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Reversibility of the Interaction of Strophanthidin Bromoacetate with the Cardiotonic Steroid Binding Site of Sodium- and Potassium-Dependent Adenosine Triphosphatase

THOMAS TOBIN, TAI AKERA, AND DAVID KU

Department of Pharmacology, Michigan State University, East Lansing, Michigan 48823

MATTHIAS C. LU

Department of Medicinal Chemistry, College of Pharmacy, University of Michigan, Ann Arbor, Michigan, 48104

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SUMMARY

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The interaction of strophanthidin bromoacetate (SBA) with rat brain ($Na^+ + K^+$)- ATPase (ATP phosphohydrolase, EC 3.6.1.3) was investigated. SBA displaced [^{3}H]ouabain from the Na^+-, Mg^{++}-, and ATP-dependent binding site on the enzyme. The apparent affinity of SBA for the cardiotonic steroid binding site of the enzyme was at least one-third of that of ouabain for this site. When SBA was allowed to bind to the cardiotonic steroid binding site in the presence of Na^+, Mg^{++}, and ATP or of Mg^{++} and P_i, [^{3}H]ouabain added at various intervals after SBA equilibrated with all the cardiotonic steroid binding sites. If SBA was allowed to bind to the enzyme in the presence of Mg^{++} and P_i or of Na^+, Mg^{++}, and UTP, its subsequent phosphorylation from [γ-^{32}P]ATP was reduced by 60–80%. Exposure of this inhibited enzyme to high concentrations of EDTA resulted in recovery of phosphorylation capacity in less than 5 min at 37°. Since SBA did not irreversibly occupy the cardiotonic steroid binding sites or give rise to irreversible inhibition of the enzyme, SBA does not appear to be an effective affinity label for ($Na^+ + K^+$)-ATPase. Because a challenge to the concept that ($Na^+ + K^+$)-ATPase may be the cardiotonic receptor for cardiotonic steroids [B. F. Roth-Schechter, G. T. Okita, R. E. Thomas, and F. F. Richardson, *J. Pharmacol. Exp. Ther.* **171**, 13–19 (1970)] is based on the supposed irreversibility of the SBA–($Na^+ + K^+$)-ATPase interaction, our results leave such an interpretation open to question.

INTRODUCTION

Affinity labeling is a method of attaching a chemical label covalently to one or more of the amino acid residues at or near a binding site on a biopolymer. Classically an

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affinity label compound contains a ligand with high affinity for a binding site on the biopolymer and another reactive group which interacts irreversibly at or near the binding site. Since the affinity group brings the reactive group to or near the binding site of the biopolymer, the probability of a site-directed reaction should be much greater than that of nonspecific interactions (1).

Because the cardiac glycosides bind specifically and with high affinity to $(Na^+ + K^+)$-ATPase (ATP phosphohydrolase, EC 3.6.1.3), attempts have been made to modify these glycosides with reactive groups that would affinity label the enzyme. This approach has been used by Hokin and coworkers (2, 3), who attached various haloacetate groups at position 3 of strophanthidin and used the resultant strophanthidin-3-haloacetates to inhibit $(Na^+ + K^+)$-ATPase. This inhibition appeared to be irreversible, and early results from their laboratory suggested that it was due to site-directed alkylation of the enzyme system (2).

Further investigation of one of these strophanthidin derivatives, strophanthidin bromoacetate, showed it to be cardiotonic, and it has since been used to investigate the relationship between $(Na^+ + K^+)$-ATPase inhibition and the inotropic action of cardiac glycosides (4). These studies showed that the positive inotropic action of SBA is rapidly reversible, in contrast to its supposedly irreversible inhibition of $(Na^+ + K^+)$-ATPase. Roth-Schechter *et al.* (4) concluded that the inotropic receptor for the cardiac glycosides must be distinct from the $(Na^+ + K^+)$-ATPase, the implicit assumption in this conclusion being that strophanthidin bromoacetate rapidly dissociates from the unidentified cardiotonic receptors.

In this report we re-examined the interaction of SBA[1] with $(Na^+ + K^+)$-ATPase *in vitro*, using ^{32}P labeling and [^{3}H]ousbain binding techniques that were not available during the original investigations on the interaction of SBA with $(Na^+ + K^+)$-ATPase (2). No evidence was obtained which supported the concept of specific site-directed, irreversible labeling or of site-directed irreversible inhibition of the $(Na^+ + K^+)$-ATPase by SBA. It appears that SBA is not an effective site-directed inhibitor (affinity label) for $(Na^+ + K^+)$-ATPase. Thus the previously reported reversibility of SBA-induced stimulation of contraction of perfused rabbit hearts (4) is entirely consistent with the rapid dissociation of SBA from $(Na^+ + K^+)$-ATPase reported here.

[1] The abbreviations used are: SBA, strophanthidin-3-bromoacetate; CS, cardiotonic steroid.

MATERIALS AND METHODS

Enzyme preparation and labeling. Rat brain ATPase, prepared by the method of Akera and Brody (5), was used throughout. Its specific activity was assayed as described by Tobin *et al.* (6), and its protein content, by the method of Lowry *et al.* (7). Total ATPase activity varied between 150 and 300 μmoles of P_i per milligram of protein per hour, and more than 95% of the activity was ouabain-sensitive.

Labeling of the enzyme from [γ-^{32}P]ATP and [^{3}H]ouabain was performed by the methods of Tobin *et al.*(6). Phosphorylation of the enzyme by [γ-^{32}P]ATP was terminated with trichloracetic acid 3 sec after the addition of [γ-^{32}P]ATP, except when UTP was present in the system. When UTP was present (Fig. 6) labeling from [γ-^{32}P]ATP was allowed to proceed for 5 sec because attainment of the steady-state level of the [^{32}P]enzyme under these conditions may be somewhat slower (8).

Because of its low water solubility, SBA was used as a solution in *N*,*N*-dimethylformamide. The concentration of dimethylformamide in any experimental system did not exceed 0.1%. Although this concentration had no significant effect on any of the parameters studied, an equivalent concentration of dimethylformamide was added to all controls. All experimental data are presented as the means ± standard errors of at least four determinations. All experiments were performed at 37°.

Reagents and chemicals. Strophanthidin-3-bromoacetate was prepared according to the procedure of Hokin *et al.* (2) with slight modification. A solution of bromoacetyl bromide (0.23 mg, freshly distilled) in purified dioxane (5 ml, freshly distilled over CaH_2) was added dropwise under a nitrogen atmosphere to a solution of strophanthidin (0.5 g, 1.24 mmoles) and pyridine (0.21 ml,

2.6 mmoles) in purified dioxane (20 ml). The mixture was stirred at room temperature for 1.5 hr under nitrogen; water (75 ml) was then added, and the dioxane was removed under vacuum at room temperature. A white precipitate was obtained, which was collected by filtration, washed with water, and dried. After recrystallization from methanol, 0.321 g of pure SBA was obtained (m.p. 195–196° with decomposition; reported m.p. 191–194°). Its structure was further established by its infrared and NMR analyses: ν_{max}^{KBr} 3500–3600 cm^{-1} [OH, 1780, 1760, 1710 (C=O), and 1610 (C=C)]; NMR ($CDCl_3$–DMSO-d_6) δ4.00 (2H, $SBrCH_2\overset{O}{\overset{\|}{C}}O$—) in addition to peaks present in strophanthidin.

[γ-^{32}P]ATP was obtained from New England Nuclear Corporation and was diluted with carrier ATP to give approximately 5×10^{-6} cpm/mole of ATP. [^{3}H]-Ouabain (also from New England Nuclear) was diluted with carrier ouabain to 500 Ci/mole. Bromoacetyl bromide was obtained from K & K Laboratories, and the nucleotide substrates were obtained from Sigma Chemical Company. All other chemicals were reagent grade.

RESULTS

The inhibition curves for both ouabain and SBA on (Na^+ + K^+)-ATPase in the standard 10-min (Na^+ + K^+)-ATPase assay are shown in Fig. 1 (6). In this test system the two drugs were indistinguishable. However, although it may be assumed that ouabain inhibits (Na^+ + K^+)-ATPase by binding to the cardiotonic steroid binding site, this assumption cannot be made for SBA. SBA may inhibit the enzyme either by binding at the CS binding site, by alkylating other reactive groups in the enzyme system, or by a combination of these mechanisms. Therefore the ability of SBA and bromoacetate to displace [^{3}H]ouabain from (Na^+ + K^+)-ATPase was tested to determine whether SBA could interact with the CS binding sites of this enzyme system.

Figure 2 shows the actions of SBA and

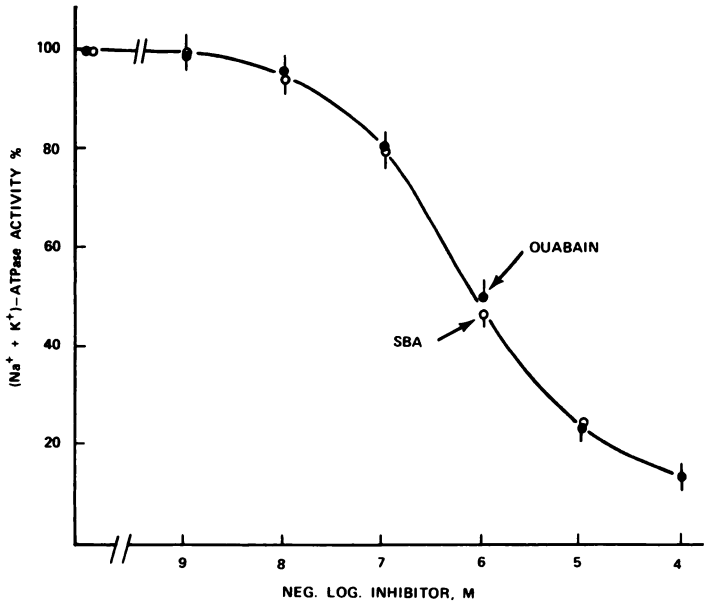

FIG. 1. *Inhibition of rat brain (Na^+ + K^+)-ATPase by ouabain and SBA*

Rat brain (Na^+ + K^+)-ATPase was incubated with concentrations of ouabain or SBA shown on the abscissa, in the presence of 100 mM Na^+, 15 mM K^+, and 5 mM MgATP, at 37° for 10 min. O——O, inhibition of (Na^+ + K^+)-ATPase activity observed in the presence of the indicated concentrations of SBA; ●——●, inhibition observed in the presence of the indicated concentrations of ouabain.

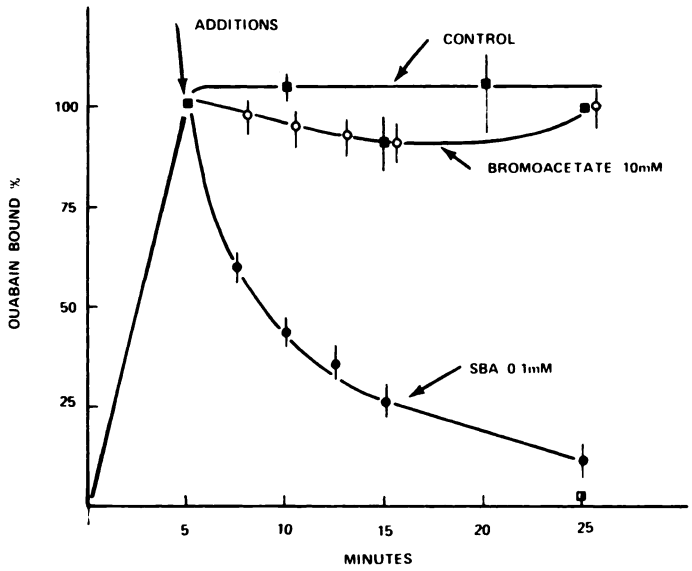

FIG. 2. *Displacement of [³H]ouabain from $(Na^+ + K^+)$ATPase by SBA*

Rat brain $(Na^+ + K^+)$-ATPase was allowed to bind 0.24 μM [³H]ouabain in the presence of 100 mM Na^+, 0.1 mM Mg^{++}, and 5 mM ATP at 37°. ■——■, [³H]ouabain binding as a percentage of that bound at 25 min, which averaged 231 ± 27 pmoles of [³H]ouabain per milligram of protein. At 5 min 0.1 mM SBA (●——●) or 10.0 mM bromoacetate (○——○) was added, and subsequent changes in the amounts of bound [³H]ouabain were monitored. □, amount of [³H]ouabain bound in the presence of 250 μM unlabeled ouabain, which represents the level of nonspecific binding.

bromoacetate on [³H]ouabain binding to $(Na^+ + K^+)$-ATPase in the presence of Na^+ and ATP. The concentration of Mg^{++} in the system was reduced to 0.1 mM to slow the rate of nonspecific Mg^{++}-ATPase activity and thus allow [³H]ouabain binding to be maintained over the period of the experiment. Five minutes after the binding reaction was started, either SBA (0.1 mM) or bromoacetate (10 mM) was added to the binding system. SBA produced an immediate response, rapidly displacing the specifically bound [³H]ouabain from the enzyme, whereas bromoacetate had little effect. The experiment shows that SBA can interact with the CS binding sites of $(Na^+ + K^+)$-ATPase and that its displacement of [³H]ouabain from the enzyme is probably not dependent on any nonspecific interaction of the bromoacetate group with this enzyme.

To test whether or not SBA could interact irreversibly with the CS binding sites on $(Na^+ + K^+)$-ATPase, the procedure of Fig. 2 was modified as shown in Fig. 3. In a routine [³H]ouabain binding experiment (6) the [³H]ouabain used consists of a mixture of carrier and tracer ouabain. If SBA interacts reversibly with the [³H]ouabain binding site it should be possible to replace the carrier ouabain with an appropriate concentration of SBA. The same amount of tracer should be specifically bound to the enzyme as in the regular [³H]ouabain binding system. When the carrier ouabain was removed from the binding system the number of counts per minute of [³H]ouabain specifically bound to the enzyme increased a little less than 3-fold. By trial and error a concentration of SBA was found which would approximately substitute for the carrier ouabain. Figure 3 shows that approximately the same equilibrium level of [³H]ouabain binding was obtained if 0.8 μM SBA ● was substituted for the 0.23 μM carrier ouabain (○). The simplest interpretation of this result is that SBA interacted reversibly with the [³H]ouabain binding sites and substituted for the carrier ouabain in the binding system. Under these conditions, therefore, SBA has

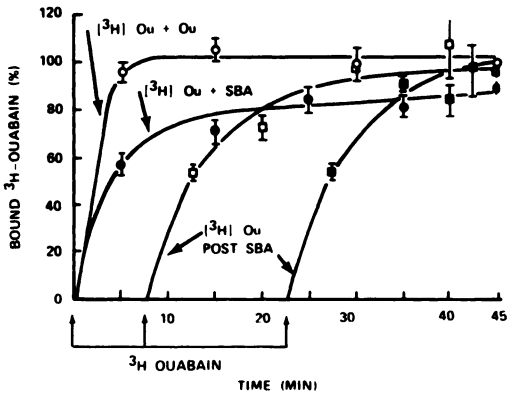

FIG. 3. *Na^+- and ATP-dependent binding of [^3H]ouabain to rat brain ($Na^+ + K^+$)-ATPase in the presence of carrier ouabain or SBA*

○——○, binding of [^{3}H]ouabain to rat brain ($Na^+ + K^+$)-ATPase under the conditions of Fig. 2 when the system contained tracer ouabain and 0.23 μM carrier ouabain; ●——●, binding when tracer ouabain was added to the system simultaneously with 0.8 μM SBA; □——□ and ■——■, binding of the same amount of tracer of [^{3}H]ouabain to the rat brain enzyme when the SBA was added at zero time and the tracer [^{3}H] ouabain at 7.5 and 22.5 min, respectively. Binding of [^{3}H]ouabain is expressed as a percentage of that observed at 45 min in the presence of labeled and unlabeled ouabain, which was 65×10^3 dpm/mg of protein. This value corresponds to 200 ± 16 pmoles of ouabain bound per milligram of enzyme protein.

about one-third the apparent affinity of ouabain for ($Na^+ + K^+$)-ATPase.

A further test for reversibility of the interaction of SBA with ($Na^+ + K^+$)-ATPase is shown in Fig. 3. Here the rat brain enzyme was incubated with Na^+, Mg^{++}, and ATP, and at zero time 0.8 μM SBA was added to the enzyme. At 0, 7.5, and 22.5 min the tracer [^{3}H]ouabain was added to the system. Either a time-dependent, irreversible occupation of the [^{3}H]ouabain binding sites by SBA should result in a time-dependent loss of [^{3}H]ouabain binding capacity, or, if the interaction of SBA with the CS binding site were reversible, the binding sites would remain accessible to [^{3}H]ouabain at all times and [^{3}H]ouabain binding would always equilibrate at the same level, independent of the order or interval between additions. As shown in Fig. 3, binding of [^{3}H]ouabain equilibrated at essentially the same level whether or not the SBA was added simultaneously with ouabain (●) or at 7.5 min (□) or up to 22 min (■) before the [^{3}H]-ouabain.

A similar experiment, except that glycoside binding was supported by Mg^{++} and P_i, is shown in Fig. 4. As found previously, prior exposure to SBA did not reduce the equilibrium level of [^{3}H]ouabain binding observed. Neither experiment suggests an irreversible interaction of SBA with the CS binding sites on this enzyme.

Although these experiments alone suggested that SBA does not interact irreversibly with the cardiac glycoside binding sites of the ($Na^+ + K^+$)-ATPase system, it was also desirable to demonstrate reactivation of the SBA-inhibited enzyme under appropriate conditions. This would be possible if the non-CS-binding site-directed interactions of SBA were insufficient to cause irreversible inhibition of the enzyme system. Figure 5 shows the inhibition of this enzyme system by SBA in the presence of Mg^{++} and P_i and the subsequent reactivation of the inhibited enzyme. In this experiment rat brain ATPase was incubated with mM Mg^{++}, 0.5 mM P_i, and 4 μM SBA at 37°. When the enzyme was challenged with Na^+, Mg^{++}, and [γ-^{32}P]ATP 9 or 24 min after the addition of SBA, the enzyme system was observed to be about 75% inhibited. However, if 10 mM EDTA was added to the system at either 9 or 24 min and the system was subsequently challenged with Na^+, Mg^{++}, and [γ-^{32}P]ATP at the indicated times, the enzyme fully recovered its ability to incorporate phosphate.

Figure 6 shows a similar experiment, in which SBA was allowed to bind via the nucleotide-stimulated pathway. Normally ATP is the nucleotide used to support binding by this pathway, but its use in this situation would result in a subsequent variable dilution of the [γ-^{32}P]ATP added to test for reactivation of the enzyme. Therefore UTP was used instead of ATP in the binding system. UTP is a very effective supporter of [^{3}H]ouabain binding to ($Na^+ + K^+$)-ATPase (8), and such binding is qualitatively similar to ATP-dependent binding (9). However, UTP is

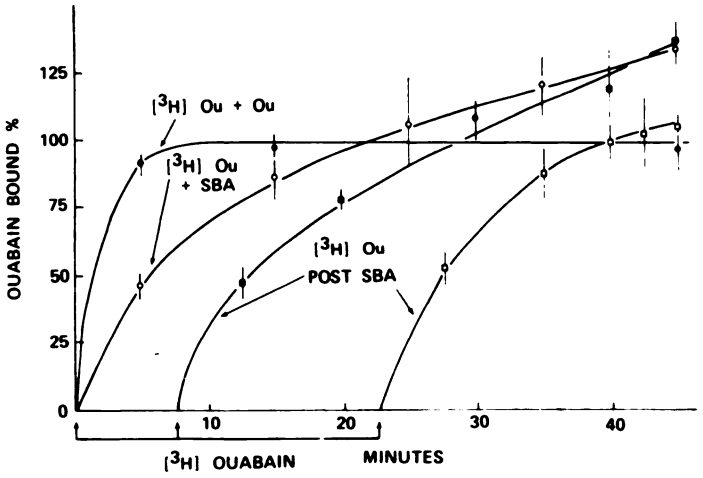

FIG. 4. *Mg^{++}- and P_i-dependent binding of [^{3}H]ouabain to $(Na^+ + K^+)$-ATPase in the presence of SBA*

The experimental conditions were the same as in Fig. 3, except that Na^+, Mg^{++}, and ATP were replaced by 4 mM Mg^{++} and 1 mM P_i . ●——●, binding of tracer ouabain with 0.23 μM carrier ouabain at indicated zero time; ○——○, binding of [^{3}H]ouabain when the tracer ouabain and 0.8 μM SBA were added together at zero time; ■——■ and □——□, binding of tracer [^{3}H]ouabain when it was added 7.5 and 22.5 min, respectively, after the addition of SBA. Binding is expressed as a percentage of that occurring in the presence of the labeled plus carrier ouabain at 45 min, which averaged 78 × 10^3 dpm/mg of protein or 239 ± 15 pmoles of [^{3}H]ouabain per milligram of protein.

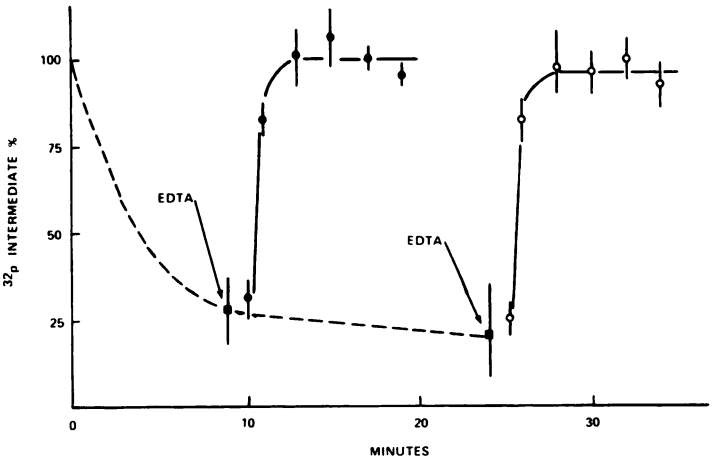

FIG. 5. *Recovery of phosphorylation after addition of EDTA to enzyme inhibited by SBA in the presence of Mg^{++} and P_i*

Rat brain $(Na^+ + K^+)$-ATPase was incubated with 5 mM Mg^{++} and 0.5 mM P_i , and 4 μM SBA was added at zero time. At 9 and 24 min after the addition of SBA, the enzyme was exposed to 100 mM Na^+, 10 mM Mg^{++}, and 0.05 mM [γ-^{32}P]ATP. The labeling reaction was stopped 3 sec later with trichloracetic acid: ■——■, amount of labeling observed. ●——● and ○——○, amount of labeling observed when 10 mM EDTA was added to the system at 9 or 24 min, respectively, and the enzyme was then allowed to react with 100 mM Na^+, 10 mM Mg^{++}, and [γ-^{32}P]ATP at the times indicated. Labeling is expressed as a percentage of that observed when the Na^+, Mg^{++}, and [γ-^{32}P]ATP mixture was added to an enzyme system in the presence of 4 mM Mg^{++}, 0.5 mM P_i , 4 μM SBA, and 10 mM EDTA. Under these conditions ^{32}P incorporation averaged 398 ± 54 pmoles/mg of protein. Labeling occurring when the Na^+ was replaced with 20 mM K^+ was deducted as background. Mg^{++} and P_i alone do not inhibit the phosphorylation of the enzyme from [γ-^{32}P]ATP under the conditions of this experiment.

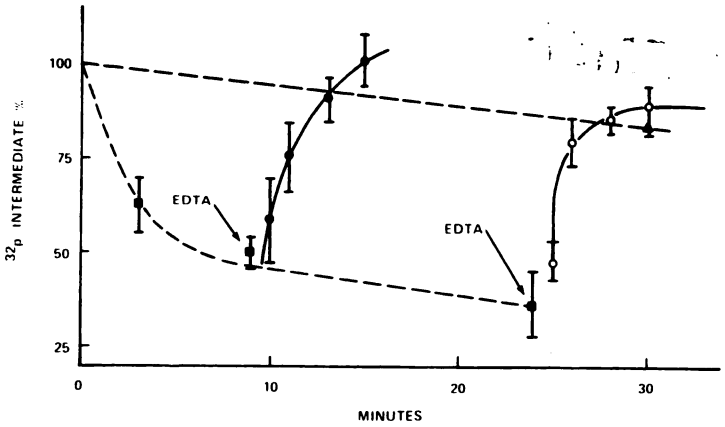

FIG. 6. *Recovery of phosphorylation after addition of EDTA to enzyme inhibited by SBA in the presence of Na^+ and UTP*

Rat brain (Na^+ + K^+)-ATPase was incubated with 20 mM Na^+, 0.2 mM $MgCl_2$, and 2 mM UTP at 37°, and at indicated zero time 4 μM SBA was added to the system. At 3, 9, and 24 min 0.05 mM [γ-^{32}P]ATP was added to the system, and the labeling reactions were terminated 5 sec after each addition. ■——■, inhibition of ^{32}P incorporation observed under these conditions; ●——● and ○——○, incorporation of ^{32}P observed when 5 mM EDTA was added to the system at 9 or 24 min (arrows), respectively, and labeling from [γ-^{32}P]ATP (plus 10 mM Mg^{++}) was carried out at the indicated time points. Labeling is expressed as a percentage of that observed when the enzyme was incubated with 20 mM Na^+, 0.2 mM Mg^{++}, 5 mM EDTA, 4 μM SBA, and 2 mM UTP and labeling was carried out with 10 mM Mg^{++} and 0.05 mM [γ-^{32}P]ATP. Under these conditions labeling averaged 289 ± 32 pmoles of ^{32}P per milligram of protein. ▲, amount of labeling observed when the enzyme was incubated with 20 mM Na^+, 0.2 mM Mg^{++}, 5 mM EDTA, 0.4 μM SBA, and 2 mM UTP for 30 min and then labeled with [γ-^{32}P]ATP and Mg^{++}. Labeling occurring when the Na^+ was replaced with 20 mM K^+ was deducted as background.

able to inhibit the phosphorylation of this enzyme from ATP only under carefully selected conditions (8) and normally will not reduce the steady-state level of the phosphorylation from ATP (10). Figure 6 shows that if the rat brain enzyme is incubated with Na^+, Mg^{++}, UTP, and SBA, substantial (60%) inhibition of the phosphorylation from [γ-^{32}P]ATP occurs. However, as found previously, the addition of excess EDTA to the system results in the recovery of labeling on the addition of excess Mg^{++} and [γ-^{32}P]ATP.

The recovery of phosphorylation in this experiment was not quite complete at 30 min. This, however, was not evidence for site-directed inhibition of the enzyme by SBA, because a similar loss in phosphorylation capacity was observed if the enzyme was incubated with Na^+, UTP, and SBA in the presence of 5 mM EDTA. Under these conditions cardiac glycosides do not bind specifically to (Na^+ + K^+)-ATPase (11).

DISCUSSION

This investigation of the reversibility of the interaction of SBA with the CS binding site of (Na^+ + K^+)-ATPase was prompted by the work of Roth-Schechter *et al.*. (4). These workers showed that the inotropic action of SBA in the perfused rabbit heart, like the inotropic action of ouabain (12), is rapidly reversible. From this observation they suggested that (Na^+ + K^+)-ATPase cannot be involved in the cardiotonic actions of these drugs, since they considered that SBA interacted irreversibly with the enzyme. They explained their inability to show a reduction in the (Na^+ + K^+)-ATPase activity of microsomes from SBA-perfused hearts by assuming that the "irreversible" SBA-enzyme complex dissociated during the preparation of the enzyme. They considered that their conclusions were supported by the observation that the incubation of cardiac (Na^+ + K^+)-ATPase with high concentrations of SBA for long periods

in vitro resulted in a loss of enzyme activity (13).

A more consistent explanation for these observations would be that SBA interacts reversibly with the cardiotonic binding site of the $(Na^+ + K^+)$-ATPase. Thus SBA would produce its positive inotropic action by interacting reversibly with the CS binding site of $(Na^+ + K^+)$-ATPase, and the rapid offset of the pharmacological effect would be associated with the reversibility of this interaction. This reversibility would also explain the inability of Roth-Schechter *et al.* (4) to demonstrate inhibition of the $(Na^+ + K^+)$-ATPase from SBA-treated hearts. The inhibition observed *in vitro* by Okita (13) would be due to nonspecific binding of SBA to these preparations at the high drug concentrations used in his experiments, probably complicated by thermal denaturation of the enzyme. To distinguish between these possibilities we undertook an investigation of the ability of SBA to interact irreversibly with the CS binding site of $(Na^+ + K)$- ATPase.

The experimental approach used represents an improvement on those available when the cardiac glycoside analogues were first developed (2). It was not then possible to study the enzyme-glycoside interaction directly, and the only parameter monitored was enzyme inhibition (2). The development of techniques to study the binding of [^{3}H]-ouabain to this enzyme (6, 8) permits a direct investigation of the effects of SBA on the CS binding site. Our results show that SBA will indeed interact at the CS binding site, although the apparent affinity of the enzyme for SBA may be somewhat less than for ouabain. However, since SBA has the ability to interact nonspecifically with any of the proteins present in the binding system, the effective concentration of SBA in the binding system will always be lower than that added to the system. Therefore the true affinity of the CS binding site for SBA is probably somewhat closer to its affinity for ouabain than the 3-fold difference suggested by the data presented in Fig. 3. The apparent discrepancy between the data presented in Figs. 1 and 3 is probably due to the greater concentration of membrane protein present in a [^{3}H]ouabain binding experiment, which would permit greater nonspecific binding of SBA and thus reduce the concentration of free SBA.

Rat brain $(Na^+ + K^+)$-ATPase was selected for these experiments because the original work suggesting that SBA may be a site-directed inhibitor of $(Na^+ + K^+)$-ATPase (2) was performed with beef brain $(Na^+ + K^+)$-ATPase preparations and Fricke and Klaus (14) presented supporting data obtained with guinea pig brain $(Na^+ + K^+)$-ATPase preparations. Since the binding of glycosides to rat brain $(Na^+ + K^+)$-ATPase preparations appears to be at least as stable as in most other tissues, and is much more stable than that occurring to rabbit or guinea pig heart $(Na^+ + K^+)$-ATPase (15–17), we felt that the use of this preparation would favor any tendency for inhibition by SBA to be irreversible.

Figure 3, which shows that the prior addition of SBA to the enzyme system did not reduct the subsequent binding of [^{3}H]-ouabain by this sytem, is strong evidence for the reversibility of the interaction of SBA with the cardiotonic steroid binding site. If the binding of SBA were irreversible one would expect a progressive decrease in the subsequent binding of [^{3}H]ouabain, whereas the binding tended to increase. This effect of SBA to increase the subsequent binding of [^{3}H]ouabain was most marked when binding was supported by Mg^{++} and P_i. These observations are consistent with a covalent interaction of SBA with the enzyme at sites other than the cardiotonic steroid binding sites. This reaction of SBA at other sites reduces the concentration of SBA available to compete for the CS binding site, and specific [^{3}H]ouabain binding increases. It is interesting that Fricke and Klaus (18) observed that this enzyme is more readily inhibited irreversibly by high concentrations of SBA in the absence of Na^+ and ATP; this finding is consistent with our data, which suggest that Mg^{++} and P_i accelerate the nonspecific interaction of SBA with the enzyme system. These experiments are also in good agreement with the data of Skou and Hilberg (19), who reported that the presence of Mg^{++} and ATP serves to protect $(Na^+ + K^+)$-ATPase from sulfhydryl inhibitors.

The data discussed thus far show that SBA will interact with the CS binding site of ($Na^+ + K^+$)-ATPase (Fig. 2). This reaction may be reversible, in that SBA could substitute for carrier ouabain in the [^{3}H]-ouabain binding system (Fig. 3) and prior incubation of the enzyme with SBA did not inhibit the subsequent binding of [^{3}H]-ouabain (Figs. 3 and 4). There are, however, circumstances under which this interpretation would be in error. If SBA reacted irreversibly with the CS binding sites, the loss of specific [^{3}H]ouabain binding capacity could be masked if SBA interacted elsewhere on the enzyme at a suitable rate. In this case the loss in specific binding sites would be compensated for by the loss of free "carrier" SBA and apparent increase in the specific activity of the [^{3}H]ouabain. Although this possibility is unlikely, it would result in some site-directed irreversible inhibition of the enzyme, and it was therefore desirable to demonstrate that the enzyme could be reactivated after its interaction with SBA. Previously such experiments have required repeated washing procedures to remove SBA and allow the assay of enzyme activity. Since these procedures are slow and inevitably associated with a loss of protein and enzyme activity from the reaction system, the formation of phosphoenzyme from [γ-^{32}P]ATP was used as a measure of enzyme activity. This was possible because bound cardiac glycosides inhibit the phosphorylation of this enzyme system but unbound cardiac glycosides do not interfere with the phosphorylation step (11, 20). Figures 5 and 6 show that whether the SBA is bound in the presence of Na^+, Mg^{++}, and UTP or of Mg^{++} and P_i, the exposure of the system to a high concentration of chelator results in reactivation of the enzyme. This presumably occurs because the chelation of Mg^{++} prevents further binding of SBA and exposes the spontaneous dissociation of this drug (11, 15–17, 21). The rate of recovery of phosphorylation was remarkably rapid (16), suggesting that the SBA dissociates from this enzyme more rapidly than ouabain, and this more rapid offset of SBA is consistent with the lower apparent affinity of the enzyme for SBA observed in Fig. 3. This relatively rapid dissociation of SBA from the enzyme is consistent with the recent data of Yoda (22), which indicate that aglycones dissociate more readily from the CS binding site on ($Na^+ + K^+$)-ATPase than do glycoside-containing molecules.

The conclusion from these experiments, that SBA is not a site-directed inhibitor of the phospho form of ($Na^+ + K^+$)-ATPase, is in good agreement with recent unpublished observations reported by Hokin (3, 23). This author reports that the phosphorylated form of the enzyme is not specifically alkylated by steroid haloacetates even though these agents are specifically bound at the CS binding site of the phospho-enzyme, as reported here. Hokin pointed out that these observations are consistent with the data of Roth-Schechter *et al.* (4) and that their data are not in conflict with the hypothesis that ($Na^+ + K^+$)-ATPase is the cardiotonic receptor for cardiac glycosides. Hokin and Dahl (23) also suggested that the interaction of SBA with dephosphorylated ($Na^+ + K^+$)-ATPase is at best a mixed reaction, being due partly to alkylation at the CS binding site and partly to interactions elsewhere on the enzyme. This interpretation is consistent with earlier observations (3) that it was not possible to label ($Na^+ + K^+$)-ATPase specifically with radiolabeled SBA because of the very high background radioactivity observed. Even when the background labeling was reduced 90% by prior incubation of the enzyme with *N*-(2-bromoacetyl)aniline, the labeling of these preparations was still many times greater than the number of ^{32}P (and therefore [^{3}H]ouabain) binding sites. Hokin's observations are further supported by the data presented here, which suggest that SBA interacts reversibly with the CS binding sites of ($Na^+ + K^+$)-ATPase and that irreversible interactions occur elsewhere on the enzyme.

The ease with which nonspecific interactions may occur during the affinity labeling of relatively impure enzyme or drug receptor preparations is attested to by the recent study of Winter and Goldstein (24) on the affinity labeling of morphine receptors. Despite the fact that these authors used more sophisticated photochemical affin-

ity labeling reagents, substantial nonspecific labeling occurred and they were unable to detect any increased specificity of labeling in their experiments. These observations and the data reported here show that particular care must be taken in studies utilizing affinity labeling to ensure that any labeling and/or inhibition actually occurs at the desired receptor site.

More recently Okita (13) has reported that the inotropic response produced by SBA, ouabain, and strophanthidin in Langendorff preparations of isolated rabbit hearts is associated with a loss in the yield of $(Na^+ + K^+)$-ATPase activity from these hearts. This occurred even though a 30-min drug washout period was allowed, during which the strength of contraction of these hearts returned to the baseline. These results have been interpreted as evidence that SBA irreversibly inhibits $(Na^+ + K^+)$-ATPase (13). Okita and co-workers have recently reported these and other experiments in more detail (25), again basing their interpretations on the suggestions of Hokin (3) that SBA is a site-directed irreversible inhibitor of $(Na^+ + K^+)$-ATPase. Unfortunately, however, ouabain and strophanthidin, both reversible inhibitors of this enzyme (2, 3, 11, 12, 14–18), produced an even greater loss of $(Na^+ + K^+)$-ATPase activity under the same conditions. Because of the apparent ease with which both SBA and ouabain dissociate from $(Na^+ + K^+)$-ATPase preparations (15–17), other possible explanations for these observations should be considered.

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