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VARIATION IN SENSITIVITY OF THE CARDIAC GLYCOSIDE RECEPTOR CHARACTERISTICS OF (Na^+ + K^+)-ATPase TO LIPOLYSIS AND TEMPERATURE

JOHN S. CHARNOCK, LAURITZ P. SIMONSON and ANTHONY F. ALMEIDA

Department of Pharmacology, University of Alberta, Edmonton, Alberta T6G 2H7 (Canada)

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Summary

1. The rate of binding of [^{3}H]ouabain to untreated membrane preparations of (Na^+ + K^+)-ATPase is a temperature-dependent process displaying a thermal transition close to 25°C. The apparent energies of activation which can be calculated above and below this transition are similar to, but not identical with, those previously reported for activation of the enzyme by cations.

2. Treatment of the enzyme preparation with detergents or lipolysis with phospholipase A eliminates the thermal transition resulting in linear Arrhenius plots.

3. The number of sites available for [^{3}H]ouabain binding is not temperature dependent as the amount of [^{3}H]ouabain bound at equilibrium is not changed between 10 and 37°C.

4. Treatment of the enzyme with phospholipase A results in time-dependent changes in the number of binding sites for [^{3}H]ouabain at equilibrium.

5. Treatment of the membrane enzyme preparations with detergents reveals additional [^{3}H]ouabain binding sites which are extremely sensitive to lipolysis with phospholipase A.

6. There are a number of [^{3}H]ouabain binding sites which remain resistant to lipolysis by phospholipase A in either untreated or detergent-treated membrane preparations.

7. It is suggested that [^{3}H]ouabain binding sites exist in the membrane in at least two different environments, one of which is resistant the other sensitive to attack by phospholipase A.

Introduction

There is a continuing interest in the mechanism of action of the cardiac glycosides as this potent group of pharmacologic agents has been in continuous

therapeutic use since the pioneering work of Withering in 1785 [1]. For example, since it was first suggested a decade ago that ouabain blocks cation transport by inhibiting the turnover of a phosphorylated intermediate of $(Na^+ + K^+)$-ATPase [2—4], there have been numerous studies of the interaction of that cardiac glycoside with particulate preparations of $(Na^+ + K^+)$-ATPase obtained from a wide variety of tissues and species [5—7]. In confirmation of the earlier reports of Schwartz and his colleagues [8,9], Erdmann and Schoner [10,11] have recently produced strong evidence that the characteristics of [^{3}H]ouabain binding to membrane preparations of $(Na^+ + K^+)$-ATPase reflect the properties of a cardiac glycoside receptor.

Studies of the effect of temperature upon both cation activation and ouabain inhibition of $(Na^+ + K^+)$-ATPase in our laboratory suggest that the lipid membranes containing this enzyme exert a differential effect at the sites of cation activation and cardiac glycoside inhibition [12,13]. This paper describes a series of experiments in which this possibility is explored more directly by examining the effect of temperature upon the binding of [^{3}H]ouabain to preparations of $(Na^+ + K^+)$-ATPase following a variety of treatments known to influence membrane lipids.

Materials and Methods

Enzyme preparation. Ouabain-sensitive $(Na^+ + K^+)$-activated ATPase (EC 3.6.1.3) was prepared from both fresh or frozen beef brain by the general procedure of Charnock and Post [14]. Homogenization was carried out at 4°C in the medium described previously [12] using either (a) four strokes of a teflon-glass homogenizer, or (b) one 10 s pulse in a Polytron Homogenizer fitted with a PT-20 generator and operated at setting 8. After removal of the cellular debris by centrifugation at $1000 \times g$ for 15 min in a refrigerated Sorvall RC2-B centrifuge fitted with a SS34 rotor, mitochondrial particles were removed by centrifuging at $9000 \times g$ for 20 min. A "heavy microsomal" pellet was then isolated by centrifugation at $46000 \times g$ for 30 min. This pellet was washed twice by resuspension and sedimentation in buffer (20 mM Tris · HCl/1 mM EDTA at pH 7.6). The washed pellets were resuspended in this buffer and stored at —20°C after rapid freezing in liquid N_2. The protein content of these microsomal suspensions, which was determined by the method of Lowry et al. [15], ranged from 2 to 8 mg per ml.

Membrane enzyme preparations obtained by both method a and b were treated with 0.1% deoxycholic acid for 10 min at 4°C; with 0.1% deoxycholic acid in the presence of 3 mM ATP for 30 min at 30°C; with 0.05% deoxycholic acid and 2 mM ATP in the presence of 5 mM $MgSO_4$ and 80 mM NaCl for 5 min at 30°C; and with 0.1% sodium dodecyl sulfate plus 4 mM ATP for 30—60 min at 30°C as indicated in the text. After each of these treatments the detergent-extracted membranes were sedimented at $46000 \times g$ for 60—120 min and the pellets washed twice by resuspension in buffer.

The procedures for treatment of the enzyme preparations with phospholipase A and subsequent reconstitution was phosphatidylserine were adopted from Imai and Sato [16] and have been described previously [12]. The ratio of phospholipase A to enzyme preparation were determined by preliminary

experiments which monitored the extent of lipolysis by continuous titration of free fatty acid release. Maximum lipolysis usually occurred within 10 min of addition of phospholipase A. Further addition of phospholipase A did not result in further liberation of free fatty acid. Usually this procedure yielded a product having a specific ouabain-sensitive ATPase activity from 20 to 30% of the untreated control preparation. Variations from this time of lipolysis are described in the text.

[^{3}H]Ouabain binding studies. Binding studies were performed in an incubation medium of 100 mM glycylglycine, 2 mM $MgSO_4$, 80 mM NaCl, 2 mM ATP and 0.2 mM H_4EDTA adjusted to pH 7.6 with 1 M Tris/base. Except for some preliminary experiments which are discussed later in the text, the final concentration of [^{3}H]ouabain was $5 \cdot 10^{-7}$ M. The specific radioactivity of the [^{3}H]-ouabain was maintained between 300 and 400 dpm per pmol ouabain.

The bound [^{3}H]ouabain was determined by a rapid Millipore filtration method similar to that employed by others [17,18]. At rapid intervals (as short as 5 s at higher temperatures) 1-ml aliquots were removed from the incubation vessel and filtered on 0.8 μm Millipore filters. Protein retention was virtually complete under all experimental conditions employed, as analysis of filtrates revealed no detectable protein. Assay for ATPase activity in the filtrates also failed to reveal any enzyme activity.

To negate non-specific drug binding to the filters, they were prewashed with 2 ml of a wash solution whose composition was identical to the binding medium but without ATP or radioactivity. The temperature of the binding medium and its respective wash solution was identical. After the initial filtration step, two washes of 2 ml each ensured removal of unbound drug. Increasing the number of washes did not reduce the levels of [^{3}H]ouabain bound.

1-ml aliquots of the incubation mixture were removed for binding analysis at various times after the addition of the enzyme preparations. Binding times were taken to the time the aliquot was applied to the filter. At 37°C the aliquots were removed at 5-s intervals; at lower temperatures the intervals were increased until at 9°C the whole operation took about 250 s. However, at all temperatures samples were removed at much longer intervals (up to 60 min), so that equilibrium levels of [^{3}H]ouabain could be obtained.

Rates of [^{3}H]ouabain binding were determined from the slopes of regression analyses of the levels of bound drug, using the Olivetti program No. 681009. Assays were always in duplicate or more. Individual assays varied less than ±5%, and the mean values were reproducible upon repeated assay of samples stored at −20°C.

The filters were dried in air, disintegrated in 1 ml of methanol and dissolved in 10 ml of 13.5% (v/v) toluene-dioxane fluor and counted to 3% error in a Beckmann LS-100 liquid scintillator.

ATPase activity measurement. All enzyme preparations used in the [^{3}H]-ouabain binding study were routinely assayed for both ouabain-sensitive and ouabain-insensitive ATPase activity under similar conditions to the binding studies.

Enzyme activity was measured by a coupled optical assay system using a Gilford 2400 recording spectrophotometer equipped with a jacketed ethylene glycol/water constant temperature bath [9,19,20]. The temperature of the

spectrophotometer cell was controlled to ±0.2° C. Reactions were performed in 100 mM glycylglycine (pH 7.6) containing 2 mM $MgSO_4$, 80 mM NaCl, 20 mM KCl with 250 mM sucrose added to prevent protein settling. The assay ingredients included 3.14 mM phosphoenolpyruvate (sodium salt) and 64 units of pyruvate kinase, 19 units lactate dehydrogenase and 0.2 mg NADH in a final volume of 3 ml. The enzyme preparation was added to the reaction cuvette and allowed to thermally equilibrate before the reaction was initiated by the addition of ATP to a final concentration of 0.4 mM. Monitoring of NADH oxidation at 340 nm commenced immediately after mixing of the cuvette contents. Mg^{2+}-ATPase activity was followed in the presence of 0.4 mM ouabain, and was subtracted from the total ATPase activity obtained in the presence of Mg^{2+}, Na^+ and K^+ to give the ouabain-sensitive ($Na^+ + K^+$)-ATPase activity.

Determination of activation energies. The effect of temperature on ouabain-sensitive ($Na^+ + K^+$)-dependent ATPase activity, and on the rate of [^{3}H]ouabain binding to these enzyme preparations was determined by a procedure described previously [21]. The data which could be displayed as Arrhenius plots, were further analyzed by the technique of Bogartz [22] for fitting either a single or two intersecting lines, utilizing an APL/360 computer program developed in this laboratory [21]. This analysis yields values for both the apparent activation energies and the critical temperature of the system.

Materials. ATP (disodium salt), glycylglycine, L-histidine (free base), bee venom phospholipase A, phosphoenolpyruvate (sodium salt), pyruvate kinase, lactate dehydrogenase and NADH were all obtained from the Sigma Chemical Co.; [^{3}H]ouabain from New England Nuclear, sucrose (ANALAR grade) from British Drug House Ltd., (Chemical Division); and phosphatidylserine (bovine brain) from Serdary Research Laboratories (Montreal, Quebec).

Results

Preliminary experiments

Although it is well known that two different sets of experimental conditions lead to optimal binding of [^{3}H]ouabain to ($Na^+ + K^+$)-ATPase [20,23—27], we chose to examine [^{3}H]ouabain binding under those conditions which had been previously shown to lead to optimal enzyme phosphorylation, i.e. the presence of ATP, Mg^{2+} and Na^+ [3,4,7,28—30]. In addition, Erdmann and Schoner [10, 11,31] have reported that both maximum drug receptor occupancy and inhibition of ($Na^+ + K^+$)-ATPase activity occur at a ouabain concentration very near $5 \cdot 10^{-7}$ M; although many other workers have shown that the K_i for ouabain inhibition of ($Na^+ + K^+$)-ATPase activity is $5 \cdot 10^{-6}$ M [2,29,30].

We therefore commenced our study by examining [^{3}H]ouabain binding under these reported "optimising" conditions. From preliminary experiments at 37° C we found that in agreement with the reports of Taniguchi and Iida [32, 33], the rate of [^{3}H]ouabain binding only remains linear for about the first 30 s of the experiment; that at this temperature an equilibrium level of bound ouabain is established after about 2 min of incubation; and that this level remains constant thereafter. In addition the binding of [^{3}H]ouabain in the absence of ATP and other activating ligands is negligible compared to that in the presence of both ATP and Na^+. However, in contrast to the findings of Erd-

mann and Schoner [11], we found that the maximum rate of binding was not achieved at $5 \cdot 10^{-7}$ M [^{3}H]ouabain. With ouabain concentrations higher than $1 \cdot 10^{-6}$ M, the rate of drug binding was too fast to be measured experimentally, even by our rapid filtration method, since at 37°C equilibrium levels of binding were reached within 5 s of protein addition. This effect of drug concentration was apparent over the range of enzyme protein concentrations examined (90—240 μg protein/ml). We therefore continued to examine the rate of ouabain binding at a concentration of $5 \cdot 10^{-7}$ M which, although not maximal was technically convenient.

Effect of temperature and lipolysis on the rate of ouabain binding

The effect of temperature upon the rate of [^{3}H]ouabain binding was examined in experiments described in Fig. 1 where it can be seen that the rate of drug binding decreases with decreasing temperature. It is also evident that the linearity of the rate of binding was maintained under the conditions of our experiments. The mean rates of [^{3}H]ouabain binding that can be obtained from these experiments are typical of the values which were used to construct the Arrhenius plots described later in this paper.

All studies of the rate of [^{3}H]ouabain binding were conducted within the linear time period of drug uptake.

In Fig. 2 we give the results of more than 40 experiments using ($Na^+ + K^+$)-

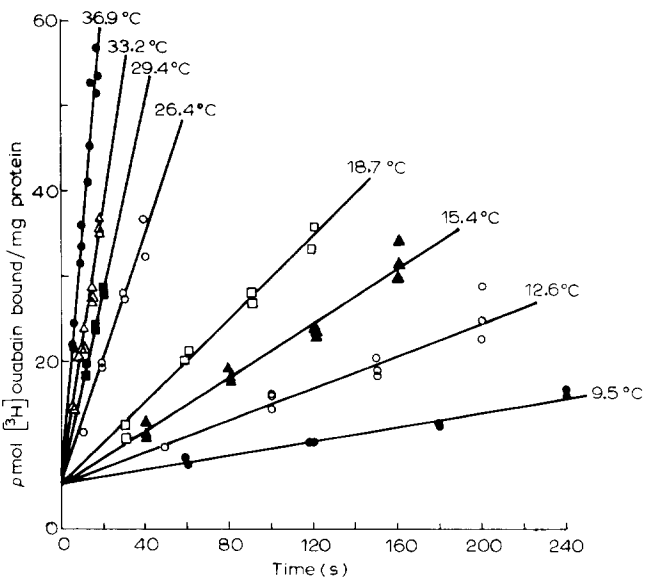

Fig. 1. The effect of temperature on the binding of [^{3}H]ouabain to an untreated membrane preparation of ($Na^+ + K^+$)-ATPase. The enzyme preparation had a specific activity of 16.5 μmol P_i/mg protein per h; 82% of the total ATPase activity of the preparation was inhibited by 0.4 mM ouabain. The protein concentration was 107 μg/ml. The rate of [^{3}H]ouabain binding was determined from the slope of the line obtained at each experimental temperature, using the Olivetti programme No. 681009. The mean initial rates determined in this experiment were from 9.5 to 36.9°C, respectively: 0.05, 0.08, 0.13, 0.20, 0.59, 0.89, 1.24 and 1.95 pmol [^{3}H]ouabain/mg protein per s. Standard error of the means of each temperature were always <10%. Assays were in duplicate or triplicate.

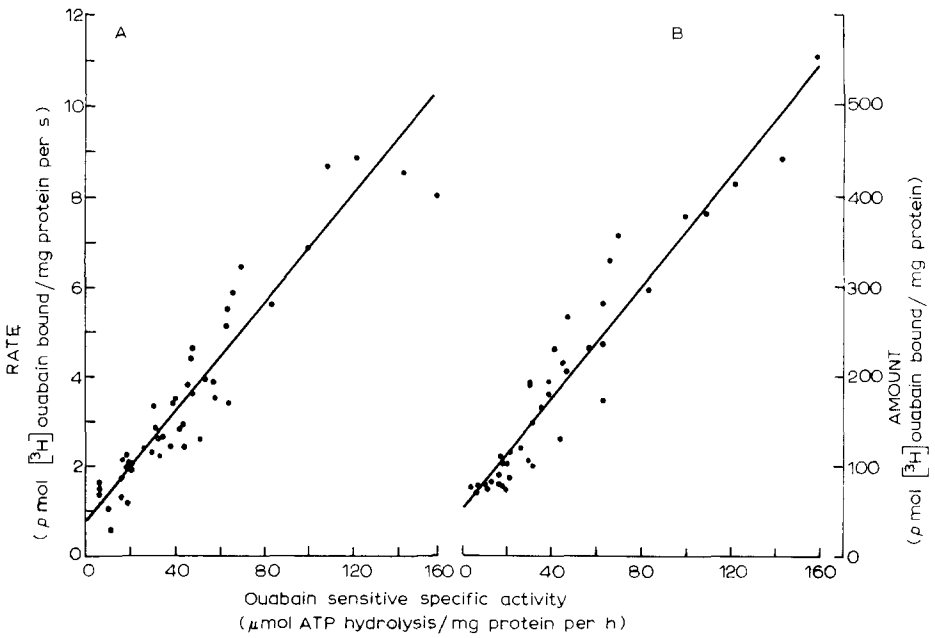

Fig. 2. Relationship of the specific activity of ouabain-sensitive ($Na^+ + K^+$)-ATPase with the rate of [^{3}H]-ouabain binding (Panel A) and the amount of [^{3}H]ouabain bound at equilibrium (Panel B). All experiments were at 37°C. Enzyme preparations of widely different specific activity were obtained from untreated preparations and following the various detergent extractions with deoxycholic acid and sodium dodecyl sulfate described under Materials and Methods. Rates were determined from aliquots taken during the first 30 s after the addition of the enzyme. Each point represents a single enzyme preparation, assays were in duplicate or triplicate. Amounts of [^{3}H]ouabain binding at equilibrium were determined after 10 min incubation at 37°C and are the means ± S.E. of duplicate assays.

ATPase preparations of widely variable specific activity obtained from both untreated and detergent-treated preparations. The relationship between the rate of [^{3}H]ouabain bound at 37°C and the specific activity of ouabain-sensitive ($Na^+ + K^+$)-ATPase activity at this temperature is shown in Panel A. It is clear that the rate of [^{3}H]ouabain binding increases with increasing specific activity of these preparations. The correlation shown is significant at the 0.05 level or better. Panel B gives the relationship between the amount of [^{3}H]ouabain bound at equilibrium and the specific activity of ($Na^+ + K^+$)-ATPase at 37°C. It is again evident that a good correlation is obtained between these latter parameters with significance at the 0.05 level or better. Because our data was obtained from both control and detergent-treated preparations, our findings both confirm and extend the earlier reports of Erdmann and Schoner [10,11].

From the information obtained in these experiments we were able to examine the effect of temperature upon [^{3}H]ouabain binding under conditions of ligand, drug and enzyme protein concentration shown to give rates of binding which were as close to the initial rates as we were able to determine by our procedure.

The experimental data obtained from a series of experiments with untreated enzyme preparations and after detergent extractions with deoxycholic acid under various conditions was first displayed as Arrhenius plots and then values

for the apparent energies of activation above and below the transition temperature were calculated [21,22]. The numerical values for these parameters are given in Table I which shows that in four experiments using untreated beef brain enzymes prepared by limited teflon-glass homogenization (Method a), it is possible to construct a non-linear Arrhenius plot for [^{3}H]ouabain binding which can be described by two straight lines intersecting at a transition temperature (T) of 25.4 ± 2.0°C. Calculation of the mean apparent energies of activation above ($E_{a,I}$) and below ($E_{a,II}$) the critical temperature yields values of 18.5 ± 2.1 and 29.1 ± 1.3 kcal/mol, respectively. Comparison of these mean values by the paired "t" test indicates statistical significance at the 0.01 level.

The mean data from another group of six experiments using untreated beef brain enzymes but prepared by Polytron disintegration (Method b) also yields a non-linear Arrhenius plot with an identical value for the transition temperature of 24.6 ± 1.40°C. Although the mean values for $E_{a,I}$ and $E_{a,II}$ which are derived from this plot (20.9 ± 0.33 and 25.7 ± 0.86 kcal/mol, respectively) are not as widely different from each other as are those seen in the previous group, comparison demonstrates statistical significance at the 0.001 level.

Mild detergent extraction of these two apparently similar enzyme preparations with 0.1% deoxycholic acid at 4°C for 10 min reveals some differences in these materials. With enzyme preparations prepared by method a, there are some obvious quantitative changes following extraction, as the mean values for $E_{a,I}$ and $E_{a,II}$ are now 11.7 ± 1.36 and 21.8 ± 2.2 kcal/mol, respectively. The thermal transition in the Arrhenius plot of [^{3}H]ouabain binding is still apparent, although the mean transition temperature is now somewhat higher than before. (T, 29.0 ± 1.3°C). The non-linearity of the temperature dependence of [^{3}H]ouabain binding has remained unchanged.

On the other hand, when enzyme preparations prepared by method b were extracted with deoxycholic acid it was found that the mean value for $E_{a,I}$ (20.1 ± 1.35 kcal/mol) was no longer significantly different ($P > 0.40$) from the mean value for $E_{a,II}$ (22.1 ± 1.59 kcal/mol). That is this mild detergent extraction of these beef brain preparations has given a product which no longer clearly displays a marked thermal transition for [^{3}H]ouabain binding. Preparations obtained by the presumably less disruptive method a are more resistent to alteration by this form of detergent treatment than are preparations obtained by method b.

In addition, Table I also shows the results obtained by treatment of beef brain enzymes (prepared by method b) with deoxycholic acid at higher temperatures as well as treatment with sodium dodecyl sulfate or phospholipase A. All these more vigorous procedures yield data which can best be described by linear Arrhenius plots, as the Bogartz [22] analysis does not reveal either statistically significant differences in the activation energies which could be calculated for $E_{a,I}$ and $E_{a,II}$, or meaningful values for the transition temperatures. It is of considerable interest that incubation of the enzyme preparation with phosphatidylserine after treatment with phospholipase A did not produce a non-linear temperature dependence for [^{3}H]ouabain binding. Clearly both more vigorous extraction of (Na^+ + K^+)-ATPase with detergents and treatment with phospholipase A gives ratios of $E_{a,I}$: $E_{a,II}$ which are close to unity, that is, plots which are best described as linear.

TABLE I

APPARENT ACTIVATION ENERGIES CALCULATED FROM THE RATE OF [^{3}H]OUABAIN BINDING TO VARIOUS ($Na^+ + K^+$)-ATPase PREPARATIONS AT DIFFERENT TEMPERATURES

	Method	n	$E_{a,I} \pm$ S.E. (kcal/mol)	$E_{a,II} \pm$ S.E. (kcal/mol)	$\frac{E_{a,I}}{E_{a,II}}$	$T \pm$ S.E. (°C)	$E_{a,I}$ vs. $E_{a,II}$ P
Untreated	a	4	18.5 ± 2.10	29.1 ± 1.30	0.64	25.4 ± 2.00	<0.01
Deoxycholic acid (4°C)	a	7	11.7 ± 1.36	21.8 ± 2.20	0.54	29.0 ± 1.30	<0.01
Untreated	b	6	20.9 ± 0.33	25.7 ± 0.86	0.81	24.6 ± 1.40	< 0.001
Deoxycholic acid (4°C)	b	3	20.1 ± 1.35	22.1 ± 1.59	0.91	27.2 ± 8.26	>0.40
Deoxycholic acid + ATP (30°C)	b	6	20.4 ± 0.56	21.9 ± 0.37	0.93	43.7 ± 3.06	>0.05
Deoxycholic acid + ATP + Mg^{2+} + Na^+ (30°C)	b	5	22.8 ± 0.37	21.5 ± 1.79	1.06	9.5 ± 2.25	>0.50
Sodium dodecyl sulfate + ATP (30°C)	b	3	21.9 ± 1.84	21.4 ± 1.06	1.02	3.15 ± 3.41	>0.80
Deoxycholic acid (4°C) + phospholipase A *	b	3	26.5 ± 2.43	25.8 ± 2.31	1.03	−27.0 ± 12.4	>0.80
Deoxycholic acid (4°C) + phospholipase A * + phosphatidylserine	b	2	22.2 ± 0.80	24.3 ± 6.25	0.91	40.2 ± 36.7	>0.70

* 5 units phospholipase A for 5 min at 37°C.

TABLE II

APPARENT ENERGIES OF ACTIVATION CALCULATED FROM THE RATE OF ATP HYDROLYSIS BY OUABAIN-SENSITIVE ($Na^+ + K^+$)-ATPase PREPARATIONS AT DIFFERENT TEMPERATURES

	Method	n	$E_{a,I} \pm$ S.E. (kcal/mol)	$E_{a,II} \pm$ S.E. (kcal/mol)	$\frac{E_{a,I}}{E_{a,II}}$	$T \pm$ S.E. (°C)	$E_{a,I}$ vs. $E_{a,II}$ P
Untreated	a	9	18.1 ± 1.10	37.0 ± 3.40	0.49	20.0 ± 1.00	<0.001
Deoxycholic acid (4°C)	a	6	21.8 ± 0.51	49.2 ± 3.90	0.44	17.8 ± 0.80	<0.001
Untreated	b	4	14.6 ± 1.95	29.8 ± 0.30	0.49	20.9 ± 1.31	<0.001
Deoxylcholic acid (4°C)	b	4	19.7 ± 1.89	41.8 ± 3.08	0.47	17.9 ± 0.93	<0.001
Deoxycholic acid + ATP (30°C)	b	5	16.4 ± 0.80	45.4 ± 5.40	0.36	16.6 ± 1.83	<0.001
Deoxycholic acid + ATP + Mg^{2+} + Na^+ (30°C)	b	3	15.1 ± 0.93	32.8 ± 5.30	0.46	19.2 ± 1.55	<0.05
Sodium dodecyl sulfate + ATP (30°C)	b	3	13.6 ± 1.68	30.2 ± 1.20	0.45	20.0 ± 1.27	<0.01
Deoxycholic acid (4°C) + phospholipase A *	b	3	26.9 ± 1.30	20.3 ± 4.20	1.32	30.8 ± 5.00	<0.20
Deoxycholic acid (4°C) + phospholipase A * + phosphatidylserine	b	2	17.6 ± 2.10	35.9 ± 3.45	0.49	22.7 ± 2.40	<0.05

* 5 units phospholipase A for 5 min at 37°C.

By contrast, Table II gives the data for the temperature dependence of ATP hydrolysis by these enzyme preparations which were obtained in paired experiments. As we have demonstrated previously [12,13], there is a marked non-linear response to temperature with all untreated and treated preparations of $(Na^+ + K^+)$-ATPase except following lipolysis with phospholipase A. Again we have been able to demonstrate that this effect of phospholipase A on the rate of ATP hydrolysis can be overcome by reconstitution of the treated enzyme preparations with phosphatidylserine [12,13]. However, under conditions of our experiments (3 mg phosphatidylserine/mg protein at 37°C for 10 min) reconstitution of phospholipase A-treated enzyme preparations with phosphatidylserine only restored the non-linearity to the Arrhenius plots for ATP hydrolysis and not to those for [^{3}H]ouabain binding (cf. Tables I and II). Nevertheless, it should be noted that the ratio of $E_{a,I}$: $E_{a,II}$ which is obtained for [^{3}H]ouabain binding after treatment with phosphatidylserine shows a tendency towards non-linearity.

We can conclude from these studies of the effect of temperature on the rate of [^{3}H]ouabain binding to $(Na^+ + K^+)$-ATPase that this characteristic of the drug-receptor interaction is lipid dependent, and is more susceptible to change by detergent extraction than is the hydrolysis of the substrate by the enzyme. Apparently membrane lipids are involved in both processes but our preliminary attempts at reconstitution with phosphatidylserine imply that different membrane lipids are associated with these different characteristics of the enzyme receptor system.

Effect of temperature and lipolysis on the amount of ouabain binding

The effect of temperature on the amount of [^{3}H]ouabain which was bound to the enzyme preparation at equilibrium was also examined. The results of a study using untreated enzyme preparations is given in Table III. At true equilibrium temperature does not affect the amount of drug which can be bound; that is, temperature does not alter the number of ouabain receptors available for binding.

TABLE III

EQUILIBRIUM LEVELS OF OUABAIN BOUND TO BEEF BRAIN $(Na^+ + K^+)$-ATPase AT VARIOUS TEMPERATURES

Binding reactions were started by the addition of protein to a final concentration of 225 μg/ml and terminated by filtration after 30 min, except for the lowest temperature * where equilibrium was not reached until 60 min after protein addition. The concentration of ouabain was $5 \cdot 10^{-7}$ M in all experiments. The values given are the means ± S.D. of four experiments.

Temperature (°C)	[^{3}H]ouabain bound pmol/mg protein ± S.D.
36.8	87 ± 5.7
33	90.6 ± 2.3
29.3	85 ± 2.5
26.5	94.6 ± 5.2
18.5	94.3 ± 5.2
15.3	89 ± 3.6
12	85 ± 4.8
9.3	93.2 ± 4.2 *

However, it is possible to alter the number of ouabain binding sites under certain experimental conditions. For example, although mild treatment of the enzyme preparations with deoxycholic acid at 4°C only leads to a relatively small increase in the specific activity of the enzyme and in the amount of [^{3}H]-ouabain bound at equilibrium, more pronounced detergent treatment with either deoxycholic acid or sodium dodecyl sulfate at 30°C in the presence of ATP produces a marked increase in both enzyme specific activity and the amount of [^{3}H]ouabain bound at equilibrium (Table IV). Apparently, additional binding sites are exposed after detergent extraction, indicating that lipid-lipid or lipid-protein interactions previously prevented ready access of [^{3}H]-ouabain to a potential binding site. It is important to note from the data in Fig. 2 that the increase in [^{3}H]ouabain binding which follows treatment with detergents is proportional to the increase in ouabain-sensitive ($Na^+ + K^+$)-ATPase which also occurs after this treatment.

In contrast to the increase in the amount of [^{3}H]ouabain bound after detergent extraction, Taniguchi and Iida [32,33] have reported that treatment of the enzyme with phospholipase A resulted in a loss of activity and a reduction in the initial rate of [^{3}H]ouabain binding. These workers also reported that the "binding capacities" of the ouabain binding site showed no remarkable change as a consequence of treatment with phospholipase A.

In our experiments we found that the effects of phospholipase A varied with both the time of lipolysis and with pretreatment of the enzyme with detergents. For example, if untreated enzyme preparations are exposed to phospholipase A for only brief periods there is a biphasic effect. This is shown by an initial increase in specific activity and drug binding which is followed by a reduction in both these parameters to near control levels after about 10 min of exposure. This can be seen in Fig. 3 where treatment with 20 units of phospholipase A was followed for 40 min. Under these conditions it is clear that the action of phospholipase A does not result in a marked reduction in the amount of [^{3}H]ouabain bound to ($Na^+ + K^+$)-ATPase.

On the other hand, if the enzyme preparations were first extracted with deoxycholic acid at 4°C, the biphasic effect of phospholipase A was lost. This is shown in Fig. 4 where it can be seen that while the initial levels of both ouabain-sensitive ($Na^+ + K^+$)-ATPase activity and [^{3}H]ouabain binding are

TABLE IV

COMPARISON OF THE SPECIFIC ACTIVITY AND EQUILIBRIUM LEVELS OF [^{3}H]OUABAIN BOUND TO ($Na^+ + K^+$)-ATPase AFTER VARIOUS TREATMENTS WITH DETERGENTS

Specific activity given as μmol ATP hydrolysis/mg protein per h at 37°C. Equilibrium level given as pmol [^{3}H]ouabain bound/mg protein.

Treatment	*n*	Specific activity (mean ± S.E.)	Equilibrium level (mean ± S.E.)
None	14	15.2 ± 2.5	90.5 ± 6.1
Deoxycholic acid at 4°C	16	31.6 ± 3.2	146.5 ± 12.7
Deoxycholic acid + ATP at 30°C	3	96.9 ± 18.0	342 ± 55
Deoxycholic acid + ATP + Mg^{2+} + Na^+ at 30°C	7	60.9 ± 4.8	295 ± 26
Sodium dodecyl sulfate + ATP at 30°C	3	133 ± 17	457 ± 51

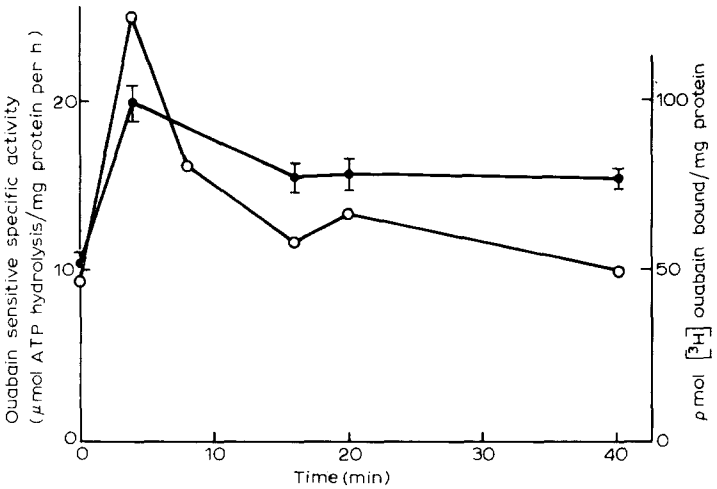

Fig. 3. Effect of progressive treatment at 37°C with 20 units of bee venom phospholipase A/mg protein on the specific activity and binding of [^{3}H]ouabain at equilibrium to otherwise untreated membrane preparations of (Na^+ + K^+)-ATPase. ●, [^{3}H]ouabain binding given as pmol/mg protein, values are the means ± S.E. of triplicate determinations from 1-ml aliquots taken after 5 min incubation at 37°C; ○, specific activity given as μmol P_i/mg protein per h at 37°C; values are means of duplicate assays. 1 unit of phospholipase A hydrolyses 1 μmol of L-α-lecithin to lysolecithin and fatty acid per min at pH 8.5 at 37°C.

much higher than those of untreated enzyme preparations, the action of phospholipase A is to produce an immediate and progressive decline in the specific activity of the preparations which was accompanied by a fall in the amount of [^{3}H]ouabain bound to these preparations under equilibrium conditions. However, under these conditions the amount of [^{3}H]ouabain bound to the prepa-

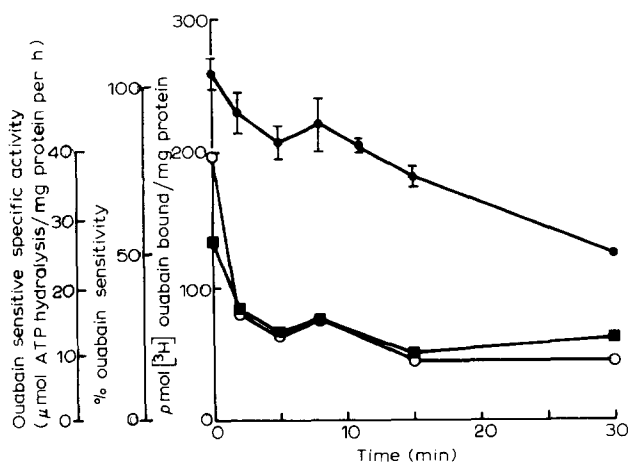

Fig. 4. Effect of progressive treatment at 37°C with 5 units of bee venom phospholipase A/mg enzyme protein on the specific activity and binding of [^{3}H]ouabain at equilibrium to membrane preparations of (Na^+ + K^+)-ATPase after 10 min extraction with 0.1% deoxycholic acid at 4°C. ●, [^{3}H]ouabain binding gives as pmol/mg protein, values are means ± S.E. of four times 1-ml aliquots taken after 5 min incubation at 37°C; ○, specific activity given as μmol P_i/mg protein per h at 37°C; values are means of duplicate assays; ■, ouabain sensitivity expressed as percent of the total ATPase activity of the preparation assayed in duplicate ± 0.4 mM ouabain in the medium as described under Materials and Methods. Note the 4-fold reduction in concentration of phospholipase A from the experiments described in Fig. 3.

TABLE V

EFFECT OF PHOSPHOLIPASE A ON SPECIFIC ACTIVITY, RATE AND EQUILIBRIUM BINDING OF [^{3}H]OUABAIN TO ($Na^+ + K^+$)-ATPase

Specific activity given as μmol ATP hydrolysis/mg protein per h at 37°C. Rate [^{3}H]ouabain binding given as pmol [^{3}H]ouabain bound/mg protein per s at 37°C. Equilibrium level [^{3}H]ouabain binding given as pmol [^{3}H]ouabain bound/mg protein.

Experiment	Control			Phospholipase A		
	Specific Activity	Rate [^{3}H]ouabain binding	Equilibrium level [^{3}H]ouabain binding	Specific activity	Rate [^{3}H]ouabain binding	Equilibrium level [^{3}H]ouabain binding
a	38.7	3.40	194	5.4	2.57	157
b	44.4	3.80	216	8.3	2.28	155
c	29.4	3.34	192	4.6	1.97	133
d	19.6	1.92	102	2.4	0.80	70.7
e	17.6	2.25	102	2.09	0.92	77.4
f	15.8	2.13	79.1	2.05	0.83	57.3
g	18.9	2.07	74.0	2.12	0.59	50.6
Mean ± S.E.	26.3 ± 4.3	2.70 ± 0.29	137 ± 23	3.85 ± 0.90	1.42 ± 0.31	100 ± 17.6
Inhibition (%)	Nil	Nil	Nil	86.2 ± 1.0	50.8 ± 6.1	27.3 ± 1.7

ration does not fall in parallel with the reduction of enzyme activity. This is shown by the data from seven experiments given in Table V, where the reduction in specific activity which occurs after 5 min incubation with 5 units of phospholipase A is compared to the rate and amount of [^{3}H]ouabain binding. Under these conditions the activity of the enzyme has been reduced to 14% of the mean control values, the mean rate of [^{3}H]ouabain binding is 49% of the controls while the mean amount of [^{3}H]ouabain which can be bound at equilibrium is only reduced to 73% of the control level. That is both the biochemical activity of the enzyme and the function of the binding sites are more susceptible to lipolysis with phospholipase A than are the number of sites available.

Discussion

Although there are many reports in the literature concerning the binding of cardiac glycosides to particulate preparations of ($Na^+ + K^+$)-ATPase, much of the data has been conflicting [24—27,31—39]. One contributing factor to this conflict has been the variable methodology employed by the numerous investigators. The studies of Erdmann and Schoner [10,11] have confirmed that under conditions of optimal phosphorylation of the enzyme viz. the presence of ATP, Mg^{2+} and Na^+, binding experiments conducted at equilibrium reflect the number of available cardiac glycoside binding sites per unit mass of enzyme, while very short term rate studies reflect the affinity of these sites rather than the number of sites available. When these distinctions are borne in mind some of the apparent differences reported in the literature can be resolved.

In recent years several laboratories have demonstrated that treatment of membrane preparations of ($Na^+ + K^+$)-ATPase with lipases destroys the thermal transitions which can be observed in the hydrolysis of ATP by this enzyme [12, 13,33,40—42], as well as reduces the rate of [^{3}H]ouabain binding to the preparations in the presence of various combinations of ligands [32,36].

In the present work we have shown that the temperature dependence of the rate of [^{3}H]ouabain binding to untreated enzyme preparations also displays a non-linear relationship with a break in Arrhenius plots at about 25° C. However, the differences in activation energy for this process which can be calculated above and below the critical temperature are somewhat less than the differences in the activation energies for ATP hydrolysis which were determined in paired experiments.

Our conclusions concerning the non-linear effect of temperature upon the rate of [^{3}H]ouabain binding do not agree with those of Siegel and Josephson [23] or Schwartz and his colleagues [35,43]. The experiments by Siegel and Josephson [23] were conducted after 15 min of incubation which under our conditions, would neither reflect the initial rates nor the equilibrium levels of drug binding, particularly in the lower temperature range examined.

Conversely, the experiments reported by Schwartz and his colleagues [35,43] should provide data reflecting the affinity of cardiac glycoside binding sites similar to that observed in our experiments. Although in the experiments of Wallick and Schwartz [43] there are an insufficient number of data points for the construction of Arrhenius plots suitable for the Bogartz [22] analysis

we employ, inspection of their data reveals that their results might also be described as non-linear. Recalculation of their data suggests that like our own findings, the differences in activation energies for [^{3}H]ouabain binding above and below the critical temperature are not as marked as is the case with ATP hydrolysis by these membrane enzyme preparations. Presumably, both our findings and those of Wallick and Schwartz [43] indicate that the binding of [^{3}H]ouabain to (Na^+ + K^+)-ATPase is less influenced by the physical state of the membrane lipids than is activation of the system by cations [12,13,44].

However, it is apparent that lipids do play at least some part in the temperature dependence of [^{3}H]ouabain binding to (Na^+ + K^+)-ATPase as detergent treatment of the preparations resulted in the loss of the discontinuity in temperature dependence and the observation of linear Arrhenius plots. The comparative ease with which detergents remove this effect suggests that the lipids which influence [^{3}H]ouabain binding are not as closely associated with the membrane protein as are those which are responsible for modulation of the temperature dependence of ATP hydrolysis [12,13]. Perhaps the former is a so-called "bulk" lipid phenomenon while the latter is more likely to be the immobilized boundary lipid type recently described by Metcalfe and his colleagues [45,46] for Ca^{2+}-ATPase. In the limited number of experiments we attempted here, it is of considerable interest that although reconstitution of phospholipase A-treated preparations with phosphatidylserine is able to regain the characteristic non-linear temperature dependence for ATP hydrolysis that we have reported before [12,13], that is not necessarily the case for [^{3}H]-ouabain binding. As only one set of experimental conditions for reconstitution with phosphatidylserine was employed here (3 mg phosphatidylserine/mg protein at 37°C for 10 min) it is clear that these attempts at reconstitution will have to be extended before the role of phosphatidylserine in [^{3}H]ouabain binding to (Na^+ + K^+)-ATPase can be decided.

Nevertheless, the partial dependence of [^{3}H]ouabain binding upon lipids is demonstrated by the effects of phospholipase A treatment upon the amount of [^{3}H]ouabain bound to the enzyme preparation at equilibrium at 37°C. With untreated enzyme preparations there is an initial increase in the amount of drug binding which falls off after about 5 min of incubation, but does not fall below control levels during further treatment with phospholipase A. In these experiments the pattern of specific activity of ouabain-sensitive (Na^+ + K^+)-ATPase activity parallels the changes in [^{3}H]ouabain binding which were observed.

On the other hand, if the membrane enzyme preparations are first treated with the detergents deoxycholic acid or sodium dodecyl sulfate at 30°C (in the presence of ATP) there is a very marked increase in both the specific activity of ouabain-sensitive (Na^+ + K^+)-ATPase and the amount of [^{3}H]ouabain bound to the enzyme. This increase in [^{3}H]ouabain binding is now very sensitive to treatment with phospholipase A and is reduced by about 50% in 15 min incubation with a much reduced concentration of lipase.

It should be noted that the final levels of ouabain-sensitive ATPase activity and [^{3}H]ouabain binding which are reached after treatment with phospholipase A are very similar whether detergent-treated or untreated control enzyme was used; apparently this residual enzyme activity and its associated [^{3}H]ouabain binding sites are very resistant to attack by this lipase. In agreement with the

earlier work of Taniguchi and Iida [36], and the recent conclusions of Hansen [47], which were published while this manuscript was in preparation, we also conclude that there are two different types of [^{3}H]ouabain binding sites available in membrane preparations of (Na^+ + K^+)-ATPase. Because of the marked sensitivity of only one of these sites to lipolysis with phospholipase A following detergent extraction, it seems very likely that not only are these sites located in different regions of the membrane but that only one site is closely associated with a phospholipid component of the membrane. Whether these sites are equally accessible for cardiac glycoside binding in situ, or whether either of these sites represents a more pharmacologically active "receptor" cannot be determined from the present work, but it is possible that variations in membrane lipids will exert differential effects on the drug-receptor interactions at these different sites. Whether either of these sites resemble the clinically important cardiac glycoside receptor of cardiac muscle remains uncertain [48–50].

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