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SHORT COMMUNICATIONS

Effects of several anions on ethacrynic acid high- and low-sensitive Mg-ATPase activities in microsomal fractions from rabbit cortical gray matter

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Ethacrynic acid (EA), a potent diuretic, is reported to be active in various biological systems [1]. Inhibition of active chloride transport by EA was clearly demonstrated in the diluting segments of rabbit renal tubules [2] and frog cornea [3]. Specific chloride-stimulated ATPase has not been demonstrated yet. EA, however, in concentration ranges similar to those required for inhibition of chloride transport (0.1 to 0.3 mM), was found recently to reduce microsomal Mg-ATPase activities in kidneys of different species, rat brain, and rat submaxillary gland [4]. We examined the effects of several anions on the EA-sensitive microsomal Mg-ATPase activity, with interest directed to interaction of anions and microsomal Mg-ATPase.

Adult white rabbits of both sexes were anesthetized with pentobarbital sodium (30 mg/kg, i.v.). After cold physiological saline perfusion from the heart, the brain was removed and placed on ice, and the cerebral cortical gray matter was separated with scissors. The tissue were homogenized in 8 vol. (v/w) of ice-cold buffer solution, containing 0.25 M sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA) and 25 mM Tris–acetate (pH 7.4), with a teflon–glass homogenizer. Centrifugal fractionation was done according to the methods of Skou [5] and Whittaker *et al.* [6]. All procedures were performed at 0–4°. The homogenates were centrifuged twice at 1,000 *g* for 15 min and the supernatant fractions were centrifuged twice at 10,000 *g* for 15 min. The resulting pellets were suspended in 1.2 M sucrose solution, containing 1 mM EDTA and 25 mM Tris–acetate (pH 7.4), and were centrifuged at 92,000 *g* for 60 min. The pellets were suspended in homogenization buffer and used as the mitochondrial fractions. The supernatant fractions of the 10,000 *g* centrifugation were centrifuged at 92,000 *g* for 60 min. The resulting pellets were suspended in homogenization buffer and used as the microsomal fractions. The mitochondrial and microsomal fractions thus obtained were stored as enzyme sources at −70°.

ATPase activity was measured as the rate of release of inorganic phosphate. In general, the reaction was carried out in 0.5 ml medium containing 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. Na,K-ATPase activity denoted the difference between paired tubes containing 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. 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 min and was stopped by the addition of 0.5 ml of ice-cold 10% trichloroacetic acid. The test tubes were cooled once in ice, and the mixtures were centrifuged at 3000 *g* for 5 min; a 0.3-ml sample of each supernatant fraction was used for the determination of inorganic phosphate liberated. The amount of inorganic phosphate was measured by the method of Chen *et al.* [7].

Monoamine oxidase activity was estimated by the method of Weissbach *et al.* [8] using kynurenamine as substrate measuring the rate of initial decrease in 360 nm absorbance.

Protein concentration was estimated by the method of Lowry *et al.* [9], using bovine serum albumin as standard. EA (free acid), provided by Merck, Sharpe & Dohme (West Point, PA), was alkalized to pH 7.4 with Tris for dissolution.

Other details of experimental procedures were as described elsewhere [10, 11].

Effects of EA on the activities of microsomal Mg-ATPase, microsomal Na,K-ATPase and mitochondrial Mg-ATPase are shown in Fig. 1. The activities of marker enzymes, monoamine oxidase and Na,K-ATPasem were 0.192 and 0 (not detectable) μmoles·(mg protein)$^{-1}$·hr^{-1}, respectively, in the mitochondrial fraction and 0.014 and 14.2 μmoles·(mg protein)$^{-1}$·hr^{-1}, respectively, in the microsomal fraction used. Na,K-ATPase activity was not inhibited by 0.03 to 1 mM EA, and mitochondrial Mg-ATPase activity was stimulated slightly. Both of the activities were inhibited in EA concentrations ranging from 3 to 50 mM, an apparent K_i being 4 mM. On the other hand, Mg-ATPase activity in the microsomal fraction was inhibited by EA at concentrations over 0.003 mM and showed a plateau between 0.05 and 1 mM; inhibition proceeded with an increase in the concentration of EA. Eadie–Scatchard plots (Fig. 1, inset) of the data for the apparent microsomal Mg-ATPase activity could be resolved into two components with clearly different slopes. Apparent K_i values of the two components were 0.016 and 5 mM.

Effects of several anions on Mg-ATPase activities in microsomal fractions are shown in Table 1. Mg-ATPase activity in the absence or presence of 0.3 mM EA was described as total or EA low-sensitive activity respectively. The difference between total Mg-ATPase activity and EA low-sensitive activity was defined as EA high-sensitive

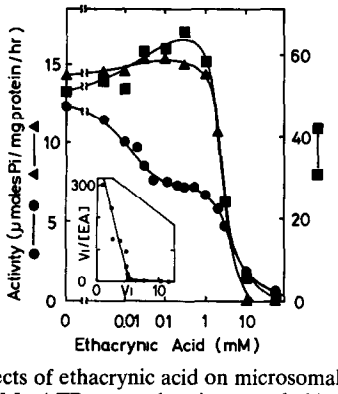

Fig. 1. Effects of ethacrynic acid on microsomal and mitochondrial Mg-ATPase and microsomal Na,K-ATPase activities in rabbit cortical gray matter. Mg-ATPase and Na,K-ATPase activities were measured as described in the text. Key: (●——●) microsomal Mg-ATPase activity, (■——■) mitochondrial Mg-ATPase activity, and (▲——▲) microsomal Na,K-ATPase activity. Inset: Eadie–Scatchard plots of inhibition of microsomal. Mg-ATPase activity by ethacrynic acid. Vi: ethacrynic acid-induced decrease in Mg-ATPase activity, i.e. (total Mg-ATPase activity in the absence of ethacrynic acid) − (Mg-ATPase activity at a definite ethacrynic acid concentration).

Table 1. Effects of anions on total, ethacrynic acid (EA) high-sensitive or low-sensitive Mg-ATPase activity in rabbit cortical gray matter*

Salts added (m-equiv./l)	Mg-ATPase activity [μmoles $P_i \cdot (mg\ protein)^{-1} \cdot hr^{-1}$]		
	Total	EA high-sensitive	EA low-sensitive
NaCl	11.3 ± 0.8 (100)	5.6 ± 0.3 (100)	5.6 ± 0.6 (100)
$NaNO_3$	6.5 ± 0.1† (58)	1.7 ± 0.5† (30)	4.9 ± 0.6 (92)
NaSCN	6.8 ± 0.1† (60)	3.1 ± 0.7† (55)	3.7 ± 0.7‡ (62)
NaF	7.8 ± 0.5† (69)	5.0 ± 0.8 (89)	2.7 ± 0.6† (49)
Na_2SO_4	9.4 ± 0.7‡ (84)	4.0 ± 0.1† (71)	5.4 ± 0.7 (96)
$Na(CH_3COO)$	10.2 ± 0.1 (90)	4.7 ± 0.4‡ (81)	5.4 ± 0.5 (96)
NaI	10.3 ± 0.9 (92)	4.6 ± 0.5‡ (81)	5.8 ± 0.6 (102)
NaBr	10.8 ± 0.6 (96)	5.2 ± 0.5 (93)	5.7 ± 0.6 (100)

* The medium for ATPase assay contained 50 mM Tris–acetate (pH 7.4), 1 mM EDTA, 3 mM magnesium acetate, 3 mM ATP–Tris, a sample (25–100 μg protein) of microsomal fractions of cortical gray matter and 50 m-equiv./l of various anions, as sodium salts. Total activity: Mg-ATPase activity measured in the absence of ethacrynic acid. EA high-sensitive activity: Mg-ATPase activity reduced by 0.3 mM ethacrynic acid. EA low-sensitive activity: Mg-ATPase activity remaining in the presence of 0.3 mM ethacrynic acid.

Values of activities are means ± S.D. of three determinations. Student's *t*-test was used to determine the statistical significance of the difference between the control activity with NaCl and the activity with replaced anions. Percent activity is given in parentheses taking the value with NaCl as 100%.

† $P < 0.01$.

‡ $P < 0.05$.

Mg-ATPase activity. In comparison with the activity with chloride salt, the total activity was reduced markedly when Cl^- was replaced with NO_3^-, SCN^-, F^- or SO_4^{2-}. From the viewpoint of sensitivity to EA, the reduction of Mg-ATPase activity differed. Substitution of NO_3^- or SO_4^{2-} for Cl^- significantly reduced EA high-sensitive activity and had lesser effects on EA low-sensitive activity. On the other hand, substitution of F^- for Cl^- reduced the latter, but had little effect on the former. In contrast, SCN^- inhibited both EA high- and low-sensitive activities. I^- and CH_3COO^- were shown slightly to reduce EA high-sensitive activity with no significant change in EA low-sensitive activity. Potassium salts of the anions described above yielded essentially the same results (data not shown). Figure 2A–C shows inhibition curves of sulfhydryl reagents for both EA high- and low-sensitive Mg-ATPase activities. The EA high-sensitive Mg-ATPase activity was rather selectively inhibited by *p*-chloromercuribenzoic acid (PCMB), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and *N*-ethylmaleimide (NEM). Loop diuretics such as furosemide and bumetanide inhibited both of the activities, in the concentration ranges over 3 mM (Fig. 2D and E).

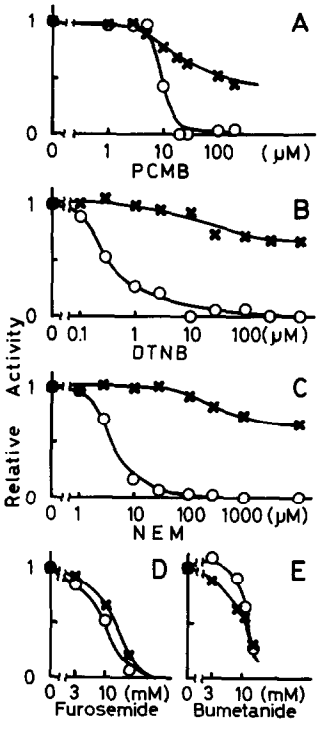

Fig. 2. Effects of sulfhydryl reagents and loop diuretics on ethacrynic acid high- and low-sensitive Mg-ATPase activities. Key: ○——○ or ×——× represents ethacrynic acid high- or low-sensitive Mg-ATPase activity respectively. (A) *p*-chloromercuribenzoic acid (PCMB); (B) 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB); (C) *N*-ethylmaleimide (NEM); (D) furosemide; and (E) bumetanide.

Our results indicate that the microsomal fractions of rabbit cortical gray matter possess both EA high- and low-sensitive Mg-ATPase activities. The former was reduced significantly by several anions in a series of potencies ($NO_3^- > SCN^- > SO_4^{2-} > I^-, CH_3COO^-$), and also by sulfhydryl reagents. The EA low-sensitive activity was reduced by F^- and SCN^-. The EA high-sensitive Mg-ATPase activity seemed to be inhibited by EA reacting with thiols.

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Effect of chirality in *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA) on adenosine deaminase inhibition

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Inhibitors of adenosine deaminase (ADA*; adenosine aminohydrolase, EC 3.5.4.4) act as immunosuppressive agents and potentiate the cytotoxic effects of a number of adenosine analogs. Two classes of adenosine deaminase inhibitors have attracted the greatest attention: the antibiotics coformycin and deoxycoformycin which have been proposed to act as transition state analogs [1] and 9-(hydroxyalkyl)adenines, such as *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA), which presumably bind to a hydrophobic region adjacent to the catalytic site [2]. The development of EHNA by Schaeffer *et al.* [3, 4] offers a classic example of the rational design of enzyme inhibitors.

Coformycin and deoxycoformycin are tight binding inhibitors of ADA with K_i values in the range of $2.5–15 \times 10^{-12}$ M, whereas EHNA is categorized as a semi-tight binding inhibitor with K_i values on the order of $2–4 \times 10^{-9}$ M [5, 6]. With the former inhibitors, spontaneous reactivation of the enzyme is exceedingly slow, i.e. $T_{1/2}$ = 8–29 hr, and in intact cells, such as erythrocytes, reactivation of inhibited ADA has not been demonstrated. With EHNA, however, the inhibition of ADA is more readily reversible, i.e. $T_{1/2} < 5$ min. In addition, EHNA has biochemical effects that are not readily explained solely on the basis of ADA inhibition [7]. Commercially available EHNA is, in fact, a racemic mixture of *erythro*-(+)-9-(2S-hydroxy-3R-nonyl)adenine and *erythro*-(−)-9-(2R-hydroxy-3S-nonyl)adenine and will be designated here as (±)-EHNA. Questions have arisen as to which chiral isomer is responsible for the inhibition of ADA and the additional biochemical effects on cellular metabolism. Therefore, chiral syntheses of these two *erythro* as well as the two *threo* isomers (THNA) have been developed, the testing of which should provide answers to these questions. This report describes the effects of the four isomers on human erythrocytic and calf intestinal ADA.

Materials and methods

Synthesis of chiral isomers of EHNA and THNA. The synthetic methods and characterizations described briefly here will be detailed elsewhere.† Starting with the sugar, L-rhamnose, the chiral *erythro* and *threo* isomers of 9-(2-hydroxy-3-nonyl)adenine have been prepared. A common known intermediate (I) was used to synthesize the chiral amines (IV, V, VI, and VII) [8]. As shown in Fig. 1, the 2,3-dideoxyrhamnose derivative (I) was converted via two isomeric tosylates at C-4 by treatment with lithium azide to compounds II and III. These were reduced catalytically to their respective amines and were then acetylated with acetic anhydride to the corresponding acetamide derivatives. Acid hydrolysis of the acetal linkage followed by chain extension with the proper Wittig reagent gave mixtures of olefins from which the saturated "tails" (C_6H_{13}) were secured by hydrogenation. Hydrolysis of the acetamide group with either hydrazine or 1 N hydrochloric acid furnished amines IV and V. The remaining amines VI and VII were obtained by inversion of the stereochemistry at C-2 when the acetamides were first treated with thionyl chloride followed by acid hydrolysis according to known procedures [9, 10]. Incorporation of the chiral amines into the adenine moiety at N-9 followed established routes [11]. All new compounds described here have been fully characterized (^{1}H NMR, ^{13}C NMR, mass spec., u.v., and $[\alpha]_D$).

Enzymatic procedures. The partial purification of human erythrocytic ADA and the spectrophotometric assay of adenosine deamination have been reported elsewhere [12, 13]. Calf intestinal ADA (sp. act. *ca.* 200 units/mg protein) was purchased from Boehringer Mannheim, Indianapolis, IN, and the Sigma Chemical Co., St. Louis, MO (Type III). The inhibition constants were determined from replots of the slopes of a double-reciprocal plot at five fixed inhibitor concentrations. A weighted linear regression analysis program was adapted from Cleland [14] to a Wang computer and extended by Dr. Sungman Cha to compute both K_m and K_i values from plots of $1/v$ vs $1/S$ at multiple inhibitor concentrations. Samples of (±)-EHNA were obtained from the Drug Development Branch of the

* Abbreviations: ADA, adenosine deaminase; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine; (±)-EHNA, racemic mixture of EHNA; THNA, *threo*-9-(2-hydroxy-3-nonyl)adenine; (+)-(2S,3R)EHNA, *erythro*-(+)-9-(2S-hydroxy-3R-nonyl)adenine; (−)-(2R,3S)EHNA, *erythro*-(−)-(2R-hydroxy-3S-nonyl)adenine; (+)-(2R,3R)THNA, *threo*-(+)-(2R-hydroxy-3R-nonyl)adenine; and (−)-(2S,3S)THNA, *threo*-(−)-(2S-hydroxy-3S-nonyl)adenine.

† G. Bastian, M. Bessodes, R. P. Panzica, E. Abushanab, S-F. Chen, J. D. Stoeckler and R. E. Parks, Jr. *J. med. Chem.*, in press.