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THE INSECT BRAIN ($Na^+ + K^+$)-ATPase

BINDING OF OUABAIN IN THE HAWK MOTH, *MANDUCA SEXTA*

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(1) A quantitative study has been made of the binding of ouabain to the ($Na^+ + K^+$)-ATPase in homogenates prepared from brain tissue of the hawk moth, *Manduca sexta*. The results have been compared to those obtained in bovine brain microsomes. (2) The insect brain ($Na^+ + K^+$)-ATPase will bind ouabain either in the presence of Mg^{2+} and P_i, ('Mg^{2+}, P_i' conditions) or in the presence of Na^+, Mg^{2+}, and an adenine nucleotide ('nucleotide' conditions) as is the case for the bovine brain ($Na^+ + K^+$)-ATPase. The binding conditions did not alter the total number of receptor sites measured at high ouabain concentrations in either tissue. (3) Potassium ion decreases the affinity (increases the K_D) of ouabain to the *M. sexta* brain ($Na^+ + K^+$)-ATPase under both binding conditions. However, ouabain binding is more sensitive to K^+ inhibition under the nucleotide conditions. In bovine brain ouabain binding is equally sensitive to K^+ inhibition under both conditions. (4) The enzyme-ouabain complex has a rate of dissociation that is 10-fold faster in the *M. sexta* preparation than in the bovine brain preparation. Because of this, the *M. sexta* ($Na^+ + K^+$)-ATPase has a higher K_D for ouabain binding and is less sensitive to inhibition by ouabain than the bovine brain enzyme. (5) This data supports the hypothesis that two different conformational states of the *M. sexta* ($Na^+ + K^+$)-ATPase can bind ouabain.

The ($Na^+ + K^+$)-ATPase is the enzyme responsible for the maintenance of sodium and potassium gradients across cell membranes [1]. An especially high concentration of the enzyme in nervous tissue is required to maintain sodium and potassium concentration gradients in nerve cells in the face of the high passive flux of this ions during impulse activity [2]. The central nervous system of the hawk moth, *Manduca sexta* is of interest since the relative morphological simplicity of this system may allow us to understand how ($Na^+ + K^+$)-ATPases from different cell types of the nervous system interact to maintain the extracellular ion concentrations within strict limits. Our previous work [3] showed that ($Na^+ + K^+$)-ATPase activity present in microsomes isolated from the ventral nerve cord of pharate adult *M. sexta* had properties similar to those observed in other preparations including the few insect neural preparations that have been examined to date [4–6]. However, we demonstrated that the ($Na^+ + K^+$)-ATPase from the *M. sexta* nerve cord had a different sensitivity to the potassium ion concentration. In order to extend and to clarify these findings, we have examined the effect of various ionic conditions on the kinetics of ouabain binding to the *M. sexta* brain ($Na^+ + K^+$)-ATPase and have compared these kinetics to those found for the enzyme in bovine brain tissue.

The binding of ouabain to the ($Na^+ + K^+$)-ATPase is specific and can be described by the equation [7].

$$E + O \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EO$$

where E is the ($Na^+ + K^+$)-ATPase, O is ouabain and

EO is the enzyme-ouabain complex. The rate constants for the reaction in the forward and reverse directions, represented by k_1 and k_{-1}, can be used to calculate the equilibrium dissociation constant, K_D, by the equation:

$$K_D = \frac{k_{-1}}{k_1}$$

The binding reaction requires magnesium ion, although at least two different binding conformations are known. These conformations have been distinguished experimentally by observing the effects of sodium ion on ouabain binding using different ligand conditions [8]. In the presence of a nucleotide such as ATP or ADP, sodium stimulates the binding rate. With Mg^{2+} alone or in the presence of inorganic phosphate, sodium inhibits the binding rate [9]. In addition, potassium inhibits ouabain binding under both conditions probably by favoring the formation of potassium-bound intermediates that have less affinity for ouabain [10]. Our present studies on the *M. sexta* brain $(Na^+ + K^+)$-ATPase indicate that these effects can be demonstrated here as well, although ouabain binding to this enzyme is more sensitive to potassium ion inhibition in the presence of sodium and an adenine nucleotide than in their absence in this preparation. Also, we have found the debinding reaction to be considerably faster in the insect system, accounting for the decreased sensitivity of the enzyme from this source to inhibition by ouabain.

Materials and Methods

Brains from pharate adult *M. sexta*, kindly provided by Dr. J.W. Truman, were dissected out under a stereomicroscope into 3 mM imidazole/1 mM EDTA, pH 7.3, and were stored frozen in the same buffer at −70°C for up to 60 days. Homogenates were prepared in Teflon-glass homogenizers using a motor-driven Eberbach homogenizer. Usually ten dissected brains were homogenized in 1 ml of buffer to a protein concentration of 1.5 mg/ml. The final concentration in the binding assay was 15 μg/100 μl.

Microsomes were prepared from bovine brain cortex as previously described [11]; they were lyophilized, and stored at −18°C. On the day of an experiment, microsomes were resuspended in the imidazole/EDTA buffer to a protein concentration of 1.5 mg/ml and were present in the binding assay at 15 μg/100 μl.

The rate of association of ouabain with the $(Na^+ + K^+)$-ATPase was measured by adding 200 μl of temperature-equilibrated $1.5 \cdot 10^{-7}$ M [^{3}H]ouabain (final concn. $1.5 \cdot 10^{-8}$ M) to 1.8 ml of binding medium containing tissue sample at either 20 or 37°C. Binding media contained either 50 mM imidazole/1 mM inorganic phosphate/3 mM $MgCl_2$/0.5 mM EDTA/0.4% bovine serum albumin, pH 7.3 [12] ('Mg^{2+}, P_i' conditions), or 10 mM imidazole/100 mM NaCl/4 mM $MgCl_2$/3 mM ADP/0.5 mM EDTA/0.4% bovine serum albumin, pH 7.3 ('nucleotide' conditions). When indicated, 3 mM ATP was substituted for ADP. Bovine serum albumin was included to stabilize the $(Na^+ + K^+)$-ATPase. At the appropriate times, 100 μl of the reaction mixture was withdrawn, vacuum-filtered through 0.45-μm nitrocellulose filters on a Millipore sampling manifold, washed with 15 ml of 10 mM imidazole, pH 7.3 at 0°C, dried, and the radioactivity was quantitated by liquid-scintillation spectrophotometry in a toluene-based counting solution. Using this scintillation fluid we routinely obtain reproducible counting efficiencies which are not improved by using solubilizing agents. Controls contained all of the components except the enzyme suspension. Apparent association rate constants, k_1, were calculated from initial rate data.

The rate of dissociation of ouabain from the $(Na^+ + K^+)$-ATPase was determined by adding 50 μl of 10^{-2} M unlabelled ouabain to a suspension containing the enzyme-[^{3}H]ouabain complex formed as described above. The final concentration of ouabain in the dissociation reaction media was $2.5 \cdot 10^{-4}$ M [13]. At appropriate times, 100 μl aliquots were removed and filtered. Controls were run as before. Apparent dissociation constants, k_{-1}, were calculated from the slope of the curves of the log [^{3}H]ouabain bound vs. time.

To determine the total number of binding sites present in the tissue, samples were incubated in binding medium at ouabain concentrations between 10^{-8} and 10^{-5} M for 30 min–1 h, and sampled on the filters as before. The data were plotted as Scatchard plots from which the total number of binding sites could be graphically determined [14]. For these

experiments, the filtrations were done at 4°C to minimize the problem of enzyme denaturation and hence dissociation of ouabain that may occur on the filter itself.

$(Na^+ + K^+)$-ATPase activity was determined by a modification of the radioactive procedure previously described [11]. The reaction mixture in a final volume of 100 µl contained: 38 mM Tris-HCl, pH 7.6, 0.4 mM EDTA, 1 mM EGTA, 100 mM NaCl, 20 mM KCl, 3.0 mM $MgCl_2$, 2.0 mM [γ-^{32}P]ATP ±1 mM ouabain. Reactions were started by the addition of the [^{32}P]ATP at 37°C and were stopped after 15 min by the addition of 50 µl of 5% ammonium molybdate, 1 mM H_3PO_4 in 2 M H_2SO_4. $(Na^+ + K^+)$-ATPase activity was defined as the difference in hydrolysis for reactions in the absence and presence of 1 mM ouabain. The I_{50} for ouabain was determined graphically after preincubating the enzyme for 90 min in reaction buffer, with the addition of 3 mM ADP, and ouabain concentrations between 10^{-9} and 10^{-4} M.

Ouabain was purchased from Sigma Chemical Company. Inorganic $^{32}P_i$ and [^{3}H]ouabain were purchased from New England Nuclear. [γ-^{32}P]ATP was prepared by the method of Buetler and Guinto [15].

Results

The nucleotide ouabain-binding conformation of the *M. sexta* brain and bovine brain $(Na^+ + K^+)$-ATPases can be formed either in the presence of ATP or ADP. As can be seen in Fig. 1, Na^+ stimulates ouabain binding in the *M. sexta* brain with either nucleotide (B and C). In contrast, Na^+ inhibits ouabain binding in the presence of Mg^{2+} and P_i. Therefore, in this study we have used ADP as the nucleotide for the nucleotide-binding conditions in order to avoid the problem of ATP hydrolysis that would inevitably occur in a crude preparation.

Fig. 2 shows the results of a typical ouabain-binding experiment in *M. sexta* brain homogenates (A and B) and bovine brain microsomes (C and D), under the Mg^{2+}, P_i and nucleotide-binding conditions. Equilibrium is reached in about 1 h at 37°C at all potassium concentrations in both preparations. As can be seen in Fig. 2, potassium concentrations as low as 1 mM significantly inhibit ouabain binding at

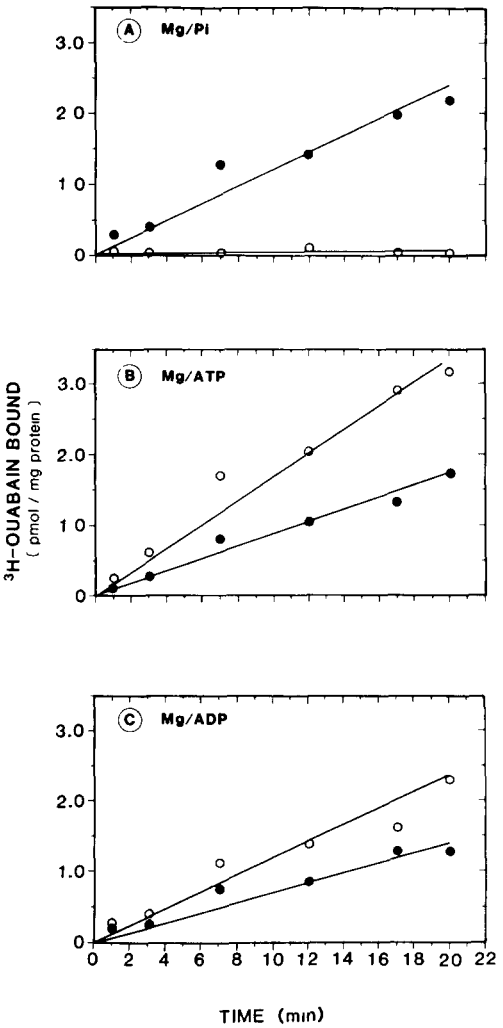

Fig. 1. Effect of 100 mM sodium on initial rate of ouabain binding in *M. sexta* brain homogenates. The binding of [^{3}H]-ouabain ($1.5 \cdot 10^{-8}$ M) was measured at 20°C in the presence (○) and absence (●) of 100 mM NaCl. (A) 50 mM imidazole/1 mM P_i/3 mM $MgCl_2$/0.5 mM EDTA/0.4% bovine serum albumin, pH 7.3, (B) 10 mM imidazole/4 mM $MgCl_2$/3 mM ATP/0.5 mM EDTA/0.4% bovine serum albumin, pH 7.3, and (C) 10 mM imidazole/4 mM $MgCl_2$/3 mM ADP/0.5 mM EDTA/0.4% bovine serum albumin, pH 7.3.

equilibrium for both the *M. sexta* and bovine brain preparations. In *M. sexta*, however, the effects of potassium are more pronounced using the nucleotide-binding conditions than using the Mg^{2+}, P_i conditions (Fig. 2A, B). In order to determine whether this differential effect of K^+ on equilibrium ouabain

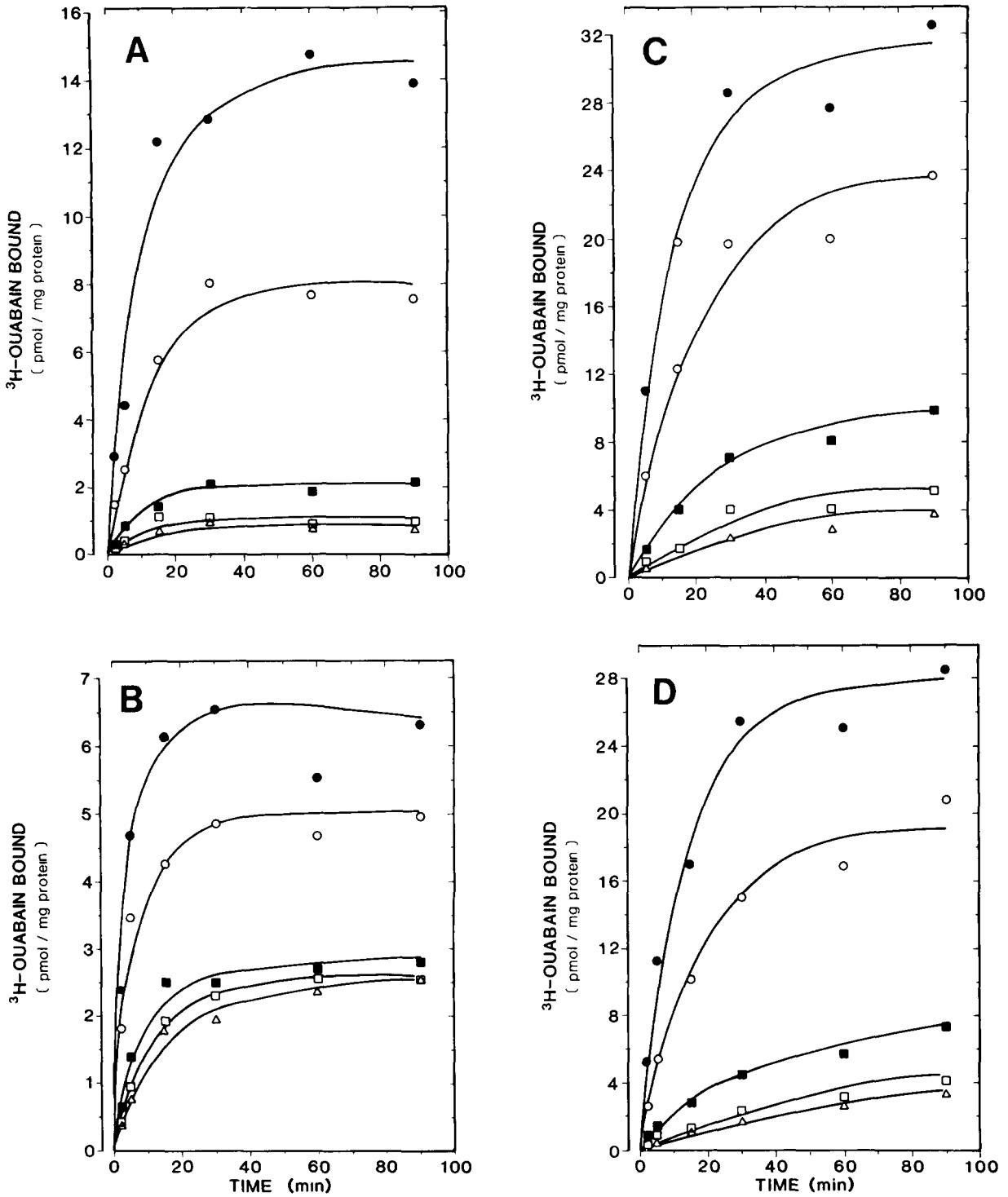

Fig. 2. Effect of potassium on ouabain binding to *M. sexta* brain homogenates and bovine brain microsomes. The binding of [^{3}H]-ouabain was measured at 37°C in the presence of 0 mM KCl (●), 1 mM KCl (○), 5 mM KCl (■), 20 mM KCl (□), and 50 mM KCl (△). (A) *M. sexta* brain homogenates using nucleotide binding conditions. (B) *M. sexta* brain homogenates using Mg^{2+}, P_i binding conditions. (C) Bovine brain microsomes using nucleotide binding conditions. (D) Bovine brain microsomes using Mg^{2+}, P_i binding conditions.

binding in *M. sexta* was due to an effect on the association rate, the dissociation rate, or both, we performed the experiments shown in Fig. 3. Here the temperature has been lowered to 20°C to slow the association reaction. As can be seen, the greater inhibitory effect of K^+ on ouabain binding to the insect brain ($Na^+ + K^+$)-ATPase under the nucleotide conditions than under the Mg^{2+}, P_i conditions is due to an increased inhibition of the association rate under the former conditions (Fig. 3A, B). The association rate is inhibited by 67% in the presence of 1 mM K^+ under the nucleotide conditions but only by 30% under the Mg^{2+}, P_i conditions. These differences correlate well with the differences in equilibrium binding. In contrast, the association rate is equally inhibited by K^+ under both conditions in the bovine brain system. This is consistent with the effects of K^+ observed at equilibrium with this enzyme.

Fig. 4 shows the effects of potassium on the rate of dissociation of the enzyme-ouabain complex in the two systems. Potassium acts to stabilize the enzyme-ouabain complex in *M. sexta* in a manner similar

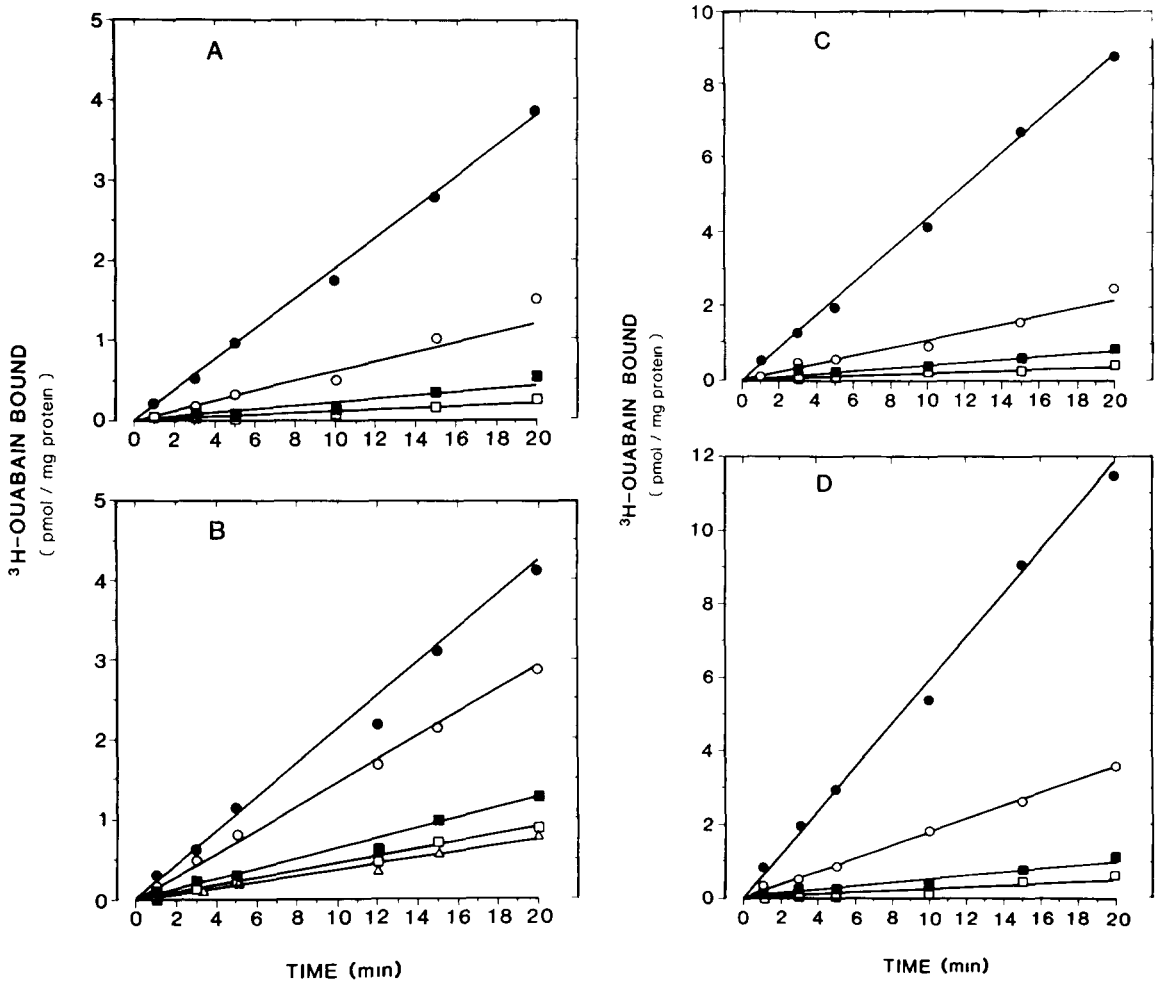

Fig. 3. Effect of potassium on the initial rate of ouabain binding in *M. sexta* brain homogenates and bovine brain microsomes. The binding of ouabain was measured at 20°C in the presence of 0 mM KCl (●), 1 mM KCl (○), 5 mM KCl (■), 20 mM KCl (□), and 50 mM KCl (△). (A) *M. sexta* brain homogenates using nucleotide binding conditions. (B) *M. sexta* brain homogenates using Mg^{2+}, P_i binding conditions. (C) Bovine brain microsomes using nucleotide binding conditions. (D) Bovine brain microsomes using Mg^{2+}, P_i binding conditions.

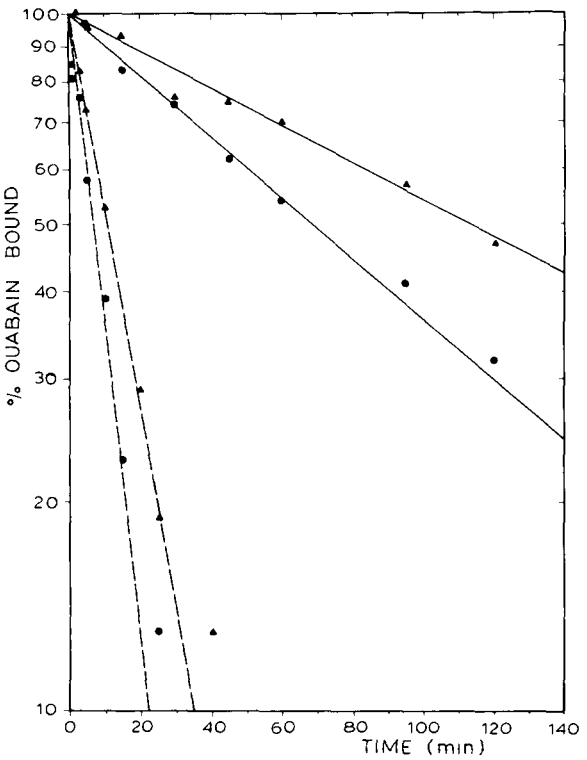

Fig. 4. Effect of potassium on the rate of dissociation of ouabain from *M. sexta* brain homogenates (------) and bovine brain microsomes (———) under the nucleotide binding conditions. Enzyme-ouabain complex were formed by incubating the tissue for 1 h at 37°C in the presence of $1.5 \cdot 10^{-8}$ M [^{3}H]ouabain. The dissociation reaction was started by addition of unlabeled ouabain to a final concentration of $2.5 \cdot 10^{-4}$ M. (•) 0 mM KCl; (▲) 20 mM KCl.

to its effect in the bovine brain system and other mammalian systems that have been examined [16]. The half-life of the enzyme-ouabain complex in *M. sexta* is increased 1.6–2.7-fold in the presence of potassium, from 6 min at 0 mM KCl to 16 min at 20 mM KCl in the presence of Mg^{2+} and P_i (data not shown), and from 7 min to 11 min in the presence of Na^+, Mg^{2+}, and ADP (Fig. 4). In the bovine brain preparation, the half-life of the complex is increased from 70 min at 0 mM K^+ to 81 min at 20 mM K^+ under the Mg^{2+}, P_i conditions (data not shown). Under the nucleotide conditions, the change is from 66 min to 108 min (Fig. 4).

The *M. sexta* brain and bovine brain ($Na^+ + K^+$)-ATPases also differ in their sensitivity to inhibition by ouabain. This can be shown by comparing the inhibition of ($Na^+ + K^+$)-ATPase activity in the two preparations for a range of ouabain concentrations. After a 90-min preincubation in the reaction buffer which contained both potassium and ADP to allow association of ouabain, the reaction was started by the addition of [γ-^{32}P]ATP. The bovine brain ($Na^+ + K^+$)-ATPase, with a half-inhibition constant (I_{50}) of 150 nM in the presence of 20 mM K^+, is about 6-times more sensitive to ouabain inhibition than the *M sexta* brain ($Na^+ + K^+$)-ATPase, which has an I_{50} of 900 nM under those conditions. The association rate constants, k_1, for ouabain are very similar for the two enzymes while the dissociation rate constants, k_{-1} differ by a factor of about 10 (Table I). Therefore, the difference in sensitivity of the two ($Na^+ + K^+$)-ATPase preparations to ouabain inhibition is due primarily to the difference in dissociation rate of the enzyme-ouabain complex [17,18].

Fig. 5A and B show the effects of various ligands on the affinity and total number of ouabain-binding sites present in *M. sexta* brains. As in the bovine brain (data not shown, see also Ref. 16), the ligand conditions strongly influence the affinity (K_D) of the *M. sexta* brain ($Na^+ + K^+$)-ATPase for ouabain, but have little or no effect on the total number of binding sites in the tissue (see also Fig. 2). Our data indicate that *M. sexta* brains contain between 80 and 90 pmol of high-affinity ouabain-binding sites per mg protein, compared to a value of between 60 and 80 pmol high-affinity sites per mg in the bovine brain. There is a suggestion of a lower affinity binding site in both preparations, but we have found the data difficult to interpret at these high ouabain concentrations. The slopes of the lines in Fig. 5 are proportional to the K_D values for the enzyme-ouabain complex. Again these data show the more pronounced inhibitory effect of K^+ ouabain binding in the *M. sexta* system under the nucleotide conditions (Fig. 5A). This is not the case for the bovine brain enzyme where the change in slope that occurs with the addition of K^+ is nearly the same for the two conditions (data not shown).

Discussion

Evidence has accumulated in recent years concerning the role of specific enzymes and cell types in the maintenance of electrolyte and non-electrolyte

TABLE I

OUABAIN-BINDING CONSTANTS FOR THE BOVINE BRAIN AND *M SEXTA* BRAIN ($Na^+ + K^+$)-ATPases

Constants were obtained as described in Materials and Methods. Binding assays were run in duplicate. Apparent association rate constants, k_1, were calculated from initial rate data at 20°C. The apparent dissociation constants, k_{-1}, were calculated from the slope of the curves of log [^{3}H]ouabain bound vs. time

Ligands	K^+ (mM)	K_D (nM)	k_1 (nM^{-1} min^{-1})	k_{-1} (min^{-1})
Bovine brain				
Mg^{2+}, P_i	0	5.9	$2\,53\ 10^{-3}$	0.015
	1	17.7	$7\,34\ 10^{-4}$	0.013
	5	66.6	$1.80 \cdot 10^{-4}$	0.012
	20	100.9	$1\,09 \cdot 10^{-4}$	0.011
	50	–	–	0.010
Na^+, Mg^{2+}, ADP	0	12 1	$2.40\ 10^{-3}$	0.017
	1	25 4	$5.52\ 10^{-4}$	0.014
	5	67.7	$1.92 \cdot 10^{-4}$	0.013
	20	92.6	$1.08 \cdot 10^{-4}$	0.010
	50	–	–	0.007
M sexta				
Mg^{2+}, P_i	0	229.8	$8.40\ 10^{-4}$	0.193
	1	273.8	$5.88\ 10^{-4}$	0.161
	5	601.0	$1.93 \cdot 10^{-4}$	0.116
	20	738.8	$1.34 \cdot 10^{-4}$	0.099
	50	754.2	$1.18\ 10^{-4}$	0.089
Na^+, Mg^{2+}, ADP	0	118.7	$1.12\ 10^{-3}$	0.133
	1	375 7	$0.37\ 10^{-3}$	0.139
	5	1 109.1	$0.11\ 10^{-3}$	0.122
	20	1 603.7	$0.53\ 10^{-4}$	0.085
	50	–	–	0.071

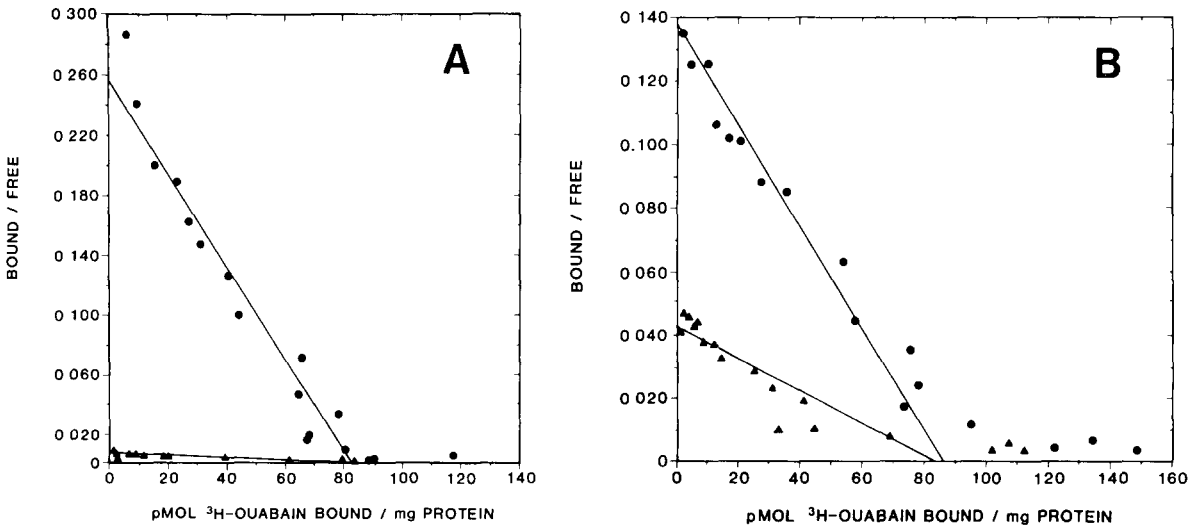

Fig. 5. Scatchard plot of ouabain binding to *M. sexta* brain homogenates in the presence (▲) and absence (●) of 20 mM KCl. The enzyme-ouabain complex was formed by incubating the tissue 1 h at 37°C with 10^{-8}–10^{-5} M ouabain under (A) nucleotide binding conditions, or (B) Mg^{2+}, P_i binding conditions. Samples were then filtered at 4°C, washed and counted as described in Materials and Methods.

homeostasis in cellular and extracellular compartments. In the mammalian nervous system, it has been suggested that the $(Na^+ + K^+)$-ATPase, the enzyme responsible for the sodium and potassium gradients across nerve cell membranes which form the basis for action potential generation, may also be responsible for regulating the concentrations of these ions in the neural extracellular fluid. High levels of the enzyme have been found in the antiluminal membranes of cerebal microvessels, for example, and thus may be involved in regulating the flux of these cations between blood and brain [20]. The enzyme probably serves a similar function in the choroid plexus where it has been found on the basolateral membrane [21]. Certain phytophagous insect species must maintain greater sodium and potassium gradients between their hemolymph and neural extracellular space to support normal cell function [22,23] than is required between blood and extracellular space in mammals. The hawk moth, *M. sexta*, is one such species. This animal maintains a hemolymph sodium concentration of about 25 mM while requiring a much higher concentration (100 mM) in the neural extracellular space [24]. We decided, therefore, to investigate the $(Na^+ + K^+)$-ATPase in the *M. sexta* nervous system in order to clarify its role in this phenomenon.

In our previous study, we determined the optimal conditions for activity of the *M. sexta* nerve cord $(Na^+ + K^+)$-ATPase, noting the basic similarities of this enzyme to its mammalian counterpart in its sensitivity to pH, magnesium, cardiac glycosides, and ATP, as well as the apparent differences in monovalent cation sensitivity [3]. In the current study, we have extended these observations in the hawk moth by examining the kinetics of the ouabain binding and debinding reactions and comparing these kinetics to the better understood bovine brain enzyme. Ouabain binding had previously been detected in the adult eye of *M. sexta* [25]. As we have shown, the rates of association between ouabain and the $(Na^+ + K^+)$-ATPase are similar in the bovine brain and insect brain. However, the rates of dissociation are quite different. The *M. sexta* enzyme-ouabain complex dissociates with half-times of 6 and 7 min under Mg^{2+}, P_i and nucleotide conditions, respectively, while the bovine brain complex is about 10-times more stable with dissociation half-times of 70 and 66 min under the same conditions. We attempted to clarify the molecular basis of this difference by comparing I_{50} ratios for cardiotonic steriods plus and minus the glycoside residue and with a lactone ring that had or had not been reduced for the two enzymes (data not shown), but the results were ambiguous. Nevertheless, while the precise molecular basis for the difference in dissociation rates is unknown, it is clear that the dissociation rate is the parameter that is primarily responsible for the differences in the equilibrium dissociation constants, K_D, for the two preparations. For *M. sexta*, the K_D values as determined from the association and dissociation rate constants are 229.8 and 118.8 nM for the Mg^{2+}, P_i and nucleotide conditions, respectively, while those measured in the bovine brain are 10–40-fold lower, i.e., 5.9 and 12.1 nM for the same conditions. Other investigators have also found that differences in dissociation rates of ouabain from enzymes from different sources will account for differences in the K_D values. Tobin et al. [26] found very similar rates of association for several mammalian $(Na^+ + K^+)$-ATPase preparations while the rates of dissociation varied from a half-time of 2 min in the guinea-pig heart to 2 h in the dog kidney. They attributed these differences in cardiac glycoside sensitivity to differences in dissociation rates. Erdmann and Schoner [27] also found that the larger equilibrium dissociation constant of the guinea-pig kidney compared to other preparations such as the dog heart ($1.62 \cdot 10^{-7}$ M vs. $4.3 \cdot 10^{-9}$ M) was due primarily to an increased dissociation rate constant for the guinea-pig kidney ($2.45 \cdot 10^{-3}\ s^{-1}$, as opposed to $2.31 \cdot 10^{-4}\ s^{-1}$ for the dog heart).

While the differences in K_D (hence I_{50}) values in different $(Na^+ + K^+)$-ATPase preparations can be attributed to differences in dissociation rates, the effect of potassium ion of decreasing the affinity of ouabain to a particular $(Na^+ + K^+)$-ATPase is due entirely to an effect on the association rate. The potassium effect on the dissociation rate is in fact in the direction of increasing the affinity (i.e., lowering the K_D) because the enzyme-ouabain complex is actually stabilized. These data are shown in Table I. The association rate constants for bovine brain are reduced about 25-fold under both the Mg^{2+}, P_i and the nucleotide conditions between 0 and 20 mM KCl. In the *M. sexta* system, the k_1 is reduced about 8-fold under Mg^{2+}, P_i conditions and 20-fold under the nu-

cleotide conditions (Table I). The lower sensitivity of the *M. sexta* enzyme to potassium under the Mg^{2+}, P_i conditions is similar to what had previously been found with the bovine kidney enzyme [19] and emphasizes the fact that the two ligand conditions put the enzyme into different conformational states. This is further supported by the data shown in Fig. 1 where totally different effects of sodium on the initial ouabain-binding rates in the *M. sexta* brain are observed depending on whether or not an adenine nucleotide is present. Siegel and Josephson [9] made a similar observation on the $(Na^+ + K^+)$ from the eel electroplax organ where in the presence of Mg^{2+} and ATP or ADP, sodium stimulated ouabain binding, while in the presence of Mg^{2+} alone it was inhibitory.

Our data suggest that the *M. sexta* brain $(Na^+ + K^+)$-ATPase, like other $(Na^+ + K^+)$-ATPases that have been studied, has at least two different conformational states that can bind ouabain. Our efforts are now directed to localizing the enzyme in insect nervous tissue in the hope that we can clarify the role of the $(Na^+ + K^+)$-ATPase in the regulation of the neural extracellular environment.

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