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INHIBITION OF RENAL Na^+,K^+-ACTIVATED ADENOSINE TRIPHOSPHATASE ACTIVITY BY ETHACRYNIC ACID*

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Abstract—The inhibition by ethacrynic acid (EA) of Na^+,K^+-activated, Mg^{2+}-dependent adenosine triphosphatase (ATPase) activity in a heavy microsomal preparation from guinea pig kidney was studied. The mean control activity of the preparation was $11{\cdot}9 \pm 0{\cdot}5$ μmoles P_i/mg protein/15 min at 37° in the presence of Na^+, K^+ and Mg^{2+}, and $4{\cdot}1 \pm 0{\cdot}1$ in the presence of Mg^{2+} alone. The effect of EA, unlike the selective action of ouabain, was to decrease nonselectively both total NaKMg-ATPase and Mg-ATPase activity. Inhibition by EA was nearly two orders of magnitude less than that obtained by ouabain and *p*-hydroxymercuribenzoate (POMB). Cysteine added to the incubation medium partially blocked ATPase inhibition by either EA or the known sulfhydryl inhibitor, POMB. The extent of the block by cysteine increased as the cysteine:drug molar ratio increased. There was an apparent relationship between sulfhydryl reactivity assayed by Ellman's reagent (dithio-bis-nitrobenzoate) and ATPase inhibition for ethacrynic acid and its cyclic analogues. These data support the interaction between EA and enzyme sulfhydryl groups originally suggested by others. Preincubation of EA and the enzyme prior to dilution for the usual incubation produced an inhibition corresponding to the high drug level of the preincubation period rather than the final diluted concentration. This lack of dissociation of the EA–enzyme complex upon dilution, in contrast to the dissociation observed in similar experiments with POMB, a known reversible inhibitor, suggests an irreversible interaction between EA and ATPase *in vitro*.

THERE has been growing evidence in the past decade supporting the view that the membrane enzyme, Na^+,K^+-activated-adenosine triphosphatase (ATPase), is closely associated with the active transport of sodium ions across biological membranes.[1] The participation of this enzyme system in the renal tubular reabsorption of sodium has been recently reviewed.[2] Inhibition of renal NaK-ATPase has been suggested as a possible basis for the diuretic effect of ethacrynic acid.[3] Limitations on the concept of this enzyme as the diuretic receptor for ethacrynic acid have been stated.[4, 5] This paper presents further studies on the nature of the inhibition of NaK-ATPase by ethacrynic acid *in vitro*.

METHODS

Kidneys from male albino guinea pigs (350–400 g) were removed immediately after sacrifice by cervical dislocation and minced in 8 vol. of ice-cold 0·25 M sucrose

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containing 5 mM EDTA, 0·1% deoxycholic acid and 30 mM Tris at pH 7·5. Homogenization was carried out in a glass homogenizer immersed in ice with ten strokes of a Teflon pestle at 400 rpm and 0·18 mm clearance. All subsequent operations were performed at 0–4°. The homogenate was centrifuged at 12,000 *g* for 30 min in a refrigerated Spinco preparative ultracentrifuge to sediment cell debris, nuclei and mitochondria. The supernatant was carefully removed and centrifuged at 35,000 *g* for 30 min. The resultant pellet, a heavy microsomal fraction, was gently resuspended by hand in the original homogenizing medium and recentrifuged at the same speed. The final pellet was resuspended in the homogenizing medium and stored at 0° for 2–3 days prior to use.

NaKMg-ATPase activity was determined by using a mixture containing- Tris-ATP (Sigma), 3 mM; $MgCl_2$, 3 mM; NaCl 100 mM; KCl, 15 mM; Tris-HCl, 30 mM at pH 7·5; and 0·2 ml of the heavy microsomal suspension in a total volume of 2·0 ml with incubation at 37° for 15 min. Mg-ATPase activity was assayed in the presence of 115 mM choline in place of the omitted Na and K. NaK-ATPase activity was calculated as the difference between NaKMg-ATPase and Mg-ATPase values. Under these conditions, ATP hydrolysis was a linear function of time and enzyme concentration. In all experiments, drugs to be tested were included in the Mg as well as in the NaKMg tubes. The incubation was terminated by the addition of 1·0 ml of 1·5 N perchloric acid. After removal of the precipitated protein by centrifugation, an aliquot of the incubation medium was assayed for inorganic phosphate by the method of Fiske and SubbaRow[6] with correction by means of appropriate blanks for nonenzymatic hydrolysis. The protein content of the preparation was determined by a modified biuret technique.[7]

The free sulfhydryl content of cysteine solutions in the presence of *p*-hydroxymercuribenzoate (POMB), ethacrynic acid and the cyclic analogues of ethacrynic acid was determined by the method of Ellman.[8] A 0·1-ml portion of the respective drug solutions (0·01 M) was added to 0·1 ml of 0·002 M cysteine and 2·7 ml of 0·1 M K_2HPO_4. After 5 min, 0·1 ml of Ellman's reagent (dithio-bis-nitrobenzoate; DTNB)[8] and 7·0 ml water were added. The absorbance of the samples at 412 mμ as an index of free cysteine sulfhydryl content was determined 5 min after DTNB addition with correction for cysteine-free blanks.

Ouabain, *p*-hydroxymercuribenzoate and 5,5′-dithio-bis-(2-nitrobenzoic acid) were purchased from Sigma Chemical Co., St. Louis, Mo. Ethacrynic acid and the cyclic analogues of ethacrynic acid were kindly supplied by Dr. John E. Baer of Merck and Dr. John G. Topliss of Schering respectively.

Statistical comparisons were made by Student's *t*-test for paired data as described by Snedecor.[9]

RESULTS

Control ATPase activity in the presence of Na, K and Mg was 11·9 $\pm$ 0·5 μmoles P_i/mg protein/15 min at 37° as compared with 4·1 $\pm$ 0·1 obtained in the presence of Mg alone, i.e. a 3-fold increase above Mg-dependent ATPase activity as a result of NaK activation. Relative inhibitions of the components of ATPase activity by ethacrynic acid as compared with inhibitions by two other known inhibitors of NaK-ATPase, ouabain and POMB, are shown in Fig. 1. Each value listed is the mean of five independent experiments. Each individual experiment within such a series was

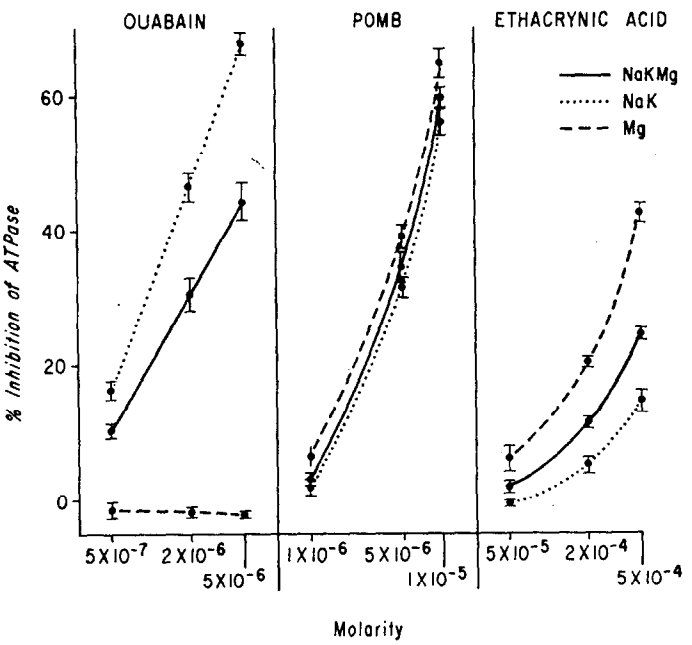

FIG. 1. Inhibition of ATPase activity of heavy microsomal fraction of guinea pig kidney by ouabain, POMB and ethacrynic acid. ATPase activity was assayed by the liberation of P_i from the substrate ATP in 15 min at 37°. NaK-ATPase activity was calculated as the difference between activities in the presence of 100 mM Na, 15 mM K and 3 mM Mg (NaKMg-ATPase) or 115 mM choline and 3 mM Mg (Mg-ATPase). Values are means (±S.E., expressed as vertical bars) of four or five experiments.

performed on a different day with microsomal suspensions from different animals and independently prepared drug solutions. The per cent inhibition values in a single experiment were calculated on the basis of daily control activity. Ouabain was noted to inhibit specifically the NaK-activated component of ATPase activity with no effect on the basal Mg-dependent activity. A different pattern was seen for POMB, with depression of both the NaK-activated component and the Mg-dependent ATPase. Ethacrynic acid was even less selective than POMB as a specific inhibitor of the NaK-activated portion of ATPase activity, as shown by the relatively greater inhibition of Mg-ATPase at all concentrations of ethacrynic acid studied. Depression of Mg-dependent ATPase activity by ethacrynic acid has been previously noted by Duggan and Noll.[3] Ethacrynic acid was shown to be a relatively weak ATPase inhibitor with concentrations as high as 5×10^{-4} M exerting less influence on the enzyme activity than did ouabain or POMB at concentrations two orders of magnitude smaller, i.e. 5×10^{-6} M.

The presence of cysteine in the incubation mixture was shown to block the inhibition of ATPase activity by ethacrynic acid or the known sulfhydryl reagent, POMB. Values for enzyme inhibition by ethacrynic acid or POMB in the absence of cysteine are presented in Table 1. When either of these drugs was premixed with cysteine before addition of the enzyme, partial blockade of the inhibition resulted. The extent of the block by cysteine increased as the cysteine:inhibitor molar ratio increased, i.e. a partial

block at a ratio of 0·5:1, with a more complete block at a ratio of 5:1. These data are consistent with the view that ethacrynic acid may inhibit NaK-ATPase by virtue of interaction with enzyme sulfhydryl groups.

Inhibition of ATPase activity by the cyclic analogues of ethacrynic acid is shown in Table 2. Analogue I (5-carboxy-methoxy-2-methylindone)at 5×10^{-4} M produced inhibition of the components of ATPase activity similar in magnitude to those values

TABLE 1. INHIBITION OF RENAL ATPASE ACTIVITY BY POMB AND ETHACRYNIC ACID IN THE PRESENCE OF CYSTEINE

Drug	Cysteine concn (M)	% Inhibition of ATPase*		
		NaKMg	NaK	Mg
POMB	0	60·0 ± 1·5	56·0 ± 2·1	65·0 ± 2·1
(1×10^{-5} M)	5×10^{-6}	30·0 ± 3·4†	13·0 ± 4·7†	59·0 ± 2·7
(n = 4)	5×10^{-5}	6·4 ± 0·5†	4·4 ± 2·6†	9·8 ± 3·6†
EA	0	24·0 ± 0·9	16·0 ± 1·4	38·0 ± 2·2
(5×10^{-4} M)	$2·5 \times 10^{-4}$	20·0 ± 0·4†	14·0 ± 1·0	31·0 ± 2·9
(n = 6)	$2·5 \times 10^{-3}$	10·0 ± 0·5†	12·0 ± 1·2	5·3 ± 1·3†

* Incubation conditions as described under Fig. 1. Values are means ± S.E. POMB = *p*-hydroxy mercuribenzoate; EA = ethacrynic acid.
† Differed from value in absence of cysteine at $P < 0·05$.

TABLE 2. INHIBITION OF RENAL ATPASE ACTIVITY AND SULFHYDRYL REACTIVITY WITH ETHACRYNIC ACID AND ITS CYCLIC ANALOGUES*

Drug	n	% Inhibition of ATPase			% Cysteine bound†	Reported saluretic activity[10]
		NaKMg	NaK	Mg		
EA	5	24·0 ± 0·9‡	14·0 ± 1·7‡	43·0 ± 1·4‡	78·0 ± 1·3	high
Analogue I§	3	18·0 ± 1·9‡	2·2 ± 1·2	58·0 ± 1·1‡	99·0 ± 0·7	slight
Analogue II‖	3	2·1 ± 1·0	1·4 ± 0·9	3·8 ± 1·5	20·0 ± 0·9	slight

* ATPase assay conditions as described under Fig. 1. Drug concentrations were 5×10^{-4} M in the ATPase assay and 1×10^{-4} M in the sulfhydryl assay. Values are means ± S.E.
† Calculated as total cysteine (2×10^{-5} M) minus free sulfhydryl, assayed by the method of Ellman.[8]
‡ Differed from control value at $P < 0·5$.
§ 5-Carboxymethoxy-2-methylindone.
‖ 2-Carboxymethoxy-8,9-dihydro-6-methyl-5H-benzocyclohepten-5-one.

obtained with ethacrynic acid. Analogue II (2-carboxymethoxy-8,9-dihydro-6-methyl-5H-benzocycycloheptен-5-one) did not inhibit ATPase activity at concentrations as high as 5×10^{-4} M. Also shown in Table 2 are the results of the DTNB assay for thiol content of cysteine solutions in the presence of ethacrynic acid and its cyclic analogues. Analogue I, an inhibitor of ATPase, had considerable affinity for the sulfhydryl group of cysteine, as did ethacrynic acid. Analogue II, which does not inhibit ATPase, exhibited only slight sulfhydryl affinity by binding only 20% of total cysteine under the same conditions. These data further support the suggestion that a sulfhydryl interaction is involved in the inhibition of renal ATPase *in vitro*.

In order to examine the possibility that the interaction between ethacrynic acid and

NaK-activated ATPase might be irreversible, the following preincubation–dilution experiments were performed. A 0·2-ml portion of the heavy microsomal suspension and 0·1 ml of an ethacrynic acid or POMB solution were placed in the incubation tube at 37° for 15 min. The POMB and ethacrynic acid concentrations in the 0·3-ml preincubation volume were $1·3 \times 10^{-5}$ M and $6·7 \times 10^{-4}$ M respectively. At the end of the preincubation period, the enzyme reaction was initiated by the addition of 1·7 ml, containing the substrate ATP, appropriate cations and buffer. Upon this dilution to the 2·0-ml final incubation volume, the POMB and ethacrynic acid concentrations were 2×10^{-6} M and 1×10^{-4} M respectively. Values for the inhibition of NaKMg-ATPase by POMB and ethacrynic acid, calculated relative to control ATPase activity in the absence of inhibitors in the preincubation–dilution procedure, are shown in Table 3. The observed value of 8·0 ± 0·7 per cent inhibition

TABLE 3. PREINCUBATION OF POMB OR ETHACRYNIC ACID AND RENAL ATPASE WITH SUBSEQUENT DILUTION PRIOR TO ENZYME ASSAY*

Drug		Concn (M)	% Inhibition of NaKMg-ATPase	
			Expected	Observed
POMB	During preincubation	$1·3 \times 10^{-5}$	(>70)	8·0 ± 0·7
	Upon final dilution	2×10^{-6}	(10)	
EA	During preincubation	$6·7 \times 10^{-4}$	(25)	25·0 ± 1·5
	Upon final dilution	1×10^{-4}	(5)	

* The preincubation–dilution procedure for the ATPase assay is described in the text. Control experiments showed no significant increase in enzyme inhibition as a function of preincubation duration during the 15-min interval. Values are means ± S.E. of four experiments. Figures in parentheses indicate expected values at the stated concentrations on the basis of data in Fig. 1. For abbreviations, see Table 1.

by POMB closely approximated the inhibition expected at the final incubation concentration of 2×10^{-6} M, and was considerably less than the inhibition expected at the preincubation concentration of $1·3 \times 10^{-5}$ M. POMB, known to be a reversible inhibitor of NaK-ATPase,[1] apparently dissociated from the enzyme upon dilution. The preincubation–dilution procedure with ethacrynic acid, however, provided a different result. The 25·0 ± 1·5 per cent inhibition by ethacrynic acid closely approximated the inhibition expected at the preincubation concentration of $6·7 \times 10^{-4}$ M and was considerably greater than the inhibition expected at the final incubation concentration of 1×10^{-4} M. It appears that the ethacrynic acid–enzyme complex, which formed at the relatively higher preincubation concentration, failed to dissociate upon dilution to the 2·0-ml final incubation volume. Preincubation time as a possible factor in the observed ethacrynic acid–enzyme interaction was eliminated when control

experiments showed no significant increase in inhibition as a function of preincubation duration in the 15-min interval. These preincubation–dilution data therefore indicate a degree of irreversibility in the interaction between ethacrynic acid and NaK-ATPase.

DISCUSSION

NaK-ATPase in several tissues may contain sulfhydryl groups that are essential for enzyme activity.[11-13] Sulfhydryl reagents such as POMB, *N*-ethylmaleimide and 2,4-dinitrofluorobenzene are known inhibitors of NaK-ATPase.[1] The inhibition of NaK-ATPase by organomercurials such as POMB can be reversed by the presence of cysteine.[12] The data on cysteine blockade of ethacrynic acid inhibition of NaK-ATPase in this report support the suggestion by Duggan and Noll[3] that ethacrynic acid may inhibit NaK-ATPase by the same mechanism as the organomercurials, i.e. presumably by interaction with enzyme sulfhydryl groups. An important weakness in this argument, however, is that cysteine could simply be decreasing the amount of free ethacrynic acid available for the interaction between ethacrynic acid and enzyme, which may or may not include a sulfhydryl mechanism. The present results on sulfhydryl reactivity for ethacrynic acid and its cyclic analogues in the DTNB assay in comparison with the ATPase inhibition data provide additional circumstantial evidence for involvement of sulfhydryl groups in the mechanism of enzyme inhibition by ethacrynic acid.

The observed irreversibility of ethacrynic acid interaction with NaKMg-ATPase *in vitro* is difficult to relate to characteristics of ethacrynic acid action *in vivo*. Irreversibility of action would seem inconsistent with the relatively brief duration of diuresis following ethacrynic acid administration *in vivo*. One possible correlate on this irreversibility *in vivo*, however, involves the relative reversibility of the actions of mercurials and ethacrynic acid upon administration of sulfhydryl compounds. Mercurials produce diuresis reversible by thiol compounds, whereas thiol reversal of an ethacrynic acid diuresis is difficult.[14] Although the difference in cysteine effectiveness against mercurials and ethacrynic acid could be accounted for by selective sulfhydryl affinities, the irreversibility of interaction between ethacrynic acid and the enzyme reported here may be an important contributing factor.

There are a number of limitations on acceptance of the renal NaKMg-ATPase system as the site of the diuretic action of ethacrynic acid. The magnitude of renal ATPase inhibition by ethacrynic acid is quite similar in the dog, guinea pig and rat;[3] however, ethacrynic acid produces marked, weak or no saluresis, respectively, in these species.[14] Other inconsistencies include ATPase inhibition by nondiuretic mercurials[5] and lack of ATPase inhibition by known diuretics such as thiazides, spironolactone and xanthines.[2] With awareness of such limitations, the inhibition of the renal ATPase enzyme system remains for further consideration as a possible mechanism of diuretic action.

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