Effects of Ethacrynic Acid upon Membrane ATPase of Dog Kidney *in Vivo* and *in Vitro* (36233)

DANIEL E. DUGGAN AND RONALD M. NOLL
(Introduced by C. G. Van Arman)
Merck Institute for Therapeutic Research, West Point, Pennsylvania 19486

Inhibitions by ethacrynic acid (EA) of Na–K–Mg-dependent or "membrane" ATPase preparations derived from renal cortex of several species have been described (1–4). Correlations of the respective saluretic and *in vitro* inhibitory activities of EA and two series of its congeners (1), and the inactivity of EA with respect to unmodified ATPase preparations from the rat (2), a species uniquely insensitive to EA (5) and to cardiac glycosides (6), are consistent with the concept of membrane ATPase as a receptor for EA in the kidney.

The ATPase inhibitory activity of EA *in vitro* can be enhanced manyfold by preincubation of drug and enzyme (1, 3), by modifications of the ionic environment (4), and by the exclusion of endogenous soluble thiols, thus precluding determination of definitive I_{50} values under any but the most rigidly defined experimental conditions. Since even minimal values for I_{50} are orders of magnitude in excess of total drug bound to membrane fractions isolated from kidneys undergoing saluretic response (7), they would appear to be without pharmacological relevance (8, 9) unless the ability of the intact kidney to concentrate the drug at appropriate receptors greatly exceeds that of *in vitro* preparations. The present study was undertaken in an attempt to resolve this specific aspect of the problem of membrane ATPase as receptor for ethacrynic acid.

Materials and Methods. Ethacrynic acid-2-^{14}C (1.41 μCi/μmole) was prepared by Merck Sharp & Dohme Research Laboratories, Rahway, NJ and used undiluted in all *in vivo* experiments. When added at higher concentrations to tissue homogenates and microsomes, it was diluted with appropriate levels of unlabeled carrier.

Male beagle dogs (9–12.5 kg) were anesthetized with pentobarbital (35 mg/kg), and both ureters were exposed and cannulated, while infusing mannitol–phosphate medium (1) at 5 ml/min. After collecting urine for 20 min, the right (control) kidney was removed, and the cortex was homogenized in 4 vol of 0.25 *M* sucrose containing histidine (15 m*M*), sodium EDTA (1 m*M*) and sodium desoxycholate (0.1%). Immediately thereafter, ethacrynic acid-^{14}C was injected via the infusion system at 10 mg/kg or directly into the left renal artery at 1 mg/kg. After 15 min, the left (experimental) kidney was removed and treated as above. An aliquot of the homogenate was counted to determine total EA concentration, and various multiples thereof were added to aliquots of the control kidney homogenate. Thereafter, all samples were treated identically.

Following 2 min exposure to 10 kHz ultrasound, at 0°, light microsomal fractions were isolated by conventional centrifugation (2), washed once, and resuspended at a level of 10 mg of protein/ml as determined by the Sutherland procedure (10). Assays for Na–K–Mg-dependent ATPase activities were performed immediately by the procedure previously described (1), and aliquots of the assay system were withdrawn for determination of total and bound EA-^{14}C by ultrafiltration (7).

In those *in vitro* inhibition experiments where EA was added directly to microsomes, either of two protocols were followed. Microsomes were added directly to an otherwise complete system at zero time, or were preincubated for 60 min with drug, each at 10 times the ultimate assay concentration, then

TABLE I. Inhibition of Dog Kidney ATPase After 1 mg/kg of Ethacrynic Acid Via Renal Artery.[a]

	P_i (μmoles/hr/mg of protein)				EA in assay system	
	$+K^+$	$-K^+$	Δ	%I	Total	Bound
Control	41.2	24.4	16.8	—	—	—
In vivo	31.8	23.0	8.8	48	5.0×10^{-8}	4.5×10^{-8}
1X	40.8	23.8	17.0	0	2.3×10^{-8}	6.0×10^{-9}
10X	36.0	22.4	13.6	19	2.7×10^{-7}	3.0×10^{-8}
100X	30.6	21.0	9.6	43	1.6×10^{-6}	4.7×10^{-7}
300X	27.6	19.6	8.0	53	6.2×10^{-6}	1.1×10^{-6}
1000X	24.0	19.2	4.8	71	1.5×10^{-5}	2.2×10^{-6}

[a] Original 1:10 homogenate of experimental (left) kidney contained 2.45 μg EA equiv/ml. Indicated multiples of this concentration were added to aliquots of the control kidney homogenate, and all samples thereafter were treated identically. All reaction rates were determined by best straight lines through origin, 7.5, and 15 min time points. Drug concentrations refer to the final assay system, samples at 10 min for determination of binding.

added to 9 vol of an otherwise complete medium. In each case, aliquots of the complete assay system were taken for measurement of microsomal binding by membrane filtration.

Fractionation of ethacrynic acid and metabolites was accomplished by adjustment of samples to pH 1 with HCl, and successive extractions by equal volumes of benzene, ethyl acetate, then *n*-butanol, twice each. Radioautography of paper chromatograms developed in *n*-butanol:benzene:methanol:water, 2:1:1.25:1 revealed the benzene extract to consist exclusively of unchanged EA (R_f 0.74), the ethyl acetate fraction of three components with R_f values 0.64, 0.55, and 0.34 (the first superimposable upon the cysteine adduct of ethacrynic acid), and the butanol extract, two components at R_f values 0.60 and 0.29, both unidentified. In light of these results, radioactivity recoverable by two successive extractions with benzene was used as a measure of unchanged ethacrynic acid.

Results. The natriuretic response to EA, whether injected intravenously or directly into the renal artery, peaks within the interval 10–30 min, and is ipsilateral in the case of the latter route, demonstrating that the drug acts directly upon the kidney (5). Fifteen minutes postdrug was therefore chosen as an appropriate interval for removal of experimental kidneys.

In each of two dogs receiving EA-^{14}C at 10 mg/kg iv, plasma and urine levels of total radioactivity and of unchanged drug were followed over 4 hr for calculations of their respective half-lives, volumes of distribution, and renal clearances (11). Total ^{14}C declined biexponentially with half-lives of 11 and 147 min with apparent volumes of distribution of 25 $\pm$ 3.5% body volume, and ^{14}C/creatinine clearance ratios of 1.05 $\pm$ 0.07. Corresponding values for benzene extractable radioactivity were 12 and 126 min, 192 $\pm$ 24% body volume, and clearance ratios 2.71 $\pm$ 0.26. In each of three terminal experiments at 10 mg/kg iv, initial plasma decay curves were not significantly different than the above, and kidney:plasma ratios, with respect to total radioactivity were fairly constant at 2.75 $\pm$ 0.4 (absolute values 32–45 μg/g of cortex), and with respect to unchanged drug, 5.30 $\pm$ 0.95. Tissue levels after renal arterial injection (1 mg/kg) were 19.6 $\pm$ 1.4 μg/g of cortex, and tissue:plasma ratios, 13.5 $\pm$ 6.0. Ultimate concentrations of total EA-derived radioactivity in microsomes isolated from cortex was remarkably constant at ca. 5×10^{-10} moles/mg of protein in all experiments.

The resulting concentration in the ATPase assay system (ca. 5×10^{-8} *M*) of which approximately 90% was bound (ultrafiltration), effected significant inhibition of the Na–K-dependent fraction relative to that derived from the contralateral kidney. To achieve a comparable inhibition of the con-

TABLE II. Inhibition of Dog Kidney ATPase After 10 mg/kg of Ethacrynic Acid, iv.[a]

		P_i (μmoles/hr/mg of protein)					
		No pretreatment		Cysteine (1×10^{-4} *M*)		Cysteine (1×10^{-4} *M*) from 2 hr	
	EA total	—K	Δ	—K	Δ	—K	Δ
Control		23.1	20.0	23.1	23.2	23.2	20.8
In vivo	7.0×10^{-8}	23.5	11.6	23.0	13.6	23.0	10.6
1X	3.4×10^{-8}	23.0	20.8	23.0	21.9	23.5	20.8
10X	3.6×10^{-7}	22.2	16.8	21.9	18.4	23.2	15.2
100X	5.8×10^{-6}	22.4	9.4	21.7	12.2	22.8	10.0

[a] Original 1:10 homogenate of experimental kidney contained 3.2 μg EA equiv/ml. Cysteine was either added to otherwise complete assay media at a final concentration of 1×10^{-4} *M*, or preincubated 2 hr at 37° before addition of ATP, with all components at ½ their ultimate concentrations. Other notations as for Table I.

trol preparation, an approximately 100-fold excess of drug had to be added to the original homogenate, yielding a total drug concentration in the final assay system approximately 30-fold higher. Representative inhibition experiments at 1 mg/kg via renal artery and 10 mg/kg i.v. are summarized in Tables I and II, respectively, and inhibition vs concentration curves based upon five experiments are in Fig. 1.

The concentration requirements for drug added directly to the final incubation system, or preincubated with microsomes for 60 min prior thereto, were respectively, 4500-fold and 660-fold that of microsomes labeled *in vivo* (Fig. 1). In terms of *unchanged* ethacrynic acid (free plus bound), these ratios would be approximately 8-fold higher owing to the disproportionate abundance of unchanged drug in the *in vitro* system. The relative abundances of free drug and metabolites in relevant body fluids and *in vitro* preparations are summarized in Table III.

Discussion. Traditionally, "membrane" ATPase is designated as the increment in enzyme activity attainable upon addition of

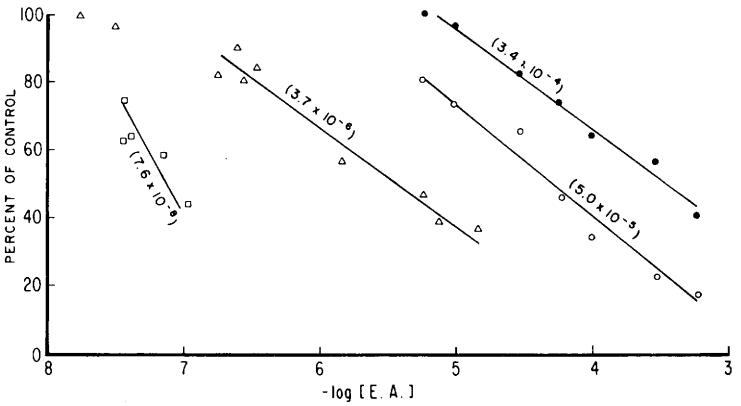

FIG. 1. Inhibition vs concentration curves for dog kidney microsomes: (□—) drug injected, 10 mg/kg iv (3 animals) or 1 mg/kg renal artery (two animals); (△—) drug added to original homogenate prior to isolation of microsomes (3 animals); (○—) drug and control microsomes preincubated 60 min at 10-times ultimate assay concentrations; (●—) microsomes added to otherwise complete medium at zero time. Concentrations refer to total drug derived radioactivity in final assay system.

TABLE III. Relative Abundances of Ethacrynic Acid and Metabolites in Dog Tissues and *in Vitro* Systems.[a]

	Total ^{14}C extractable into:			
	ϕH	EtOAc	BuOH	Residue
Control (0.1 *N* HCl)	94.5	5.2[b]	—	—
Kidney homogenate (*in vivo*)	33.5	29.3	24.8	12.8
Microsomes (*in vivo*)	11.8	29.4	51.7	7.1
(*in vitro*)[c]	92.8	5.9	1.0	0.3
Renal tubules, *in vitro*[d]				
Tissue	68.0	19.0	9.6	3.4
Medium	21.6	56.2	20.8	1.3
Plasma, 15 min	16.8	49.5	9.5	24.2
Urine, 0–15 min	46.3	39.5	11.8	0.4

[a] All values represent total recoveries for three successive extractions from pH 1 by equal volumes of solvents preequilibrated with 0.1 *N* HCl.

[b] Represents labeled impurity in starting material for which subsequent EtOAc-extractable values are not corrected.

[c] Dog renal microsomes at 1.0 mg of protein/ml incubated 15 min with EA-^{14}C (1×10^{-5} *M*) at 37°, pH 7.4.

[d] Suspension of rabbit renal tubules, 5% (v/v) incubated 15 min at 37° with EA-^{14}C (1×10^{-5} *M*).

exogenous Na^+ and K^+, equal to the maximum decrement subsequently attainable with cardiac glycosides. Evidence for the role of membrane ATPase in the maintenance of cation gradients is largely indirect, deriving from correlations of the levels of activity in various tissues with their respective capacities for ion transport, and the sensitivity of intact transport sytems to cardiac glycoside (16, 17).

That membrane ATPase constitutes a portion of the total cation reabsorptive capacity of the kidney, whether directly as carrier, or as part of an energy coupling system, appears to be reasonably well established; and that the natriuretic response to ouabain is related to inhibition of this sytem (8). The implication of membrane ATPase inhibiton in the mechanisms of action of mercurials, furosemide, and ethacrynic acid is more tenuous. The, then current, status of this problem has been reviewed recently (8, 9, 12).

The saluretic effects of ethacrynic acid are clearly additive with those of thiazides (5) and acetazolamide (5), but are not superimposable upon maximal responses to ouabain (7), furosemide (13), or organomercurials (5), suggesting a possible common mode of action with the latter agents. The I_{50} values for ouabain and mercurials with respect to membrane ATPase preparations *in vitro* are of an order reasonably corresponding to concentrations obtaining in microsomes isolated from kidneys during saluretic response. Corresponding values for ethacrynic acid were found to be manyfold lower (ca. 3×10^{-8} moles/mg of protein), and have been interpreted as insufficient to produce a significant inhibition *in situ* unless concentrated at a critical site of cation reabsorption (7). Direct measurement of enzyme levels in these preparations was, unfortunately, not reported.*

In the present study, the larger dosages employed resulted in even lower concentrations of EA-derived radioactivity associated with renal microsomal protein ($5.0 \pm 0.6 \times 10^{-10}$ moles/mg), but were consistently found to have effected significant inhibitions of ATPase relative to control kidneys removed prior to injection of the drug. Such

* *Note added in proof*: These authors have subsequently demonstrated a dose-dependent inhibition of dog renal ATPase *in vivo* (18).

inhibition was, furthermore, specifically of the K-dependent fraction of total ATPase, and was not significantly reversible by cysteine, even when preincubated with thiol at a concentration vastly in excess of that of the EA–enzyme complex. This essentially irreversible nature of the *in vivo* labeling is in marked contrast to that of ATPase inhibited *in vitro* (1, 15) and of adducts of EA with a number of purified sulfhydryl enzymes, which undergo spontaneous dissociation and reactivation upon dilution (14).

The apparent ability of the intact organ to concentrate the drug at appropriate receptor(s), as suggested by these results, is further emphasized by comparisons with the ultimate effects of drug added to disorganized mixtures of cellular components. Concentrations of ethacrynic acid, equal to that present in the experimental kidney, have no effect upon either ATPase fraction when added to control homogenate prior to fractionation. Approximately 100-fold excesses added at this stage ultimately produce an inhibition of "membrane" ATPase comparable to that in the experimental kidney, in addition to a significant inhibition of the Mg-dependent fraction; total concentrations of radioactivity present in and bound to such microsomal fractions are, respectively, 32-fold and 2-fold higher than in the preparation labeled *in vivo*. Assuming a gross heterogeneity of the isolated microsomal population with respect to origin and function, such a lack of correlation between drug concentration and inhibition would be expected if the drug were disproportionately concentrated *in vivo* at appropriate receptors. Thus, the disparity between respective concentrations of EA required for inhibition *in vitro*, and prevailing *in vivo*, does not, of itself, preclude involvement of membrane ATPase in the mechanism of action of ethacrynic acid.

As direct evidence for such an involvement, the present findings should be qualified to the extent that the dosages employed are supramximal with respect to saluretic effect at 15 min. Through intravenous dosage levels up to 25 mg/kg, however, a clear dose–response relationship is evident with respect to *duration* of response (5). The irreversible nature of enzyme inhibition, as effected *in vivo*, while inconsistent with the transient aspect of the initial saluretic response, might thus have a role in the mechanism of the sustained effect of the drug. Measurement of membrane ATPase at extended intervals following dosage is indicated.

Summary. Na–K–Mg-dependent ATPase preparations isolated from dog kidneys undergoing ipsilateral saluretic response to ethacrynic acid contained quite reproducible levels of drug-derived radoactivity (ca. 5×10^{-10} moles/mg of protein), and were significantly inhibited relative to contralateral kidneys removed immediately prior to dosage. Inhibition was specifically of the Na–K-dependent fraction of total ATPase, and was essentially irreversible by soluble thiol, and upon dilution and prolonged incubation. With respect to the final assay system, I_{50} in microsomes labeled *in vivo* was 7.6×10^{-8} *M;* respective values following introduction of drug at various stages of isolation ranged from 50- to 4500-fold higher, emphasizing the ability of the intact organ to concentrate drug disproportionately at ATPase receptor(s).

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