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RELATION BETWEEN DIGITALIS BINDING *IN VIVO* AND INHIBITION OF SODIUM, POTASSIUM-ADENOSINE TRIPHOSPHATASE IN CANINE KIDNEY*

JULIUS C. ALLEN,† M. MARTINEZ-MALDONADO, G. EKNOYAN, W. N. SUKI and ARNOLD SCHWARTZ‡

Division of Myocardial Biology and Department of Internal Medicine, Baylor College of Medicine and V. A. Hospital, Houston, Texas, U.S.A.

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Abstract—Digoxin was infused into a dog renal artery, using the contralateral kidney as control. At peak diuretic effect, sodium, potassium-adenosine triphosphatase (Na^+,K^+-ATPase) activity of both experimental cortex and medulla was significantly depressed compared to control. The inhibitory effect *in vitro* of ouabain on the control Na^+,K^+-ATPase from both medulla and cortex was time dependent; no regional difference in sensitivity was noted. The binding *in vitro* of ^3H-ouabain to Na^+,K^+-ATPase from both experimental cortex and experimental medulla was significantly lower than that for control enzymes, suggesting previous occupation of drug-binding sites. The data represent further indication that digoxin-induced diuresis is related to inhibition of Na^+,K^+-ATPase.

THE PRECISE role of the ouabain-sensitive Na^+,K^+-ATPase§ in the process of sodium reabsorption by the kidney has not been clearly defined. Numerous investigators have isolated this enzyme preparation from kidney tissue,[1–9] and it has been suggested that various diuretic compounds may act by inhibiting the enzyme activity.[10–17] It has been known for some time that direct renal arterial administration of cardiac glycosides, which are potent and specific inhibitors of the Na^+,K^+-ATPase system, induce a profound diuresis.[18,19] Recently, we presented studies suggesting that the diuretic effect appears to be directly related to inhibition of the Na^+,K^+-ATPase system.[20–22] Other investigators have corroborated these results.[23–26] The present studies are concerned primarily with the interaction of glycosides with Na^+,K^+-ATPases from both cortical and medullary regions of the dog kidney. Isotopically labeled drugs were employed for specific binding experiments. Detailed methodology of isolation and assay of the enzyme is also presented.

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† Postdoctoral Fellow, USPHS, National Heart Institute (1-FO2-HE-43042-01).

‡ Career Research Development Awardee, National Heart Institute (K_3 HE 11, 875-05).

§ Abbreviations: Na^+,K^+-ATPase, sodium, potassium-adenosine triphosphatase; C_{Na}, sodium clearance; C_{osm}, osmolar clearance; $T^C_{H_2O}$, free water reabsorption; PK, pyruvate kinase; LDH, lactate dehydrogenase; DOC, Na-deoxycholate; PEP, phosphoenol pyruvic acid.

METHODS

Physiological studies

Mongrel dogs of either sex were fasted and thirsted for at least 24 hr prior to the experiment. One hr prior to pentobarbital anesthesia (30–50 mg/kg), 5 units of pitressin tannate was administered intramuscularly. The animals also received 50–60 mU/kg/hr of aqueous pitressin throughout the experiment, along with a 10 per cent mannitol infusion at 1 ml/min. After anesthesia, the trachea was intubated, and the animal ventilated with room air by means of a Harvard respirator. Peripheral veins and the femoral artery were cannulated for infusions and blood collection, respectively. The ureters were catheterized through a suprapubic incision. A curved needle was placed into the left renal artery through a flank incision for either saline infusion (1 ml/min) during control periods or digoxin in saline (0·01 ml/min). In four control dogs the saline infused into the renal artery did not contain digoxin. A comparison between the results in these control animals and the experimental animals showed that infusion of digoxin into one kidney did not significantly affect the other organ either physiologically or biochemically. Hence, the contralateral (uninfused) kidney was employed as an internal control. Control urine samples were collected from both kidneys for at least 30 min, after which the infusion of digoxin was begun. An increase in urine flow in the experimental organ was maximal between 60 and 100 min after the beginning of the drug infusion. In control animals a waiting period of at least 90 min was observed (30 min saline control plus 60 min saline "infusion") before removing the kidneys. When the maximum effect was noted (no further increase in urine volume in two successive 10-min collection periods), the kidneys were removed, separated into cortex and medulla, and placed into the isolation medium. Physiologic parameters, sodium clearance (C_{Na}), osmolar clearance (C_{osm}) and free water reabsorption ($T^C_{H_2O}$) were measured as previously detailed.[22,27]

Isolation of Na^+,K^+-ATPase

All procedures were carried out at 0–4° and were identical for both cortex and medulla. The isolation method was a modification of the procedure for cardiac tissue[28] and salivary gland.[29] The tissue was finely minced with scissors in a relatively small volume, placed in 9 vol. of 0·25 M sucrose, 1 mM Tris-EDTA, pH 7·0, and treated for two 20-sec periods with a Polytron (Brinkman Instruments) at a rheostat setting of 2. It was found that a Sorvall Omnimixer or Waring Blendor yielded an inactive preparation. The homogenate was filtered through 4 and then 8 layers of cheesecloth; the filtrate was centrifuged at 10,000 *g* for 15 min in a Sorvall RC2-B refrigerated centrifuge. The supernatant was discarded and the pellet resuspended in 8 vol. of 0·25 M sucrose, 1 mM Tris-EDTA, 0·1 per cent sodium deoxycholate (DOC), by means of the Polytron, for six 30-sec periods, with a 15-sec rest in ice after each step. The suspension was kept in ice for 30 min after the addition of 1 drop of octyl alcohol to decrease foaming, and stirred periodically. Another drop of octyl alcohol was usually added after 10–15 min, if the foam was excessive. The resulting mixture was centrifuged at 10,000 *g* for 30 min and the supernatant carefully separated through 8 layers of cheesecloth (pellet discarded) and centrifuged at 100,000 *g* for 1 hr. The resulting high speed pellet was suspended by means of a glass homogenizer and Teflon pestle in 1·5 vol. (relative to the original tissue weight) of 0·25 M sucrose, 1 mM Tris-EDTA, 0·05 per cent DOC and centrifuged for 20 min at 20,000 *g*. The resulting

supernatant was then centrifuged at 100,000 *g* for 1 hr. This pellet was then suspended in 1 mM Tris-EDTA and used for Na^+,K^+-ATPase assay and ^3H-ouabain binding studies.

At the outset of these studies, a sodium iodide treatment was carried out to further purify the Na^+,K^+-ATPASE.[28] Although this treatment was successful, it was not routinely used, since the DOC enzyme yielded the same qualitative results as the "more purified" preparations.

Spectrophotometric assay of Na^+,K^+-ATPase

The pyruvate kinase–lactate dehydrogenase (PK-LDH)-linked enzyme assay procedure employed was identical to that already published in detail.[30] Preliminary experiments on control kidneys, using both this procedure and the standard colorimetric assays,[28] clearly indicated that the concentrations of Na^+ (100 mM), K^+ (10 mM), Tris (25 mM, pH 7·4), Mg^{2+} (2·5 mM) and ATP (2·5 mM) used for rat kidney,[31] beef and dog heart and salivary gland[28–30] were optimal for dog kidney (data not presented). Briefly, the PK-LDH method consists of monitoring NADH oxidation at 340 mμ with a recording spectrophotometer. The basic reaction sequence is:

$$(1)\quad \text{ATP} \xrightarrow{Na^+,K^+\text{-ATPase}} \text{ADP} + \text{inorganic phosphate}$$

$$(2)\quad \text{PEP} + \text{ADP} \xrightarrow{\text{PK}} \text{ATP} + \text{pyruvate}$$

$$(3)\quad \text{Pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}^+ .$$

Since all additions but the Na^+,K^+-ATPase were in excess, NADH oxidation was directly proportional to the ATP hydrolyzed. Na^+,K^+-ATPase is expressed as that activity which was completely ouabain sensitive (activity in the absence of ouabain minus activity in the presence of 5×10^{-4} ouabain). Results were identical using digoxin. All experiments were carried out at 37°. Specific activity is expressed as micromoles P_i per milligram of protein per hour to avoid any confusion. Justification for this step has already been published in detail.[30]

^3H-digitalis binding studies

The procedures were the same as those previously published.[32,33] Experiments using ^3H-digoxin, rather than ^3H-ouabain, yielded the same results.

RESULTS

Na^+,K^+-ATPase

The activity of Na^+,K^+ATPase in control medulla was significantly higher than in control cortex (Fig. 1, control: specific activity, cortex and medulla). It is important to note that there is no difference between contralateral kidneys of experimental dogs and kidneys of control animals (data not presented). Hence, the contralateral kidneys of experimental animals may be employed as controls.

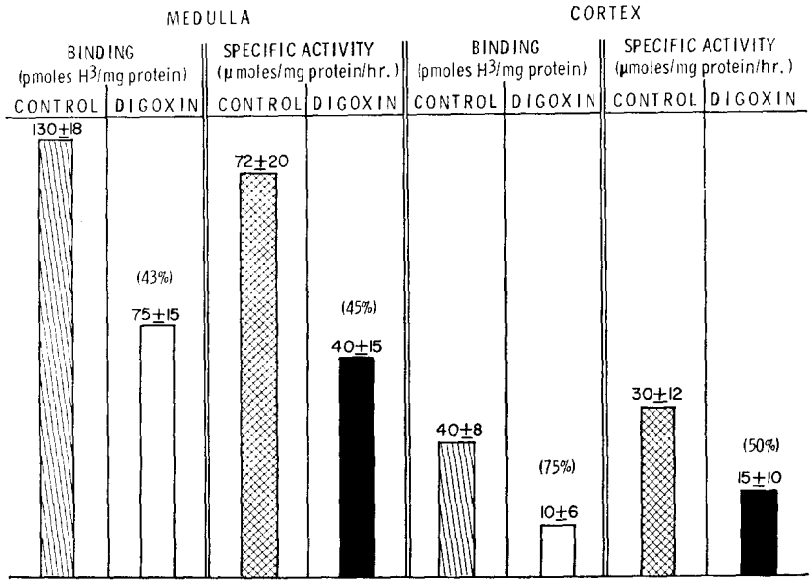


FIG. 1. Relation between ^{3}H-ouabain binding and Na^+,K^+-ATPase activity. Details of the binding procedure are presented in Table 3. Details of the Na^+,K^+-ATPase assay are presented in Methods and in Fig. 2. The binding differences noted are those of the Na^+-stimulated binding.

It is of interest that inhibition by ouabain of the Na^+,K^+-ATPase from control cortex and medulla is time dependent (Fig. 2 A and B). This is consistent with data previously presented and is characteristic of species that are sensitive to cardiac glycosides.[30,31]

There appeared to be a correlation between the effects of direct renal administration of cardiac glycosides and Na^+,K^+-ATPase activity (Table 1). It can be seen that two

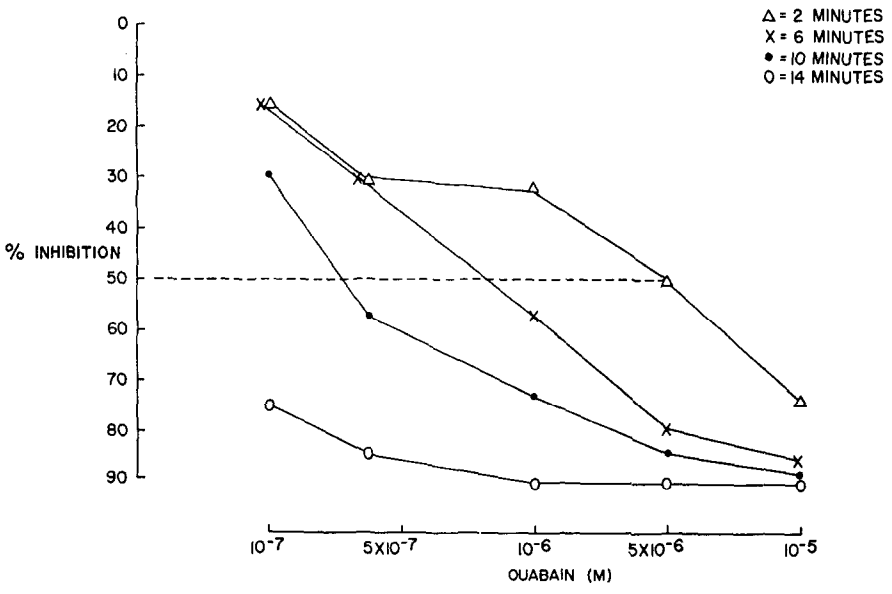
TABLE 1. RELATION BETWEEN Na^+,K^+-ATPASE ACTIVITIES AND RENAL FUNCTION PARAMETERS*

Na^+,K^+-ATPase activity (μmoles P_i/mg protein/hr)					Renal function (ml/100 ml GFR)		
Cortex		Medulla			Control		Digoxin
Control	Digoxin	Control	Digoxin		Right	Left	Left
38 ± 10	22 ± 7†	90 ± 21	41 ± 18†	C_{Na}‡	1·6 ± 0·1	1·8 ± 0·2	9·5 ± 0·8†
				C_{osm}	4·4 ± 0·4	4·3 ± 0·5	14·5 ± 2·1†
				$T^C_{H_2O}$	2·6 ± 0·2	2·7 ± 0·4	3·4 ± 1·1

* Saline was infused into the left renal artery for 30 min. Thus the values for control renal function are for the right kidney throughout the entire experiment, and for the left kidney up until the point of the beginning of digoxin infusion. After that point in time, the left kidney was the experimental organ. Na^+,K^+-ATPase isolation and assay were performed as described in the text. All renal function parameters were corrected for 100 ml glomerular filtration rate (GFR) and expressed as per cent. The enzyme activities are expressed in μmoles P_i/mg protein/hr. Each figure represents the mean values ± S.E.M. of seven experiments.

† The means of the differences are statistically significant at $0{\cdot}001 < P < 0{\cdot}01$.

‡ C_{Na} = sodium clearance; C_{osm} = osmolar clearance; $T^C_{H_2O}$ = free water reabsorption.



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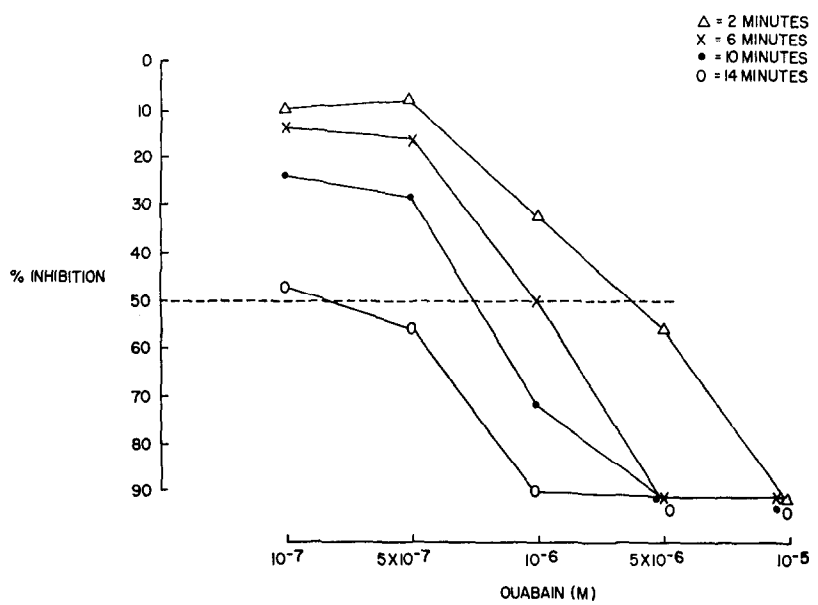

b

FIG. 2. Ouabain inhibition of cortical and medullary Na^+,K^+-ATPase. Na^+,K^+-ATPase was assayed as described in the text, using the Gilford model 2000 recording spectrophotometer, allowing four cuvettes to be continually and simultaneously monitored. One cuvette was always the control (without added ouabain), and ouabain was added to the other three in volumes less than 0·05 ml. The reaction was begun after a 5 min equilibration period by addition of the various enzyme preparations. Total quantity of enzyme protein varied between 10 and 50 μg per cuvette, depending on the specific activity of the enzyme. Since the control reaction is linear, the quantity of inorganic phosphate hydrolyzed is directly proportional to the quantity of NADH oxidized. The reaction mixtures contained (in final concentrations) 2·5 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl, 25 mM Tris HCl (pH 7·4), 2·5 mM ATP, 0·4 mM NADH (Sigma Chemical Company), 2·5 mM phosphoenol-pyruvic acid and 0·02 ml of a pyruvate kinase–lactate dehydrogenase suspension (Sigma Chemical Company). a, cortex; b, medulla.

TABLE 2. EFFECT OF DIGOXIN ON URINARY EXCRETION OF Na^+ *

	Right kidney	Left kidney
Control	68·3 ± 14·6	75·2 ± 13·1
Experimental	78·2 ± 24·6	347·1 ± 71·4 ($P < 0.01$)

* Same as Table 1 renal function studies. This table depicts the actual increase in Na^+ excretion. N = 7. Values are expressed as microequivalents/min.

measurements of Na^+ diuresis showed a marked increase, while Na^+,K^+-ATPases from both cortex and medulla were significantly inhibited and $T^C_{H_2O}$ was unchanged. Table 2 shows the actual increase in Na^+ excretion in seven experiments.

^{3}H-ouabain binding

Control studies. Earlier reports from this laboratory showed that ^{3}H-ouabain specifically binds to Na^+,K^+-ATPase preparations; the binding is maximal in the presence of ATP, Mg^{2+} and Na^+.[30–33] Studies *in vitro* yielded qualitatively the same results for both dog kidney cortical and medullary control preparations (Table 3).

Experimental studies. If digoxin infusion into the renal artery causes diuresis by an inhibition of the Na^+,K^+-ATPase, the glycoside should bind to the inhibitory site *in vivo*, and could conceivably remain bound throughout the isolation procedure, since our previous experiments suggest a tight bond formation between drug and enzyme.[31] Thus, in the experimentally treated enzyme, digitalis sites should be occupied and, therefore, subsequent binding *in vitro* to these isolated preparations should be lower quantitatively than in Na^+,K^+-ATPases isolated from control kidneys. Both control cortex and medullary preparations with high specific activities bound more ouabain (*in vitro*) than their experimental counterparts (Fig. 1). This is further evidence that digitalis glycosides bind to the experimental enzyme *in vivo*.

TABLE 3. ^{3}H-OUABAIN BINDING TO DOG RENAL Na^+,K^+-ATPase*

	−Na^+		+50 mM Na^+	
Additions†	Cortex	Medulla	Cortex	Medulla
1·25 mM ATP	0	0	1·1 ± 0·2	2·4 ± 0·5
1·25 mM $MgCl_2$	0	3·5 ± 0·5	0·5 ± 0·3	7·6 ± 2·5
Mg^{2+} + ATP	1·2 ± 0·8	11·4 ± 1·3	40·5 ± 10·6	130·8 ± 20·7

* Specific activities: cortex = 25·5; medulla = 72·0.

† Cortical or medullary Na^+,K^+-ATPase (0·25 to 0·5 mg protein/ml) was incubated with ^{3}H-ouabain at 37° for 3 min in tubes containing 50 mM Tris HCl (pH 7·4), 1 mM Tris EDTA and the additions as indicated, with and without 10^{-4} M unlabeled ouabain (in alternate tubes) in a volume of 2 ml. The unlabeled ouabain, which competes with ^{3}H-ouabain for receptor sites, is used to compute nonspecific binding.[32,33] The tubes were centrifuged at 100,000 *g* for 10 min, and the resulting pellets were dissolved in 0·3 ml of 0·2 N NaOH in a boiling water bath. The solution was then transferred to a scintillation medium containing naphthalene, 2,5-diphenyloxazole in dioxane, and the radioactivity was determined in a Beckman liquid scintillation spectrometer. The values are means ± S.E.M. of at least five experiments. Similar results were obtained using ^{3}H-digoxin instead of ^{3}H-ouabain. The binding is expressed as pmoles ^{3}H-ouabain/mg protein.

Furthermore, the conditions for binding (ATP, Mg^{2+} and Na^+) are very specific, and the use of the dilution method for determining nonspecific binding enforces the suggestion that the decrease in binding *in vitro* must be due to glycoside binding to specific sites.

DISCUSSION

Other laboratories have corroborated our earlier studies[20,21,34] indicating a correlation between glycoside-induced diuretic effect and Na^+,K^+-ATPase activity.[23,25,26] The role of Na^+,K^+-ATPase in renal concentrating and diluting mechanisms is now under close scrutiny. We have previously suggested[21] that the demonstration of inhibition *in vivo* of both cortical and medullary Na^+,K^+-ATPase, at a time when Na^+ diuresis was occurring (hence, inhibition of net tubular Na^+ reabsorption), indicates involvement of this enzyme in active Na^+ transport in both regions. In particular, after the infusion of digoxin into one renal artery, changes in free water clearance (C_{H_2O}) and free water reabsorption ($T^C_{H_2O}$), both of which are dependent on the active reabsorption of sodium in the medulla, were prevented relative to increased delivery to the distal nephron. This evidence further indicates involvement of Na^+,K^+-ATPase in renal mechanisms.[22]

The specific activities of the Na^+,K^+-ATPase preparations from both regions of the kidney are higher than previously published values,[1–8,11–17,23–26] although Leth-Jorgensen and Skou[35] have recently published data indicating very high specific activities of microgram quantities of recovered enzyme from the inner medulla of rabbit kidneys. The time dependency of the ouabain effect on enzyme preparations from both cortex and medulla is consistent with conclusions we have previously published employing other tissues.[30] Furthermore, the fact that both cortical and medullary Na^+,K^+ATPase preparations exhibit the same ouabain inhibitory profile indicates that at any given time the I_{50} values of ouabain for the two regional enzymes are the same. Any difference in regional Na^+ reabsorption in the kidney would necessitate either a different regional distribution of the enzyme or a large difference in specific activity. The latter appears to be the case.

The digitalis binding data obtained with control preparations further indicate the similarity of digitalis effects on the medulla and cortex. Both preparations are maximally stimulated by Na^+, in the presence of Mg^{2+} and ATP, and follow the standard effects previously noted for other tissues.[30–33]

The decreased ^{3}H-ouabain binding *in vitro* to Na^+,K^+-ATPase isolated from both regions of experimental organs lends credence to the suggestion that the inhibited enzyme activity is due to binding of the drug *in vivo*, and substantiates the postulated role of Na^+,K^+-ATPase in renal functions. The dilution method we have used to determine specific binding sites eliminates the possibility of nonspecific binding *in vivo* interfering with specific binding *in vitro*. Also, in all probability, the nonspecific binding which occurs *in vivo* is washed off during the enzyme isolation procedure.[30] Similar binding data have been presented using Na^+,K^+-ATPase from dog heart with positive inotropic effect as the measurable parameter.[36]

We have earlier suggested[30,37] that the Na^+,K^+-ATPase system is the pharmacologic receptor for the cardiac glycosides. The alteration of active cation movements will lead to different responses, depending on the tissue being studied. The fact that

it takes "more" digoxin to cause a diuresis in the kidney than it takes to cause an increased cardiac inotropic effect does not necessarily reflect a different affinity of the drug for the receptor. Rather it reflects a quantitative difference in the specific chain of events leading to a measurable biological response.

Hence, with this system and its internal control (contralateral kidney), we have presented specific data suggesting that Na^+,K^+-ATPase is a pharmacologic receptor for the cardiac glycosides, and have further strengthened the concept that the digitalis-induced diuresis is a direct result of the combination of glycosides with this enzyme system.

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