ON THE REVERSIBILITY OF BINDING OF CARDIOTONIC STEROIDS TO A PARTIALLY PURIFIED (Na + K)-ACTIVATED ADENOSINETRIPHOSPHATASE FROM BEEF BRAIN*

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SUMMARY

Incubation of a partially purified preparation of NaK ATPase from beef brain with various cardiotonic steroids under conditions of maximum binding of the steroid to the enzyme, followed by dilution, shows that the binding of cardenolide aglycones to the enzyme is reversible but that the binding of bufadienolide aglycones is only partly reversible. The binding of all cardiac glycosides to the enzyme is completely irreversible, suggesting that the sugar in glycosidic linkage with the 3-position of the steroid plays an important role in irreversible binding.

Cardiotonic steroids inhibit Na and K transport and the membrane-bound (Na + K)-activated adenosinetriphosphatase (NaK ATPase) which is believed to be involved in these processes[1]. This inhibition has attracted considerable attention for several reasons, among which is that it serves as an excellent model for study of drug-receptor interaction at the molecular level. Recently, several reports[2-4] have appeared on the specific binding of radioactive cardiotonic steroids to membrane preparations containing the NaK ATPase.

There has been some controversy concerning the reversibility of binding of cardiotonic steroids to the NaK ATPase[5]. This disagreement has prompted us to investigate the conditions for dissociation of the cardiotonic steroid-enzyme complex more closely.

Our testing for reversibility is based on the following technique. The enzyme is preincubated with a concentration of cardiotonic steroid which will give half-maximal inhibition (I_{50}) under standard assay conditions. The system is then diluted ten times with the assay medium, and assay is carried out. If the binding of the steroid to its site is reversible the inhibition by the cardiotonic steroid after dilution should be much less than 50% (approximately 10%);

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on the other hand, if the binding is irreversible the inhibition after dilution should be 50% (or higher if binding progresses during dilution and assay).

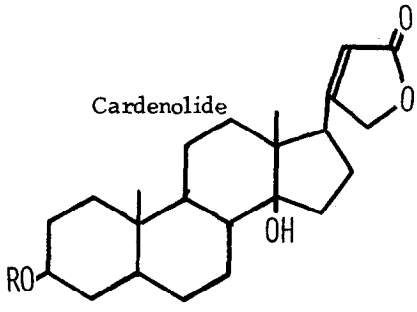
EXPERIMENTAL

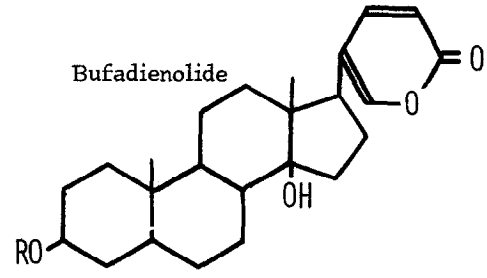
About 20 μg of partially purified beef brain NaK ATPase with a specific activity of 450-750 μmoles Pi/mg protein/hr and showing 99% ouabain-sensitivity was incubated for 10 minutes at 37° C with each steroid at a concentration to give its I_{50} and other additions as indicated, in a final volume of 0.5 ml. The I_{50} was determined by plotting percentage inhibition during assay against steroid concentration. After preincubation 2.5 mls of ice-cold water were added and 0.3 ml aliquots were mixed with 0.2 ml of assay medium. The final concentrations in the assay medium were: ATP, 4 mM; $MgCl_2$, 4 mM; KCl, 20 mM; NaCl, 140 mM; and imidazole-HCl (pH 7.3), 30 mM. The blank tube contained 0.1 mM ouabain. The liberated inorganic phosphate was measured by a modification of the method of Yoda[6]. Five-tenths ml of incubation medium (Pi> 0.01 μmoles) was added to 1 ml of a mixture of 4 volumes of 4.5% ammonium molybdate and 1 volume of 60% perchloric acid. Three mls (any suitable volume could be chosen) of n-butyl acetate were then added and the mixture was vortexed. About 2 mls of the organic layer were then clarified by centrifugation, and absorbancy of the clear butyl acetate solution was measured between 310 and 400 mμ (any wavelength between these values could be chosen depending on the concentration of inorganic phosphate). This method allows a much wider range of phosphate determinations than the usual Fiske and Subbarow method. The optical density is very stable and it is not necessary to remove protein and Lubrol if less than 20 μg and 10 μg, respectively, are present. If ATP-γ-^{32}P was used as substrate suitable aliquots of the butyl acetate solution were counted in a Geiger gas flow counter with a Mylar window.

Digitoxin, cymarin, and ouabain were obtained from S. B. Penick, Aldrich Chemicals, and Nutritional Biochemicals, respectively. Hellebrin was a kind gift of Hoffman La Roche. Digitoxigenin and strophanthidin were prepared by acid hydrolysis of digitoxin and strophanthin. Ouabagenin was obtained from ouabain by the method of Mannich[7]. Hellebrigenin and sciliglaucosidine 3-one were prepared by the microbiological method of Kupchan et al[8]. Berscillogenin was a kind gift of S. Morris Kupchan. After hydrolsis all aglycones were purified by

TABLE I

IRREVERSIBLE INHIBITION OF PARTIALLY PURIFIED NaK ATPase BY VARIOUS CARDIOTONIC STEROIDS[a]





Steroid	R	C_{19}	Substitution	I_{50} ($\times 10^{-7}$ M)	Irreversible Inhibition: Preincub. conc. ($\times 10^{-7}$ M)	Irreversible Inhibition: Inhibition after dilution %
Cardenolides						
Digitoxigenin	H	CH_3	-	1.5	2	16
					4	24
Digitoxin	D-digitoxyl	CH_3	-	5.7	10	89
					20	95
Strophanthidin	H	CHO	5 OH	3.0	2	10
					4	18
Cymarin	D-cymarosyl	CHO	5 OH	3.1	1	83
					2	85
Ouabagenin	H	CH_2OH	1 -OH 5 -OH 11 -OH	23.0	20	17
Ouabain	D-rhamnosyl	CH_2OH	1 -OH 5 -OH 11 -OH	5.4	1 2	73 88
Bufadienolides						
Hellebrigenin	H	CHO	5 -OH	0.5	0.5	41
					1	61
Hellebrin	D-rhamnosyl + D-glucosyl	CHO	5 -OH	1.3	2	88
					4	89
Sciliglaucosidine 3-one	-	CHO	4,5 C=C 3 C=O	1.5	1 2	35 40
Berscillogenin	H	CHO	4,5 C=C	1.0	0.5	33
					1.0	42

[a] During preincubation with the steroids the following were also present: 140 mM NaCl, 4 mM $MgCl_2$, 4 mM ATP, and 30 mM imidazole-HCl buffer (pH 7.3).

chromatography and/or crystallization. With the exception of strophanthidin**, all aglycones and glycosides were homogeneous on silica gel thin layer chromatography. The IR and UV spectra, melting points, and elemental analyses gave satisfactory values for all compounds. Our samples of strophanthidin, hellebrigenin, and sciliglaucosidine 3-one were coincident on thin layer chromatography with authentic samples.

RESULTS

As shown in Table I all glycosides showed strong inhibition of the NaK ATPase after dilution, indicating apparent irreversibility of binding to the enzyme. On the other hand, all cardenolide (5-membered lactone ring) aglycones derived from their parent glycosides showed negligible inhibition after dilution. Thus, among the cardenolides it can be concluded that the sugar in glycosidic linkage at the 3-position of the steroid is responsible for irreversibility and that the aglycone binds reversibly to the enzyme.

TABLE II

IRREVERSIBLE INHIBITION BY OUABAIN[a]

Addition	Percent Inhibition			
	Without Mg or K	With 4 mM Mg	With 10 mM K	With 4 mM Mg + 10 mM K
None	7	29, 31	0	3
Na (140 mM)	16, 8	13	-	-
ATP (4 mM)	6, 1	62, 60	0	29
Na + ATP	63, 51	88 (±4)	15	52
Pi (3 mM)[b]	0	90, 83	-	23
Pi + Na	0	11	-	-
p-Nitrophenyl-phosphate (4 mM)	0	40	-	-

[a]The enzyme was preincubated with 0.2 μM ouabain and 30 mM imidazole-HCl buffer (pH 7.3) and further additions as indicated.

[b]Assay was carried out by the radioactive method as described under Experimental.

**Preparations of strophanthidin consistently contain a faint spot on thin layer chromatograms in addition to strophanthidin. This contaminant was estimated to be less than 10%.

The bufadienolides (6-membered conjugated lactone rings) behaved differently: the aglycones showed some irreversibility of binding after preincubation, although not as great as the glycosides. Note that with both the cardenolides and the bufadienolides the affinity of the aglycone for the NaK ATPase was usually greater than that of the glycoside, the one exception being ouabain and ouabagenin. Thus, irreversibility cannot be explained merely by higher affinity of the cardiotonic steroid to its site but must be ascribed to some specific structural properties.

Irreversible inhibition by ouabain required the addition of certain combinations of ATP and/or ions (Table II). Maximal inhibition was achieved with ATP + Mg, ATP + Na, ATP + Mg + Na, and Mg + Pi. Considerable inhibition was also observed with Mg alone. Addition of K decreased irreversible inhibition under all of the conditions studied. Reversibly acting steroids added before ouabain or hellebrigenin antagonized the irreversible inhibition by these steroids, but were ineffective after the enzyme was inhibited irreversibly. The effects of ions and ATP on the irreversible inhibition by ouabain are similar to those reported for binding of radioactive ouabain or digoxin[2-4].

TABLE III

IRREVERSIBLE INHIBITION BY HELLEBRIGENIN[a]

Addition	Percentage Inhibition			
	Without Mg or K	With 4 mM Mg	With 10 mM K	With 4 mM Mg + 10 mM K
None	0	0	-	-
ATP (4 mM)	1	31, 27, 26[b]	-	35, 31[b]
ATP + Na (140 mM)	31, 28, 28[b]	58(±6), 55[b], 61[b]	0[b], 4	56, 65[b]
Pi (3 mM)[c]	16	57	-	50
Pi + Na[c]	0	0	-	-

[a] The enzyme was preincubated with 0.1 μM hellebrigenin.

[b] The enzyme concentration was one-twentieth the usual and the radioactive method was used.

[c] The radioactive method was used.

The effects of ions and ATP on the irreversible inhibition by hellebrigenin (a bufadienolide aglycone) were similar to those on the irreversible inhibition by ouabain except for the antagonistic effects of K (Table III). K exhibited no antagonistic effect under any condition if Mg was also present. However, there was antagonism in the absence of Mg.

DISCUSSION

The salient finding in this communication is that among the cardenolides, which constitute the majority of cardiotonic steroids, the inhibition of the NaK ATPase is reversible with the aglycones and irreversible with the glycosides. That the aglycones did bind initially but dissociated after dilution is attested by the fact that incubation of the enzyme with strophanthidin 3-[1-^{14}C] iodoacetate, ATP, Mg, and Na for a short period (no alkylation occurred under these conditions) led to maximal binding[9]. Washing of the enzyme after incubation with strophanthidin 3-[1-^{14}C] iodoacetate led to full recovery of enzyme activity.

Among the bufadienolides the story was somewhat different. The aglycones as well as the glycosides showed irreversible inhibition, although the irreversibility with the bufadienolide aglycones was not as great as with the cardiac glycosides. It would therefore appear that with the cardiotonic steroids containing the 5-membered lactone ring the sugar or sugars in glycosidic linkage at the 3-position impart irreversibility to the binding. The irreversible inhibition by the bufadienolide aglycones suggests that the mechanism of inhibition may be different here than with the cardenolide aglycones. It is possible that the reactivity of the conjugated 6-membered lactone ring of the bufadienolide is responsible for the difference.

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