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ETHACRYNIC ACID INHIBITION OF $(Na^+ + K^+)$-ACTIVATED ADENOSINE TRIPHOSPHATASE

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Abstract—Ethacrynic acid was found to inhibit microsomal preparations of sodium+potassium-activated adenosine triphosphatase obtained from guinea pig kidney cortex. The degree of inhibition of ethacrynic acid was influenced by the concentration of potassium ion present and was greatest when potassium ion was least. Examination of the effect of ethacrynic acid on the transfer of ^{32}P from γ-labeled substrate ATP^{32} to an intermediate formed during ATP^{32} hydrolysis demonstrated no inhibition by ethacrynic acid in the presence of 20 mM Na^+, but considerable inhibition in the release of inorganic ^{32}P from this complex upon the subsequent addition of K^+. Again this effect was greatest when the potassium concentration was least. The antagonism between ethacrynic acid and potassium ions may be explained by this mechanism.

THE PHYSIOLOGICAL role of sodium+potassium-activated adenosine triphosphatase (transport ATPase) in the active transport of monovalent cations across biological membranes is now generally accepted.[1–3] The inhibitory action of ethacrynic acid and other diuretic agents on this enzyme system both *in vitro* and after administration to the whole animal[4, 5] has raised the possibility that this interaction may be the basis for the pharmacological effects of diuretic agents, although such a mechanism has been questioned by some investigators.[5, 6]

In accordance with a previous suggestion by Nechay *et al.*,[6] a recent study in this laboratory has shown that isolated slices of guinea pig kidney cortex are capable of accumulating 2-^{14}C-ethacrynic acid from an incubation medium by a temperature-dependent process.† Although the accumulation of drug observed was only about 10-fold, equilibrium was not attained in these experiments and such a mechanism may explain the discrepancy between the plasma levels of this drug which are observed after therapeutic doses of ethacrynic acid to both man and experimental animals[4, 7] and the levels required to demonstrate enzyme inhibition *in vitro*.[4, 6] Thus the pharmacological significance of ethacrynic acid inhibition of transport ATPase remains of considerable interest and a more detailed understanding of the mechanism is desirable.

The experiments described in this paper were conducted to re-examine the inhibitory effect of ethacrynic acid on a sodium+potassium-activated ATPase prepared from guinea pig kidney cortex, by utilizing a sensitive test system which employs γ-ATP^{32} as substrate, and to further examine the competitive effect of K^+ on this reaction, as this ion has recently been observed to influence the ethacrynic acid inhibition of transport

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ATPase.[6] By measuring the transfer of ^{32}P from substrate to an acid-stable phosphorylated protein intermediate of sodium–potassium ATPase,[8–11] further insight into the mechanism of ethacrynic acid effects was obtained.

MATERIALS AND METHODS

Sodium–potassium-activated ATPase preparations were obtained from guinea pig renal cortex by a method described previously.[12] ATPase activity was measured by the release of inorganic ^{32}P from γ-ATP^{32} at pH 7·5. Inorganic ^{32}P content of the reaction system was determined after charcoal absorption of nucleotide phosphate by the method of Crane and Lipmann.[13] In these experiments, transport ATPase activity was defined as the hydrolysis of γ-ATP^{32} which was inhibited by 0·1 mM ouabain when enzyme preparations were incubated in the presence of 80 mM Na^+ and 20 mM K^+ ions.

The method for isolation of the acid-stable ^{32}P-labeled intermediate complex and the per cent of ^{32}P liberated has been described previously.[10] γ-Labeled ATP^{32} was obtained as the tetra-ammonium salt from the Radiochemical Centre, Amersham, Bucks, and had a specific activity greater than 1400 mc/m-mole. This material was diluted with disodium ATP (Sigma) and adjusted to pH 7·5 by the addition of solid Tris immediately before use.

Ethacrynic acid was obtained as a gift from Merck & Company Inc., (Rahway, N. J.) and was rendered water-soluble by the addition of solid Tris to give solutions of pH 7·5.

^{32}P radioactivity was determined in a Nuclear Chicago gas flow apparatus.

RESULTS

Preliminary experiments demonstrated that under the standard conditions of assay, i.e. in the presence of 80 mM Na^+ and 20 mM K^+, 85 per cent of the total ^{32}P liberated from γ-ATP^{32} could be inhibited by addition of 0·1 mM ouabain to the medium, and that the rate of ^{32}P liberation remained linear throughout the period of incubation.

The effect of varying concentrations of ethacrynic acid on ouabain-sensitive ATPase was then determined under these "standard" conditions of cation activation. The results are shown in Table 1, where it can be seen that inhibition of enzyme activity

TABLE 1. EFFECT OF ETHACRYNIC ACID ON OUABAIN-SENSITIVE ATPASE OF GUINEA PIG KIDNEY CORTEX

Ethacrynic acid (mM)	ATPase activity*		Δ†	% Inhibition
	Na + K	Na + K, ouabain		
Control	2·45	0·37	2·08	0
0·01	2·42	0·37	2·05	1
0·1	2·30	0·37	1·93	7
1·0	1·67	0·35	1·22	41
5	0·82	0·32	0·50	61
10	0·45	0·29	0·16	91

* Results are expressed as μmoles ^{32}P liberated from γ-ATP^{32}/0·1 ml of enzyme suspension (0·24 mg protein)/15 min at 37° and are the mean of five observations; 80 mM Na^+, 20 mM K^+ and 0·1 ml ouabain present where indicated.

† Calculated value for ouabain-sensitive transport ATPase, e.g. 85% of control activity is ouabain sensitive.

increased with the concentration of ethacrynic acid. At 1 mM ethacrynic acid, 41 per cent of the ouabain-sensitive component of the ATPase activity was inhibited, but there was no significant effect upon ouabain-insensitive ATPase activity. However, when 10 mM ethacrynic acid was employed, 91 per cent of the ouabain-sensitive component was inhibited and there was also more than 20 per cent inhibition of the ouabain-insensitive ATPase of these preparations.

Thus, at high concentrations, ethacrynic acid was not completely specific for transport ATPase, confirming the previous observations of Duggan and Noll.[4] Furthermore, under these conditions of assay, ethacrynic acid failed to demonstrate any greater degree of inhibition toward ouabain-sensitive ATPase than that reported by other investigators.[4, 6]

However, greatly increased sensitivity to ethacrynic acid inhibition could be demonstrated when the K^+ concentration of the assay system was decreased. Table 2

TABLE 2. EFFECT OF [K^+] ON ETHACRYNIC ACID INHIBITION OF OUABAIN-SENSITIVE ATPASE OF GUINEA PIG KIDNEY CORTEX

Expt.	[K^+] (mM)	Ethacrynic acid (mM)	ATPase activity* 80 mM Na^+	80 mM Na^+, 0·1 mM ouabain	Δ†	% Inhibition transport ATPase
A	20		2·74	0·38	2·36	
	20	1	2·04	0·38	1·66	29
	2		2·64	0·36	2·28	
	2	1	1·38	0·36	1·02	55
	0·2		1·44	0·20	1·24	
	0·2	1	0·66	0·20	0·46	63
B	20		3·00	0·46	2·54	
	20	5	2·07	0·43	1·64	36
	0·2		0·83	0·23	0·60	
	0·2	5	0·33	0·21	0·12	80

* Results are expressed as μmoles ^{32}P liberated from γ-ATP^{32}/0·1 ml of enzyme suspension/15 min at 37°. Expt. A contained 0·28 mg protein/0·1 ml; expt. B contained 0·32 mg protein/0·1 ml. Results are the mean of duplicate assays.

† Calculated value for ouabain-sensitive transport ATPase.

demonstrates that the inhibition by 1 mM ethacrynic acid could be increased from 29 to 63 per cent by lowering the [K^+] from 20 to 0·2 mM. This effect was also demonstrated when 5 mM ethacrynic acid was employed and here the degree of inhibition was increased from 36 to 80 per cent when the [K^+] was reduced from 20 to 0·2 mM. These results confirm and extend the recent observations of Nechay *et al.*,[6] who noted a 6 per cent increase in sensitivity toward ethacrynic acid when the potassium concentration of their medium was varied over a 10-fold range.

Previous work from this laboratory[14] has demonstrated that the transfer of ^{32}P from γ-ATP^{32} to an intermediate of the transport ATPase reaction[8–11] can be adequately demonstrated by the addition of 20 mM Na^+ to the medium and that dephosphorylation of this complex can be achieved by the subsequent addition of low concentrations of potassium ions. This system was thus ideal for an examination of effects of ethacrynic acid on transport ATPase in the presence of varying concentrations of K^+ ions.

The mean result of five experiments is given in Table 3, where control experiments again confirmed the effect of Na^+ and K^+ ions upon the incorporation into and the release of inorganic ^{32}P from the acid-stable intermediate of transport ATPase.[7-11] The increase in inorganic ^{32}P liberation on the addition of 20 mM Na^+ to the system is attributed to the presence of a trace amount of ammonium ions introduced with the labeled substrate.

When 5 mM ethacrynic acid was added to the system, there was an increase in the transfer of ^{32}P from substrate to intermediate, which in every case was accompanied by a decrease in the amount of inorganic ^{32}P liberated from the enzyme substrate. The increased ^{32}P transfer found in the presence of 20 mM Na^+ indicates that ethacrynic acid does not inhibit the formation of the ^{32}P-labeled complex, whereas the

TABLE 3. EFFECT OF ETHACRYNIC ACID ON THE TRANSFER OF ^{32}P FROM γ-ATP^{32} TO AN INTERMEDIATE OF (Na + K)-ATPASE*

Additions	Control		5 mM Ethacrynic acid	
	Counts ^{32}P incorporated	% ^{32}P liberated	Counts ^{32}P incorporated	% ^{32}P liberated
None	606	18	725	8
20 mM Na^+	3398	28	5647	16
20 mM Na, 5 mM K^+	515	36	805	25
20 mM Na, 0·5 mM K^+	715	38	969	27
20 mM Na, 0·05 mM K^+	1590	41	3260	22

* Results are the mean of five experiments (assays in duplicate). Counts ^{32}P incorporated given as cps/mg protein nitrogen; per cent inorganic ^{32}P liberated is equivalent to the per cent γ-ATP^{32} substrate hydrolyzed in 10 sec.

reduced liberation of inorganic ^{32}P from this complex suggests that ethacrynic acid inhibits the breakdown of this complex, which is necessary for the overall hydrolysis of ATP by transport ATPase.

This is confirmed by the increased incorporation of ^{32}P and decreased liberation of inorganic ^{32}P found when ethacrynic acid was added in the presence of K^+ ions. As in the previous demonstration of the effect of $[K^+]$ ion concentration on ethacrynic acid inhibition of transport ATPase activity (cf. Table 2), the effect of ethacrynic acid was most marked when the concentration of added potassium was least (0·05 mM).

DISCUSSION

In this paper we have confirmed that ethacrynic acid inhibits the hydrolysis *in vitro* of ATP by guinea pig kidney preparations of transport ATPase and that meaningful depression of activity cannot be measured under "standard" conditions of cation activation when the concentration of the drug is less than 10^{-4} M.[4, 6]

In addition, we have shown that higher concentrations of the drug do not specifically inhibit transport ATPase, which was defined as the ouabain-sensitive component of our test system, but also exert an inhibitory effect upon the residual ouabain-insensitive component of the total ATPase activity.[6]

Similar to the competition known to exist between K^+ and cardiac glycosides for transport ATPase inhibition,[1, 14-16] Nechay *et al.*[6] had observed a small (6%)

decrease in ethacrynic acid effect when the concentration of [K^+] was increased from 2 to 20 mM in their assay system. Thus it was perhaps possible to demonstrate an increased inhibition of transport ATPase by reducing the K^+ content of the assay system rather than by attempting to increase the concentration of drug, which was reported to be approaching maximum solubility under some experimental conditions[6] and certainly required increasingly high concentrations of Tris buffer (approaching 50 mM when 10 mM ethacrynic acid was employed) to neutralize in the experiments reported here.

This expectation was confirmed when about a 2-fold percentage increase in ethacrynic acid inhibition was observed when the concentration of [K^+] was reduced from 20 to 0·2 mM. Hence, ethacrynic acid could be expected to be a more potent inhibitor of transport ATPase in any biological situation in which the K^+ was low. Furthermore, the lack of specificity of this agent toward ATPase reactions, which is seen only at high concentrations of the drug, would not seem to be a serious problem, since both these experiments and others from our laboratory[14] suggest a factor of about 100 X in preference for the ouabain-sensitive component of the ATPase present.

Finally, our experiments with the sequential addition of cations to the reaction mixture and the isolation of a ^{32}P-labeled intermediate compound of the ATPase reaction, which has recently been demonstrated by Kahlenberg *et al.*[11] to be L-γ-glutamyl-phosphate, indicate that ethacrynic acid inhibits the K^+-dependent dephosphorylation step in the overall mechanism of ATP hydrolysis by this enzyme,[1] and thus offer an explanation for the antagonism between this diuretic agent and potassium ions.

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