycholate. (Na + K)-ATPase activity of a preparation that was not treated with DOC was low compared to that of DOC-treated preparation (6 as compared to 39, for cortex, and 8–10 compared to 80 μmol Pi/mg prot. × hr for medulla). However, Mg-ATPase activity was of comparable magnitude in DOC-treated and untreated preparation. The effect of EA was essentially the same as in preparations treated with DOC: EA at 10^{-3} M markedly inhibited Mg-ATPase, while (Na + K)-ATPase was not affected at all in both cortex and medulla.

Effect of EA-cysteine adduct. The effect of EA-cysteine adduct (10^{-3} M) on the activity of microsomal (Na + K)-ATPase was compared to the effect of 10^{-3} M EA alone. The adduct was less effective than EA alone but qualitatively had the same effect: inhibition of Mg-ATPase (by 19.4 per cent in cortex, and by 12.5 per cent in medulla) but no inhibition or even a slight enhancement of (Na + K)-ATPase (increase of 7 per cent in activity).

Replacement of Cl^- *by* SO_4^{2-}. The activity of (Na + K)-ATPase and of Mg-ATPase when all the Cl^- in the incubation medium was replaced by SO_4^{2-} was not significantly different from the activity in Cl^--medium.

The pattern of inhibition by EA under these conditions also remained the same: marked inhibition of Mg-ATPase (by 38 per cent) without any change in the activity of (Na + K)-ATPase.

Preincubation with EA. Since the inhibitory effect of SH-reagents depends on the duration of contact with the enzyme, we compared the effect of 10^{-3} M EA on the activity of microsomal (Na + K)-ATPase after 30 min preincubation with the enzyme, prior to addition of the substrate. Preincubation with 10^{-3} M EA increased the inhibition of Mg-ATPase (from 36.4 per cent to 49.1 per cent) whereas (Na + K)-ATPase was unaffected.

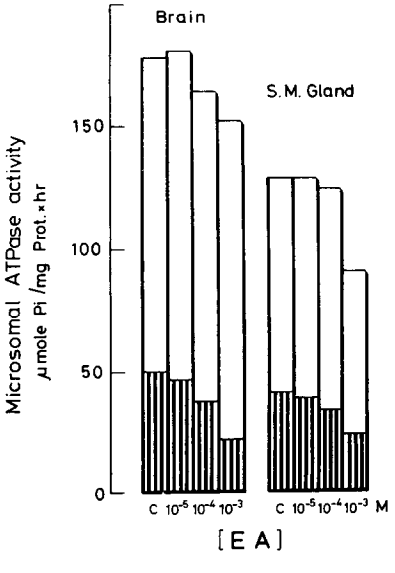

Fig. 4. Effect of EA on microsomal ATPase activity of rat brain and submaxillary gland. Hatched columns: Mg-ATPase; open columns: Na,K-ATPase. S.M.: submaxillary. Mg-ATPase was inhibited at EA = 10^{-5} – 10^{-3} M in both brain and submaxillary gland. Na,K-ATPase was inhibited only at [EA] = 10^{-3} M in submaxillary gland.

DISCUSSION

EA is a potent diuretic of established clinical value and has been shown to be an effective drug in several species [1]. However, the molecular mechanism for its action has not yet been elucidated [16]. As for its physiological mode of action there are at least two different suggestions: Burg and Green [7] suggest inhibition of a chloride pump in the thick ascending loop, affected specifically by the EA-cysteine adduct in the luminal surface of the nephron. Whittembury and Proverbio [6] suggest inhibition of a sodium-pump independent of the Na,K-ATPase mechanism. Since both of these theories require an energy-dependent transport mechanism, one would assume the involvement of an energy supplying mechanism. That this mechanism does not involve the Na,K-ATPase has been suggested by various indirect evidence [5, 6, 17]. The present report also shows that at EA concentrations up to 10^{-4} M there was even some enhancement of Na,K-ATPase activity rather than inhibition of this activity (Figs. 2 and 3). Such enhancement can also be seen in Fig. 1 of Inagaki *et al.* [17] at EA concentrations between 10^{-4} and 10^{-3} M. Na,K-ATPase was inhibited by EA only at rather high concentrations, above 10^{-3} M, in the present study, corroborating a similar finding of Charnock *et al.* [4]. It seems doubtful whether such concentrations are commensurate with those achieved *in vivo* under effective diuretic doses. However, one should keep in mind the possibility that in some particular part of the nephron rather high concentrations of EA could be achieved, i.e. in the proximal tubule. We have recently reported that EA can accumulate in kidney cortex slices more than 10-fold compared to the medium [18]. This could lead to high intra-cellular EA concentration and could affect the function of these cells in a non-specific way, acting as an inhibitor of mitochondrial and/or cytosolic enzymes involved in energy supply [8, 19, 20]. Such a mechanism may be the cause for the drastic effect of EA on Na-extrusion from cortical slices compared to a small effect on medullary slices as reported in our recent study [9], since medullary slices could not concentrate EA. The negligible effect of EA on medullary slices was also reported by Law [21].

The present study shows that in a series of various species, rodents and non-rodents, EA inhibited Mg-ATPase considerably more effectively than Na,K-ATPase. The Mg-ATPase studied in our experiments was a membranal enzyme, being part of a "microsomal" fraction. Considerable inhibition was observed at 10^{-4} M and some inhibition of Mg-ATPase could already be seen at 10^{-5} M. This inhibition was not augmented by conjugating EA to cysteine, in contrast to the increased effectiveness of the adduct reported in the study on isolated thick ascending limb of rabbit, when the drug was applied intra-luminally [7]. Furthermore, replacement of Cl^- by sulfate did not affect the inhibition of Mg-ATPase by EA, again distinguishing this phenomenon from the "chloride pump", where replacement of Cl^- abolished the electrical potential across the thick ascending limb [7].

Inhibition of membrane Mg-ATPase by EA could be either a non-specific phenomenon, similar to inhibition of various intra-cellular enzymes, or could lead to identification of a specific energy-coupling mechanism for transport. The concentration of EA effective for inhibition of various enzymes involved in glycolysis [8] was similar or higher than that reported here for inhibition of Mg-ATPase. The involvement of a Mg-ATPase in any specific cell membrane transport mechanism has not yet been demonstrated. However, uptake of catecholamines into chromaffin granules in adrenal medulla involves a Mg-ATPase which is incorporated in the granule membrane [22]. If Mg-ATPase is linked to transport across cell membrane, then one would expect EA to affect transport in many tissues, because it was shown in the present study to inhibit Mg-ATPase in various tissues. Inhibition of ion transport by EA has, in fact, been demonstrated in erythrocytes [23], in muscle [24] and in intestine [25]. However, no indication has been given to a correlation of these ion transport phenomena to Mg-ATPase. Further investigation has, therefore, to be carried out to clarify whether the inhibition of Mg-ATPase by EA represents just an additional enzyme sensitive to this drug or whether this is a common denominator for the various effects of this drug.

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