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AZASTEROIDS AND HEART ADENOSINE TRIPHOSPHATASE*

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Abstract—Azasteroids, some known to be active, others to be inactive as inotropic and antishock agents, were tested as inhibitors of a metal-stimulated heart ATPase. In every instance the biologically active azasteroids inhibited catalysis just as did ouabain and the erythrophleum alkaloids. Biologically inactive azasteroids had no effect upon the enzyme. The *in vitro* enzyme system appears to hold promise as a preliminary assay method for inotropic activity. The relationship of azasteroid action *in vivo* to that of the cardiac glycosides and to the transport enzyme has been discussed.

QUINDONIUM bromide, an isoquiniline derivative, and some of its analogs have inotropic and antishock activities.[1, 2] These substances, though structurally similar to the cardiac glycosides, differ from them in essentials. Because the effects *in vivo* of some azasteroids are similar to those of the cardiac steroids, they provide a vehicle by which the structural requirements for inotropic effects may be studied. R. E. Brown *et al.*[2] have provided information that allows generalization concerning structure–activity characteristics of several azasteroids.

Cardiac aglycones and another class of substance, the erythrophleum alkaloids, have as one primary site of action the $Na^+ + K^+$ membrane ATPase.[3, 4] It was the purpose of our present study to examine the relationship of the azasteroids to a metal-stimulated ATPase of heart muscle. The effect of these compounds upon the enzyme has been compared with that of ouabain and of erythrophleum alkaloids.

MATERIALS AND METHODS

Rabbit hearts were obtained frozen from a commercial supplier. The azasteroids were a gift of the Warner-Lambert Research Inst., Morris Plains, N.J. E. Merck, GMBH, Darmstadt, provided the erythrophleum alkaloids. Ouabain and ATP were purchased from Sigma.

Hearts were macerated in 10 volumes of Tris/HCl buffer, 0·2 M, pH 7·2, containing 0·25 M sucrose, and then homogenized in a Potter-Elvehjem mill. In this and all subsequent steps of preparation, the material was kept at 0–4°. The homogenate was centrifuged at 600 *g* (20 min) and the supernatant dialyzed for 24 hr against the Tris-sucrose buffer containing 5 mM EDTA and Tris-saturated Amberlite IRC 50 resin in suspension. The dialysate was centrifuged at 10,000, 20,000, and 80,000 *g* (each 30 min), each time rejecting the pellet, and thence at 100,000 *g* for 70 min. The final pellet,

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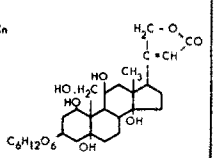
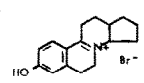
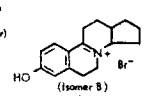
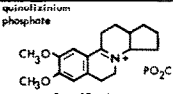
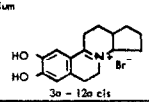
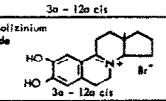
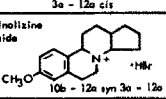
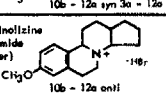
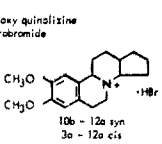
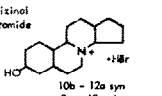
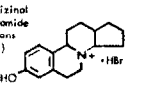
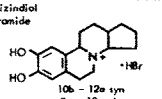
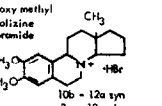
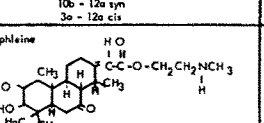
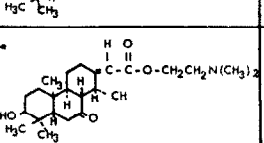
resuspended in 2 ml of buffer, constituted the enzyme. The method has been modified from that of Schwartz.[5] Protein content was determined for sample aliquots.

Reaction mixtures were incubated as a total volume of 1 ml consisting of 0·1 ml enzyme, 0·8 substrate [containing 0·3 disodium- or Tris-ATP, and KCl (0·002 M), NaCl (0·001 M), $MgCl_2$ (0·001 M), sodium deoxycholate (DOC), in Tris-sucrose buffer], and 0·1 ml of water or inhibitor in water.

Incubation was for 1 hr at 42°, after which the reaction was stopped with 0·1 ml of 50% trichloroacetic acid and the mixture centrifuged. The supernatant was decanted and assayed for inorganic phosphate.

TABLE 1. EFFECTS OF OUABAIN, THE SERIES OF AZASTEROIDS, AND OF ERYTHROPHLEUM ALKALOIDS UPON ATPASE ACTIVITY OF HEART SARCOPLASMIC RETICULUM

STRUCTURE	Biological Activity	ATPase inhibition (10^{-4} M inhibitor) % mean inhibition ± % variation	# of determinations
Ouabain 	+	38·2 ± 2·8	10
Quindonium bromide  3a – 12a cis	+	70·1 ± 20·0	6
Quindonium bromide (trans isomer)  (Isomer B) 3a – 12a trans	–	1·2 ± 1·2	10
Dimethoxy quinolizinium dichloro phosphate  3a – 12a cis	–	0·0 ± 0·8	9
Quinolizinium bromide  3a – 12a cis	±	32·1 ± 3·7	9
Methyl quinolizinium bromide  3a – 12a cis	+	26·3 ± 0·2	7
Methoxy quinolizine hydrobromide  10b – 12a syn 3a – 12a cis	+	29·3 ± 4·0	9
Methoxy quinolizine hydrobromide (anti isomer)  10b – 12a anti 3a – 12a cis	+	48·1 ± 9·3	9
Dimethoxy quinolizine hydrobromide  10b – 12a syn 3a – 12a cis	+	61·1 ± 21·1	9
Quinolizinol hydrobromide  10b – 12a syn 3a – 12a cis	–	0·0 ± 8·2	9
Quinolizinol hydrobromide (anti trans isomer)  10b – 12a anti 3a – 12a trans	+	51·2 ± 20·2	9
Quinolizindiol hydrobromide  10b – 12a syn 3a – 12a cis	–	16·5 ± 17·0	9
Dimethoxy methyl quinolizine hydrobromide  10b – 12a syn 3a – 12a cis	–	4·0 ± 12·0	10
Erythrophleine 	+	41·4 ± 4·6	6
Cassaine 	+	55·0 ± 3·2	6

RESULTS

The enzyme catalyzed the hydrolysis of ATP. This activity is inhibited by ouabain (Table 1) stimulated by Mg^{2+} and by $Na^{+} + K^{+}$ (Fig. 1). It has optimal activity when the reaction is incubated at 42° at pH 8·1.

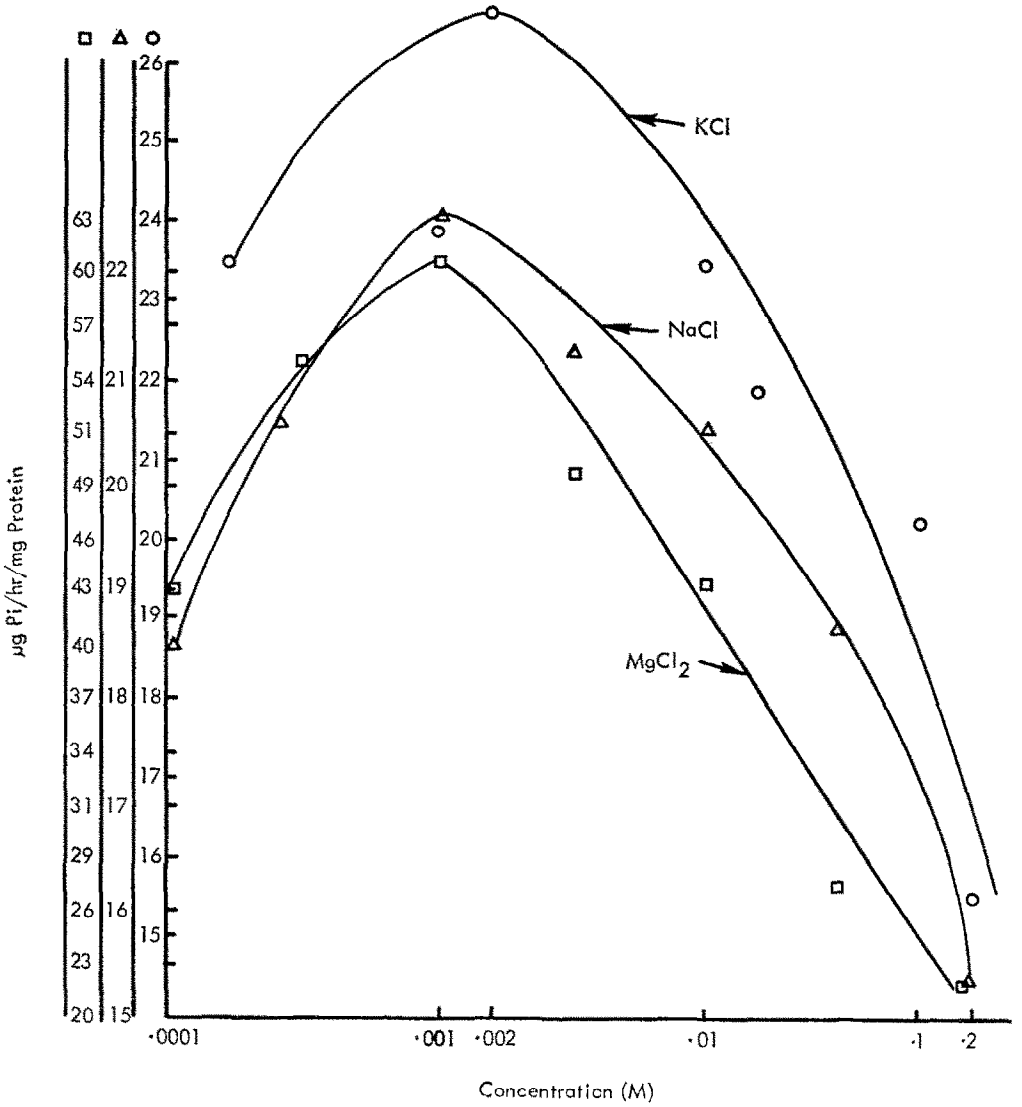


FIG. 1. The experimental results given in this figure illustrate the effect of variation in concentration of Na^{+}, K^{+}, or Mg^{++}. In each instance the other two metals were present at optimal level.

If dialysis against the Amberlite ion-exchange resin is omitted, or if the substrate mixture contains higher than the optimal amount of potassium chloride (0·01 M rather than 0·002 M), the amount of inhibition by quindonium as well as by ouabain is much less. But under these conditions synergism between quindonium and ouabain

becomes evident (Table 2). Potassium in higher concentration also lowered the effectiveness of erythrophleine sulfate. This alkaloid (10^{-4} M) in the presence of 0·002 M potassium chloride inhibited phosphate evolution 41·4 per cent (mean of six determinations); but in the presence of 0·01 M potassium chloride, the mean inhibition caused by erythrophleine was 20·2 per cent (five determinations).

TABLE 2. REPRESENTATIVE EXPERIMENTS ILLUSTRATING SYNERGISM BETWEEN QUINDONIUM AND OUABAIN WHEN HIGHER THAN OPTIMAL LEVELS OF POTASSIUM CHLORIDE (0·01 M) ARE PRESENT IN THE REACTION MIXTURE

Activity of preparation	Inhibitor	Activity in presence of inhibitor	Per cent inhibition
60·0	10^{-4} Ouabain	60·0	0·0
	10^{-4} Quindonium	58·8	2·0
	5×10^{-5} Ouabain together with 5×10^{-5} Quindonium	37·7	37·2
70·0	10^{-4} Ouabain	68·8	1·7
	10^{-4} Quindonium	64·4	8·0
	5×10^{-5} Ouabain together with 5×10^{-5} quindonium	27·7	60·0

With optimal metal levels, 10^{-4} M quindonium bromide consistently gave the maximal inhibition of the ATPase. This concentration was therefore selected for the study of the effect of the other inhibitors (Table 1). We have found that at 10^{-11} M concentration, the inhibitors were slightly stimulatory (quindonium, 8·8 $\pm$ 2·9 per cent, ouabain 13·9 per cent $\pm$ 2·1 per cent, erythrophleine sulfate 15·8 $\pm$ 6·3 per cent; figures given as the mean and variation of three determinations). This phenomenon has been reported for cardiac glycosides.[6]

DISCUSSION

We conclude from these data that biological activity and effectiveness as an inhibitor of heart membrane ATPase, by these substances, are correlatable phenomena. In every instance in which biological activity was reported, the compound effectively inhibited, in our system, ATPase-catalyzed hydrolysis. Quinolizinium bromide, described as having "questionable activity" ($\pm$) by R. E. Brown *et al.*,[2] was effective as an antagonist of the enzyme system. If it were to be assumed that enzyme antagonism and biological activity are cause and effect relationship, it would appear that the failure of the animal to consistently respond to quinolizinium involves a secondary event (perhaps, e.g., failure to reach the active site).

The cause and effect relationship between enzyme antagonism and biological activity has not, of course, been established. It has, however, been suggested[7] that ouabain, and the other cardiotonic glycosides and aglycones, have as a primary active site the $Na^+ + K^+$ transport ATPase. Whatever may ultimately prove to be the physiological mechanism of action of the cardiac glycosides, it would appear from the correlation described here that ouabain and the effective azasteroids studied have basically similar actions upon the membrane ATPase. Quindonium and the other azasteroids have sufficient structural similarity to allow, in our system, the first presumption of a common mode of action with the cardiac glycosides.

Necessity for *cis*-configuration (at the 3a–12a position; nomenclature of R. E. Brown *et al.*[2]) particularly points to the similarity of structural requirements.[8] The several ATPase inhibitors are also alike in the following regards: the concentration effects (being inhibitory at higher and stimulatory at very low concentrations) of ouabain and quindonium are similar; the several active azasteroids, like ouabain, inhibit a portion rather than the entire ATPase action of the membrane system. Further, a telling point in our view of these data, is the effect of potassium upon the inhibitory action. Both quindonium and ouabain are ineffective inhibitors in the presence of high potassium levels. This may indicate that both classes of inhibitor compete for the enzyme's potassium site.[9]

The apparent synergism of quindonium with ouabain (Table 2) in the presence of high potassium levels may be taken as a point at issue with the argument in favour of a common mode of action of the two substances. However, the synergism may instead illustrate only what is obvious from the two structures—that the substances are not identical. Even with the same sites on the enzyme, the two molecules are sufficiently different in their geometry so that under some conditions their effects may be complementary. This may be related to the thesis we have earlier presented, attributing a conformation-affecting role to the cardiac glycosides.[10] It may be noted tangentially that Osborne and Winbury[1] have observed physiological interaction of quindonium and ouabain.

The use of this heart ATPase system as a presumptive assay for the biological activity of azasteroids seems from our present observations to be a measure of practical value.

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