Biochemical Pharmacology, Vol. 17, pp. 1237–1242. Pergamon Press. 1968. Printed in Great Britain

SITE OF ETHACRYNIC ACID ACTION ON EHRLICH ASCITES TUMOR CELLS*

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(*Received* 3 *November* 1967; *accepted* 19 *January* 1968)

Abstract—The site of action of ethacrynic acid was studied in a model system consisting of respiring Ehrlich ascites tumor cells. Ethacrynic acid released oligomycin-induced respiratory inhibition and depressed the respiration of non-inhibited cells. These observations indicated that ethacrynic acid interfered with mitochondrial respiratory control of the intact cell utilizing endogenous substrate. A study of the effects of ethacrynic acid on intact mitochondria isolated from rat liver and kidney confirmed the observations with intact cells. Ethacrynic acid stimulated mitochondrial respiration in the absence of a phosphate acceptor and also released oligomycin-induced respiratory inhibition.

ETHACRYNIC acid is an extremely potent diuretic agent. The anatomical site of action of this drug has been studied extensively; apparently it exerts an effect at multiple sites along the renal tubule.[1] Studies of the cellular site of action indicated that this agent exerts an inhibitory effect on a membrane ATPase‡ preparation[2] and on a sodium transport mechanism in red blood cells.[3] The relevance of these mechanisms of action for its pharmacological consequences has been questioned on the grounds that relatively high concentrations of ethacrynic acid are required to obtain effects *in vitro*.[2,4] More recently, it has been demonstrated that ethacrynic acid also influences metabolism at the mitochondrial level.[5] The present studies were undertaken in an effort to localize the site of action of ethacrynic acid in intact, respiring cells. For this purpose, a model system consisting of K^+-depleted Ehrlich ascites tumor cells was used. Previous studies have demonstrated the feasibility of studying mitochondrial phenomena in such intact cell preparations.[6,7] Observations obtained with this system indicated that ethacrynic acid interferes with mitochondrial metabolism. The results of experiments with isolated, intact, mitochondria were consistent with this interpretation.

METHODS

K^+-depleted Ehrlich ascites tumor cells of the hyperdiploid strain were prepared by repeated washings in a K^+-free medium at 4° as previously described.[6] Only negligible quantities of intracellular K^+ remained after this washing procedure.[6,8] Suspended

* Supported in part by U.S. Public Health Service Grant AM-02598 and by a grant from the American Heart Association.

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‡ Abbreviations used: ATPase, adenosinetriphosphatase; ADP, adenosine diphosphate; ATP, adenosine triphosphate; DNP, 2,4-dinitrophenol.

cells (0·5 ml) were added to 2·5 ml of incubation medium in a chamber kept at 37°. The incubation medium was identical to the medium used for isolating and washing the cells and contained: 178 mM Na, 1·5 mM Mg, 12 mM phosphate, 154 mM Cl, and 1·5 mM SO_4. Exogenous substrate was not added to the ascites tumor cell incubations. Since there is essentially no glycogen in these cells, ATP is generated solely from the mitochondrial oxidation of other endogenous substrates. Mitochondria were prepared by a modification of the Schneider and Hogeboom technique[9] in 0·3 M sucrose–1 mM EDTA (pH 7·5) from the livers of adult, male, fed rats. After sedimentation of the nuclear fraction, the supernatant was centrifuged at 5000 *g* for 10 min. The supernatant and fluffy layer were removed and the sedimented mitochondria were resuspended and then spun at 8700 *g* for 10 min. The washed mitochondria were suspended in 0·3 M sucrose (approximately 25 mg mitochondrial protein/ml) and used without delay. Mitochondria (3·7 mg) were incubated at 30° in a volume of 3 ml containing: 0·3 M sucrose, 50 mM Tris (pH 7·5), 5·0 mM $MgSO_4$, and 3·3 mM potassium phosphate (pH 7·5). O_2 uptake of ascites tumor cell suspensions and of mitochondrial suspensions was monitored with a Clark O_2 electrode and the signal was continuously recorded on a Varian G-11 recorder. The protein content of the cells and the mitochondria was determined by a biuret method.[10] Crystalline ethacrynic acid (kindly provided by Dr. W. H. Wilkinson of the Merck, Sharp & Dohme Research Laboratory), oligomycin and valinomycin (a gift from Dr. J. C. MacDonald of the Prairie Regional Laboratory, Saskatoon, Canada) were dissolved in ethanol and added to the incubation medium in amounts not exceeding 20 μl; ethanol in this concentration had no effect on either the intact cell or mitochondrial systems. All other agents and substrates were prepared as aqueous solutions and neutralized before use.

RESULTS

Ethacrynic acid addition to K^+-depleted cells respiring in the presence of 5·1 mM K^+ resulted in an immediate depression of respiration at all concentrations between 0·1 and 1 mM (Fig. 1, closed bars). A similar but less pronounced initial response to ethacrynic acid was observed with cells respiring in the absence of K^+ (Fig. 1, open bars). A more marked inhibition of respiration was apparent upon prolonged incubation at all concentrations of ethacrynic acid under conditions of K^+ deficiency and K^+ repletion. Since previous experiments suggested that the respiratory stimulation observed upon addition of K^+ to K^+-depleted cells is probably a consequence of enhanced plasma membrane ATPase activity,[6] the effects observed upon addition of ethacrynic acid could have resulted from an inhibition of this mechanism. However, interference with mitochondrial ATP generation could produce an identical effect. Because of the inhibition of respiration beyond the increment stimulated by K^+ and because the effect was observed in the absence of K^+, ethacrynic acid interference with mitochondrial respiratory control seemed to be implicated.

In the intact Ehrlich ascites tumor cell, oxidation is tightly coupled to phosphorylation. If phosphorylation of mitochondrial ADP is blocked with the site specific agent, oligomycin, electron transport through the respiratory chain almost ceases with consequent inhibition of O_2 uptake.[6] Addition of DNP, a classical uncoupler of oxidative phosphorylation, released the oligomycin-induced inhibition in intact cells (Fig. 2) as first described by Lardy *et al.*[11] Ethacrynic acid behaved similarly in that it

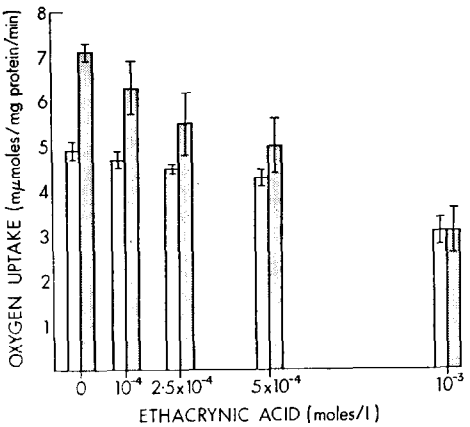

FIG. 1. Initial respiratory inhibition by ethacrynic acid in K-depleted (open bars) and K-repleted cells (closed bars). The vertical lines through the bars indicate the S.E.M. of at least 5 experiments.

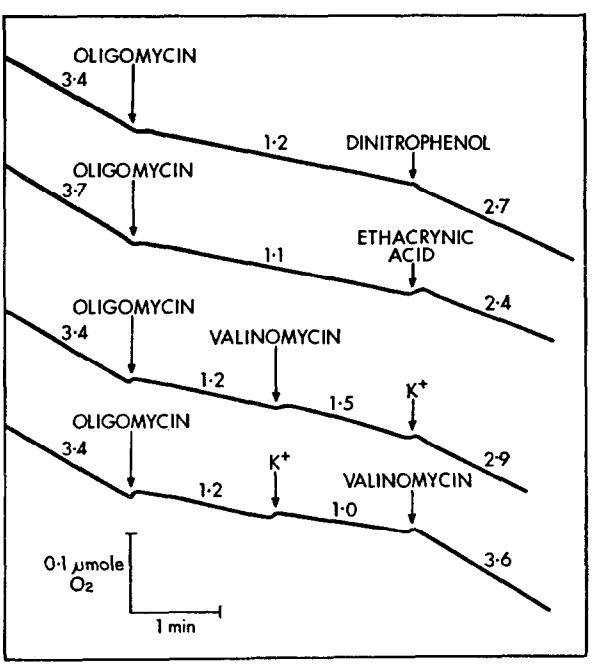

FIG. 2. Release of oligomycin-induced respiratory inhibition of Ehrlich ascites tumor cells. Final concentrations: oligomycin, 1 μg/ml; ethacrynic acid, 10^{-3} M; KCl $5{\cdot}1 \times 10^{-3}$ M; 2,4-dinitrophenol, 5×10^{-5} M; valinomycin, $3{\cdot}3 \times 10^{-7}$ M. The values represent the respiratory rate before and after each addition.

also released the oligomycin-induced respiratory inhibition (Fig. 2). This finding provided support for the hypothesis that ethacrynic acid exerted an influence on the metabolism of the intact cell at the mitochondrial level. This action of ethacrynic acid and DNP was independent of K^+, since the effect was seen with potassium-depleted cells. In contrast, the release of oligomycin-induced respiratory inhibition by valinomycin was dependent upon addition of potassium ion (Fig. 2, lower two curves).

The similarity of action of ethacrynic acid to that of DNP in its ability to release the oligomycin-induced respiratory inhibition strongly suggested that ethacrynic acid penetrated the cell membrane, made contact with the mitochondria, and acted as an uncoupler of oxidative phosphorylation in the mitochondria of the intact cell. In an attempt to confirm this finding and to explore this action of ethacrynic acid in greater detail, studies with isolated mitochondria were undertaken. The results with mitochondria isolated from rat liver were in agreement with the results obtained with the intact cell preparation. The effects of ethacrynic acid were compared with those of DNP and valinomycin on tightly coupled mitochondria (P:O ratio 1·7 and 2·6 with succinate and glutamate respectively). Mitochondria incubated in the absence of the phosphate acceptor, ADP (state 4 respiration), responded to ethacrynic acid with an increase in respiration when either succinate or glutamate was used as substrate (Table 1). A similar but more pronounced respiratory stimulation was observed in

TABLE 1. EFFECT OF ETHACRYNIC ACID, DNP AND VALINOMYCIN ON MITOCHONDRIAL RESPIRATION IN THE ABSENCE OF A PHOSPHATE ACCEPTOR (STATE 4 RESPIRATION)*

Substrate (3·3 mM)	Succinate	Glutamate
	Q_{O_2} (mμmole O_2/mg protein/min)	
Experiment 1		
Control	13·6	3.5
Ethacrynic acid	26·5	6·0
Experiment 2		
Control	12·1	3·5
DNP	48·0	10·7
Experiment 3		
Control	12·1	4·5
Valinomycin	64·8	57·3

* The experimental conditions are detailed in Methods. Concentrations of additions were as indicated in the legend of Fig. 2, except that ethacrynic acid was present in a final concentration of 5×10^{-4} M.

the presence of DNP. Addition of valinomycin resulted in an impressive respiratory stimulation in the presence of potassium ion, but not in its absence, as reported by Moore and Pressman.[12] When the phosphate acceptor, ADP was present in the medium (Table 2), state 3 respiration was inhibited with 1 μg oligomycin/ml of incubation mixture. Respiration was restored upon addition of ethacrynic acid as well as DNP or valinomycin. Again, the valinomycin effect was observed only when potassium ion was present in the incubation medium. Similar effects of ethacrynic acid and DNP on state 4 respiration in the presence of succinate were observed with mitochondria isolated from rat renal cortex. Ethacrynic acid (5×10^{-4} M) increased the respiratory rate from a control value of 19·1 to 30·8 mμmole O_2/mg mitochondrial protein/min. Dinitrophenol (5×10^{-5} M) increased the Q_{O_2} from 23·5 to 48·2.

TABLE 2. EFFECT OF ETHACRYNIC ACID, DNP AND VALINOMYCIN ON MITOCHONDRIAL RESPIRATION IN THE PRESENCE OF A PHOSPHATE ACCEPTOR (STATE 3 RESPIRATION)*

Substrate (3·3 mM)	Succinate Q_{O_2} (mμmoles O_2/mg protein/min)	Glutamate
Experiment 1		
Control	24·1	18·9
Oligomycin	12·1	6·0
Ethacrynic acid	23·4	9·5
Experiment 2		
Contril	25·6	18·5
Oligomycin	10·6	5·3
DNP	37·7	20·4
Experiment 3		
Control	23·8	17·8
Oligomycin	11·9	6·2
Valinomycin	51·1	59·6

* 5·2 μmole ADP was present in the incubation mixture. The experimental conditions are detailed in Methods. Concentrations of other additions were as indicated in the legend of Fig. 2 except that ethacrynic acid was present in a final concentration of 5×10^{-4} M.

DISCUSSION

The data with intact cells demonstrate an intracellular site of action at the level of the mitochondria for ethacrynic acid. Confirmation of an effect of ethacrynic acid on mitochondrial respiratory control was obtained with intact, tightly coupled mitochondria isolated from rat liver and kidney. In the intact cell, ethacrynic acid acts as an inhibitor of respiration. That this respiratory inhibitory effect can be dissociated from the energy demands of a plasma membrane coupled active transport of Na^+ and K^+ is seen from the data of Fig. 1. Significant respiratory inhibition is observed in the absence of K^+, suggesting an effect of ethacrynic acid on mitochondrial respiratory control. However, ethacrynic acid-induced respiratory inhibition is more pronounced in cells repleted with K^+. This response could reflect an effect on plasma membrane coupled Na^+–K^+ transport. An alternative and equally plausible argument can be made for interference with mitochondrial ATP generation by ethacrynic acid. Other evidence directing attention to a mitochondrial site of ethacrynic acid action in the intact cell was obtained with the site specific inhibitor, oligomycin. Ethacrynic acid releases the oligomycin-induced respiratory inhibition in a manner similar to that of the uncoupler, DNP.[7] If ethacrynic acid acts on mitochondrial respiratory control, as the experiments with intact cells indicate, then it should be possible to demonstrate an effect on isolated mitochondria. Indeed, an uncoupling activity is observable with isolated mitochondria.

Ethacrynic acid differs from classical uncouplers of oxidative phosphorylation in several respects. In intact ascites tumor cells, ethacrynic acid causes an inhibition rather than an enhancement of respiration, as seen with DNP.[7] Unlike DNP, ethacrynic acid has no influence on mitochondrial ATPase by itself, but actually inhibits DNP-stimulated ATPase;[18] this effect has been confirmed in our laboratory. The possibility of additional inhibitory effects of ethacrynic acid on electron transport in the mitochondrial respiratory chain cannot be ruled out.

The accumulated evidence thus far indicates that ethacrynic acid could influence energy-dependent ion transport by two mechanisms: 1) interference with ATP

production in the mitochondria, and 2) inhibition of a cation pump in the plasma membrane.[3] The former mechanism has been shown to be present in ascites tumor cells; an evaluation of the relative importance of these two mechanisms must await the demonstration of the latter mechanism in intact, respiring cells.

Acknowledgement—The skillful technical assistance of Mrs. Milagros Redinger is gratefully acknowledged.

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