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SATURABLE ADENOSINE 5′-TRIPHOSPHATE-INDEPENDENT BINDING OF [^{3}H]-OUABAIN TO BRAIN AND CARDIAC TISSUE *in vitro*

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1 Several investigators have proposed that membrane Na^+, K^+-adenosine 5′-triphosphatase (Na^+, K^+-ATPase) is a mechanism for the transmembrane transport of cardiac glycosides, rather than the receptor for pharmacological actions of these agents. This implies that the glycosides bind to an intracellular constituent (receptor) other than Na^+, K^+-ATPase.

2 In search for such a receptor site, saturable ATP-independent [^{3}H]-ouabain binding was studied in rat brain and dog and guinea-pig heart homogenates. The binding of the glucoside to this site results in a relatively unstable complex which is stabilized by K^+ to a lesser extent than is the complex formed with the ATP-dependent binding to Na^+, K^+-ATPase.

3 The ATP-independent ouabain binding sites are more abundant in rat brain tissue than in cardiac tissue, and have a lower ouabain affinity compared to the binding sites on Na^+, K^+-ATPase.

4 These results do not support the contention that there are intracellular inotropic receptors for digitalis.

Introduction

The binding of digitalis to cardiac Na^+, K^+-adenosine 5′-triphosphatase (Na^+, K^+-ATPase) observed *in vitro* in the presence of Na^+, Mg^{2+} and ATP satisfies all the criteria for 'receptor' binding (Akera, 1977). This binding and that observed *in vivo* or in isolated hearts closely parallels the positive inotropic action of these agents (Akera, Larsen & Brody, 1969; 1970; Besch, Allen, Glick & Schwartz, 1970; Prindle, Skelton, Epstein & Marcus, 1971; Akera, Baskin, Tobin & Brody, 1973; Ku, Akera, Pew & Brody, 1974; Schwartz, Allen, Van Winkle & Munson, 1974; Goldman, Coltart, Schweizer, Snidow & Harrison, 1975; Hall, Gilbart, Silverman & Goldman, 1977). Nevertheless, the concept that Na^+, K^+-ATPase is the receptor for the positive inotropic action of digitalis is not universally accepted (Okita, 1977).

Membrane sodium pump, the functional correlate of Na^+, K^+-ATPase, apparently has a reserve capacity, as indicated by the failure of an increase in Na^+ influx to cause a proportional increase in the intracellular sodium concentration (Langer, 1968). Similarly, inotropic concentrations of digitalis, which produce moderate Na^+, K^+-ATPase inhibition, fail to cause a corresponding increase in intracellular sodium (Langer & Serena, 1970; Lee & Klaus, 1971). Thus, it appears, at least superficially, that partial sodium pump inhibition *per se* observed with inotropic concentrations of digitalis does not have biochemical and physiological consequences, although the binding of digitalis to this enzyme is closely related to the action of this agent. In order to reconcile these observations, several investigators (Dutta, Goswami, Datta, Lindower & Marks, 1968; Park & Vincenzi, 1975; Fricke & Klaus, 1977) have proposed that Na^+, K^+-ATPase is involved in the transport of digitalis from an outer to an inner compartment of the cell membrane. Implicit in this hypothesis is that there is an unidentified intracellular receptor which is responsible for the positive inotropic action of digitalis.

If there is such a receptor, it should be characterized as a high affinity, ATP-independent binding site for the glycoside. The ATP-dependent binding of [^{3}H]-ouabain to tissue preparations has repeatedly been shown to be a stoichiometric binding of this agent to Na^+, K^+-ATPase (Hansen, Jensen & Nørby, 1971; Allen, Martines-Maldonado, Eknoyan, Suki & Schwartz, 1971). Preliminary experiments with rat brain homogenates indicated that there are ATP-independent, saturable binding sites for ouabain in this tissue preparation. This finding prompted us to explore whether high affinity ATP-independent binding sites for ouabain exist in cardiac tissue and whether these sites are unrelated to Na^+, K^+-ATPase.

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Methods

Tissue homogenates

Cardiac tissue was obtained from mongrel puppies (500 to 1000 g) and guinea-pigs (350 to 500 g) of either sex. A 1 g portion of the left ventricular muscle was minced and homogenized in 9 volumes of an ice-cold solution containing 0.25 M sucrose, 5 mM histidine and 1 mM disodium ethylene diamine tetraacetate (pH adjusted to 7.0 with Tris base) with a Dounce ball-type homogenizer and a motor-driven Potter-Elvehjem homogenizer. Brain homogenates were obtained from male Sprague-Dawley rats weighing 200 to 250 g. These animals were decapitated, their brains removed immediately and homogenized in 9 volumes of an ice-cold suspending solution as above with a Dounce ball-type homogenizer. All preparative procedures were carried out at 2°C. Protein concentration was assayed by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as the standard.

[^{3}H]-ouabain binding and dissociation studies

The binding of [^{3}H]-ouabain to tissue homogenates was assayed in three different incubation media: a mixture containing 5 mM ATP, 200 mM NaCl, 5 mM $MgCl_2$ and 50 mM Tris–HCl buffer (pH 7.5) for total [^{3}H]-ouabain binding; a similar mixture but without ATP, in order to estimate ATP-independent [^{3}H]-ouabain binding; and a mixture containing 1 mM non-labelled ouabain in the absence of ATP for the determination of nonsaturable [^{3}H]-ouabain binding. The protein concentration in the incubation mixture was 0.2 mg/ml and the [^{3}H]-ouabain concentration was 0.1 μM in the dog heart and guinea-pig heart studies and 0.02 μM in rat brain studies unless otherwise indicated. The binding reaction was started after a 5 min preincubation at 37°C by addition of the homogenate to the incubation mixture. Aliquots were taken at appropriate time intervals and bound [^{3}H]-ouabain was separated from unbound ouabain by the Millipore filter system. Millipore filters were dissolved in ethylene glycol monomethyl ether and the radioactivity of bound ouabain trapped on the filter was assayed by liquid scintillation counting. Counting efficiency (approx 30%) was monitored by the external standard channel ratio. [^{3}H]-ouabain binding was expressed as pmol of ouabain bound per mg protein. Saturable ATP-dependent binding was estimated as the difference between [^{3}H]-ouabain binding observed in the presence and absence of ATP, and saturable ATP-independent binding was calculated by subtraction of the binding observed in the presence of 1 mM non-labelled ouabain from that observed in the absence of ATP and non-labelled ouabain.

In one series of experiments, the dissociation of bound [^{3}H]-ouabain was monitored at 37°C. The binding reaction was stopped after 10 min of incubation at 37°C and then the mixture was centrifuged at 100,000 *g* for 60 min at 0°C. The sediment was resuspended in 10 mM Tris–HCl buffer (pH 7.5) and the dissociation reaction was immediately studied at 37°C in the presence and absence of 16.6 mM KCl. Aliquots were taken at appropriate time intervals and the amount of bound [^{3}H]-ouabain which remained undissociated was estimated as above. First order reaction kinetics were assumed and rate constants were calculated from the initial slope of the semilogarithmic plot of the [^{3}H]ouabain dissociation curve.

ATPase activity assay

In order to determine if ATP-dependent and ATP-independent ouabain binding result in enzyme inhibition, the ATPase activity of the ouabain-bound preparations was assayed from the amount of inorganic phosphate liberated from ATP (Akera *et al.*, 1969). Rat brain homogenates (0.2 mg protein/ml) were incubated at 37°C for 10 min with various concentrations of ouabain in the presence of 200 mM NaCl, 5 mM $MgCl_2$, and 50 mM Tris–HCl buffer (pH 7.5) with or without 5 mM Tris-ATP. The binding reaction was stopped by placing the incubation mixture in an ice bath and the mixture was centrifuged at 100,000 *g* for 60 min. The resulting sediment was resuspended in 10 mM Tris–HCl buffer and assayed for Na^+, K^+-ATPase activity. The incubation mixture used to assay total ATPase activity contained 100 mM NaCl, 15 mM KCl, 5 mM $MgCl_2$, 50 mM Tris–HCl buffer (pH 7.5), and 0.12 mg resuspended protein per ml. Mg^{2+}-activated ATPase activity was assayed in an incubation medium containing 5 mM $MgCl_2$, 50 mM Tris–HCl and 0.12 mg of resuspended protein per ml. After a 5 min preincubation without ATP at 37°C, ATP was added to start the reaction. The ATPase reaction was stopped 10 min later by placing the incubation mixture in an ice bath and immediately adding perchloric acid (final concentration 0.4 N). The mixture was centrifuged at 1500 *g* for 30 min. The concentration of inorganic phosphate in an aliquot of the supernatant was determined by the method of Bonting, Simon & Hawkins (1961). The Na^+, K^+-ATPase activity, expressed in μmol Pi mg^{-1} protein h^{-1}, was calculated as the difference between total ATPase activity and Mg^{2+}-ATPase activity. Results were analyzed for statistical significance by the paired *t* test. Criterion for significance was a *P* value of less than 0.05.

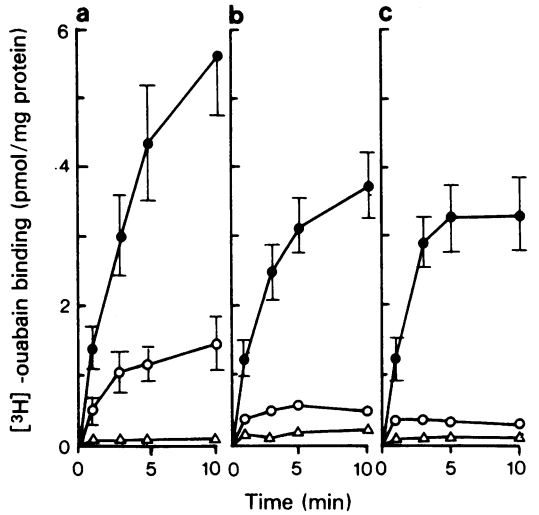

Figure 1 ATP-dependent and ATP-independent [^{3}H]-ouabain binding to tissue homogenates. (a) Rat brain homogenate (0.2 mg protein/ml) and 0.02 μM [^{3}H]-ouabain were incubated with 200 mM NaCl, 5 mM $MgCl_2$, and 50 mM Tris–HCl buffer (pH 7.5) at 37°C in the presence (●), and absence (○) of 5 mM Tris–ATP and presence of 1.0 mM cold ouabain (△). Similarly, dog heart (b) and guinea-pig heart (c) homogenates (0.2 mg protein/ml) were incubated with 0.1 μM [^{3}H]-ouabain under these three conditions. At the indicated times after the addition of homogenates, aliquots were analyzed for bound [^{3}H]-ouabain. Values are means of five (rat brain) or four (dog heart and guinea-pig heart) experiments; vertical lines indicate s.e. mean.

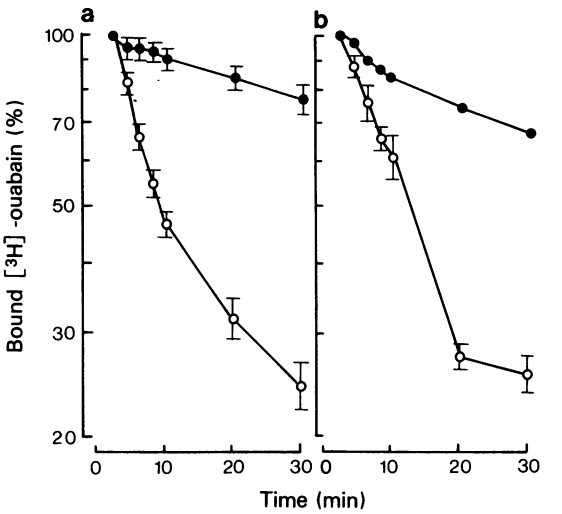

Figure 2 Effect of KCl on dissociation rates of bound [^{3}H]-ouabain due to ATP-dependent (a) and ATP-independent (b) binding. Rat brain homogenates (0.2 mg protein/ml) and 0.02 μM [^{3}H]-ouabain were incubated at 37°C for 10 min in the presence of 200 mM NaCl, 5 mM $MgCl_2$, and 50 mM Tris–HCl buffer (pH 7.5) with or without 5 mM Tris–ATP. The mixture was centrifuged at 100,000 *g* for 60 min at 0°C, and the pellet was resuspended in 10 mM Tris–HCl buffer (pH 7.5) containing no KCl (○) or 16.6 mM KCl (●). Dissociation was monitored at 37°C. Bound [^{3}H]-ouabain for each preparation at the beginning of the dissociation reaction was set at 100%. Vertical lines indicate the s.e. mean of five experiments.

Results

The purpose of the initial studies was to determine if [^{3}H]-ouabain exhibits saturable binding to tissue homogenates in the absence of ATP. Homogenate preparations from rat brain, dog heart, and guinea-pig heart were incubated at 37°C with Na^+ and Mg^{2+} in the presence and absence of ATP. Non-saturable [^{3}H]-ouabain binding was also estimated concurrently by incubating the homogenate and [^{3}H]-ouabain in the presence of 1 mM non-labelled ouabain. Figure 1 shows that three different levels of [^{3}H]-ouabain binding can be observed in several tissues depending on ligand conditions. A saturable ATP-independent [^{3}H]-ouabain binding (difference between the binding observed in the absence and presence of 1.0 mM ouabain in a medium containing no ATP) could be demonstrated in the three tissue preparations examined. When compared to the total [^{3}H]-ouabain binding observed in the presence of ATP, rat brain homogenates exhibited the largest fraction of saturable ATP-independent [^{3}H]-ouabain binding. Such binding was substantially smaller in cardiac tissues.

Characteristics of the ATP-dependent and ATP-independent [^{3}H]-ouabain binding were compared by determining the effect of potassium on the dissociation of the bound ouabain-tissue complex formed in these reactions. In these experiments, rat brain homogenates were used because of the relatively large ATP-independent ouabain binding observed. [^{3}H]-ouabain and the homogenate were incubated with various ligands for 10 min at 37°C. Subsequently, unbound [^{3}H]-ouabain was removed by centrifugation of the mixture and resuspension of the resultant pellet. Dissociation of the bound drug was monitored at 37°C in Tris–HCl buffer in the presence or absence of KCl. In order to facilitate the comparison of the dissociation rate constants, the bound [^{3}H]-ouabain for each fraction in each preparation was set at 100% at the beginning of the dissociation reaction. The rate of dissociation of both the ATP-dependent and ATP-independent complex was fairly rapid with apparent first-order dissociation rate constants of approxi-

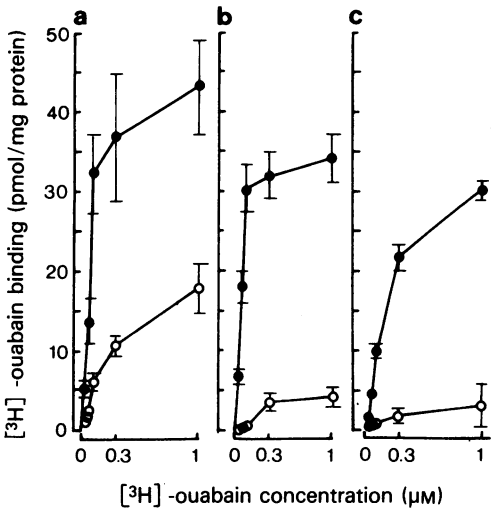

Figure 3 ATP-independent and ATP-dependent binding of [^{3}H]-ouabain to brain and heart homogenates observed with various [^{3}H]-ouabain concentrations. Binding of [^{3}H]-ouabain to homogenates (1.0 mg protein/ml) was assayed with [^{3}H]-ouabain concentrations ranging from 0.01 to 1.0 μM. See legend to Figure 1. At 20 min, aliquots of the binding mixture were taken and analyzed for bound [^{3}H]-ouabain. Saturable ATP-dependent [^{3}H]-ouabain binding (●); saturable ATP-independent [^{3}H]-ouabain binding (○). Mean of 5 experiments. Vertical lines indicate s.e. mean

mately 0.094 and 0.073 per min, respectively (Figure 2). In both instances the addition of 16.6 mM KCl to the dissociation mixture reduced the rate of release of bound [^{3}H]-ouabain. The apparent first-order dissociation rate constants for the ATP-dependent and ATP-independent complexes were decreased to approximately 0.0073 and 0.0131 per min, respectively. These data illustrate that the ATP-independent [^{3}H]-ouabain binding forms a complex which is sensitive to potassium in a manner similar to that observed for the ATP-dependent ouabain binding. The latter binding has been shown to represent the binding of ouabain to Na^+, K^+-ATPase (Matsui & Schwartz, 1968; Post, Kume, Tobin, Orcutt & Sen, 1969; Akera, 1971; Hansen *et al.*, 1971; Allen *et al.*, 1971; Post, Hegyvary & Kume, 1972). The extent of the K^+-effect, however, was somewhat greater with the ATP-dependent complex than with the ATP-independent complex.

Further studies were performed in order to determine whether the ATP-independent ouabain binding is a binding of this drug to Na^+, K^+-ATPase. If ATP-independent ouabain binding is related to Na^+, K^+-ATPase, then the binding should result in enzyme inhibition. In these experiments, rat brain homogenates were again used instead of cardiac homogenates, because of the relatively large ATP-independent ouabain binding observed in this tissue preparation. When these preparations were incubated with 0.2 μM ouabain in the presence of 200 mM NaCl, 5 mM $MgCl_2$ and 5 mM Tris-ATP for 10 min at 37°C, and Na^+, K^+-ATPase activity was assayed after a removal of excess, unbound ouabain by centrifugation and resuspension, an approximately 50% reduction in enzyme activity was observed (Table 1). In contrast, concentrations of ouabain as high as 1.0 μM failed to cause a decrease in enzyme activity after the removal of excess, unbound ouabain when ATP was absent in the incubation mixture during the ouabain binding reaction. These results, therefore, indicate that the ATP-independent ouabain binding is not a binding of this drug to the cardiotonic steroid binding site on the Na^+, K^+-ATPase molecule.

If Na^+, K^+-ATPase is the carrier for the transport of the cardiac glycosides to its receptor, there should be an intracellular digitalis binding site which has an affinity for the cardiac glycosides greater than the ATPase molecule. In the last series of experiments, ATP-dependent and ATP-independent ouabain binding at various [^{3}H]-ouabain concentrations were investigated to determine the relative affinities of the two binding sites. The binding experiments were performed as described with rat brain, dog heart, and guinea-pig heart homogenates. The binding reaction

Table 1 Effect of ouabain binding on Na^+, K^+-ATPase activity

	Ouabain conc. during pretreatment (μM)	*Na^+, K^+-ATPase activity* (μmol *Pi* mg^{-1} h^{-1})
Control	0	25.8 ± 1.2
ATP-independent binding	0.5	27.5 ± 2.0
	1.0	26.8 ± 3.2
ATP-dependent binding	0.2	13.9 ± 2.6*

* Statistically different from control at $P < 0.05$ using paired t test; $n = 4$.

Rat brain homogenates were incubated at 37°C with various concentrations of ouabain for 10 min with or without 5 mM Tris-ATP. The incubation medium contained 200 mM NaCl, 5 mM $MgCl_2$, and 50 mM Tris–HCl buffer (pH 7.5). The mixture was centrifuged at 100,000 *g* for 60 min at 0°C, the pellets resuspended in 10 mM Tris–HCl buffer, and ATPase activity assayed. Na^+, K^+-ATPase activity was calculated by subtracting Mg^{2+}-ATPase from total ATPase activity. Values are means ± s.e. of 4 preparations.

was terminated at 20 min and bound [^{3}H]-ouabain was assayed. The fraction of ATP-independent [^{3}H]-ouabain binding in both dog and guinea-pig heart homogenate was small (Figure 3). However, saturable ATP-independent [^{3}H]-ouabain binding at 20 min in rat brain homogenates was at least 30% of the saturable ATP-dependent binding even at low concentrations of the drug. It appears that the saturable ATP-dependent [^{3}H]-ouabain binding approaches a plateau at a lower concentration of [^{3}H]-ouabain (approx. 0.1 μM) than does the saturable ATP-independent [^{3}H]-ouabain binding which has not yet reached a plateau at 1.0 μM [^{3}H]-ouabain. These data indicate that the ATP-independent [^{3}H]-ouabain binding site has a lower affinity for [^{3}H]-ouabain than does the ATP-dependent binding site.

Discussion

The binding of digitalis to cardiac Na^+, K^+-ATPase may cause a positive inotropic effect by inhibiting sodium pump activity (Akera, 1977) or by inducing a specific conformational alteration of the enzyme protein (Gervais, Lane, Anner, Lindenmayer & Schwartz, 1977; Bentfeld, Lüllmann, Peters & Proppe, 1977). If one of the above theories is correct, then Na^+, K^+-ATPase is the pharmacological receptor for digitalis. Alternatively, such a binding may represent the first step of a reaction which transports the cardiac glycoside to an intracellular site of action (Dutta *et al.*, 1968; Park & Vincenzi, 1975; Fricke & Klaus, 1977). The latter theory requires the presence of an additional non-ATPase receptor for digitalis which is responsible for the positive inotropic action. Fricke & Klaus (1977) studied [^{3}H]-ouabain binding to Na^+, K^+-ATPase obtained from guinea-pig hearts under various ligand conditions, and reported the presence of two distinct binding processes of ouabain to Na^+, K^+-ATPase: a high affinity binding predominant at high Na^+ concentrations ($K_{0.5}$ value for ouabain, 0.024 μM), and a low affinity binding observable at low Na^+ concentrations ($K_{0.5}$ value for ouabain, 0.63 μM). They suggest that ouabain is translocated from the high affinity (extracellular) site to the low affinity (intracellular) site. Implicit in the hypothesis is that ouabain is released intracellularly from the latter site and interacts with the inotropic receptor. Although these investigators observed ATP-independent binding, the nature of the binding (e.g., saturability and affinity for ouabain) was not reported. Ouabain receptor sites, if present, should be identified as saturable ouabain binding sites unrelated to Na^+, K^+-ATPase. Because ATP-dependent ouabain binding has been shown to be the stoichiometric binding of this agent to Na^+, K^+-ATPase (Allen *et al.*, 1971; Hansen *et al.*, 1971), the non-ATPase ouabain binding site should be an ATP-independent ouabain binding site. The affinity of such a site for ouabain should be higher than that of Na^+, K^+-ATPase for ouabain, since the drug must move from the carrier to the receptor.

In the course of the present experiments, saturable [^{3}H]-ouabain binding was observed with tissue homogenates under binding conditions different from those yielding specific Na^+, K^+-ATPase binding. Such an ATP-independent binding is minimal in partially purified Na^+, K^+-ATPase preparations (for example, Schwartz, Matsui & Laughter, 1968) suggesting that the binding sites are unrelated to Na^+, K^+-ATPase. Alternatively, however, observed ATP-independent binding may be due to the presence of co-factor(s) in the homogenates (e.g., ATP), which may be lost during the purification process. Thus, the characteristics of the ATP-independent ouabain binding observed with tissue homogenates were studied in order to determine whether such binding sites are related to a digitalis receptor other than Na^+, K^+-ATPase.

The ouabain-ATPase complex formed in the presence of ATP, Na^+ and Mg^{2+} is relatively unstable but is sensitive to K^+. The complex could be stabilized after the termination of the binding reaction by the addition of K^+ to the mixture (Akera & Brody, 1971). The bound ouabain formed in the absence of ATP exhibited qualitatively similar characteristics; the rate of release of [^{3}H]-ouabain was relatively rapid in the absence of K^+ and the complex was stabilized by the addition of K^+. The rate constants for the dissociation reaction, however, observed in the presence and absence of K^+, as well as the magnitude of the K^+-effect, were somewhat different in two complexes, indicating that the complex formed in the absence of ATP has different characteristics compared to that formed in the presence of ATP, i.e., the complex formed with Na^+, K^+-ATPase.

A 50% decrease in enzyme activity was observed when rat brain homogenates were incubated with 0.2 μM ouabain in the presence of ATP, Na^+, and Mg^{2+}, and enzyme activity assayed after removal of the excess unbound ouabain (Table 1). This finding indicates that the ATP-dependent ouabain binding which occurred during the first incubation was a binding of ouabain to Na^+, K^+-ATPase, and that the ATPase inhibition resulting from such a binding may be observed after centrifugation and resuspension. No inhibition of the enzyme activity was observed when Na^+, K^+-ATPase activity was determined following incubation of brain homogenates with ouabain in the absence of ATP, centrifugation and resuspension (Table 1). Since the amount of ATP-independent [^{3}H]-ouabain binding at 0.5 μM ouabain was approximately equal to the ATP-dependent [^{3}H]-ouabain

binding at 0.2 μM ouabain, a 50% enzyme inhibition would be expected if the Na^+, K^+-ATPase molecule was involved in the ATP-independent binding. Even a higher concentration of ouabain (1 μM) failed to produce a significant decrease in enzyme activity following an incubation in the absence of ATP. These data indicate that ouabain does not bind to the cardiotonic steroid binding site on the enzyme in the absence of ATP, and that the binding of ouabain to Na^+, K^+-ATPase observed in the absence of added ATP is not the binding of ouabain to Na^+, K^+-ATPase supported by intrinsic co-factor(s). These results raise the possibility that ATP-independent binding is the binding of ouabain to an entity other than Na^+, K^+-ATPase which may be lost during the purification process for Na^+, K^+-ATPase.

In order to assess the relative affinity of binding sites for ouabain, saturable ATP-dependent and saturable ATP-independent [^{3}H]-ouabain binding were determined after a 20 min incubation with various concentrations of the labelled drug. Affinity is ordinarily calculated from the dissociation constant of the drug-receptor interaction. However, due to the fact that equilibrium was not attained during the observation period, a precise kinetic analysis was not possible. Thus, the relative affinity of the two types of ouabain binding sites were compared from the shape of the saturation curve. ATP-dependent [^{3}H]-ouabain binding appeared to reach a plateau (maximum) at a lower concentration of [^{3}H]-ouabain than ATP-independent binding. Since the drug concentration at half-maximal binding is an estimate of affinity, it appears that the ATP-independent site has a lower affinity for ouabain than the ATP-dependent site. The affinity of the ATP-independent ouabain binding sites in the guinea-pig heart estimated in the present study appears to be lower than that of the low-affinity ouabain binding site reported by Fricke & Klaus (1977). Additionally, ATP-independent ouabain binding is abundant in brain tissue but is relatively minor in cardiac tissue which is the site of the pharmacological action of digitalis. Therefore, while ouabain binds in a saturable-manner to a tissue moiety other than Na^+, K^+-ATPase, the present data suggest that such a binding is not related to the receptor for the positive inotropic action of the cardiac glycosides.

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