J. Enzyme Inhibition, 1997, Vol. 12, pp. 53–58
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DIFFERENTIATION BETWEEN VARIOUS TYPES OF INOTROPES THROUGH DISCOVERY OF DIFFERENCES IN THEIR ABILITY TO DETECT ISOFORMS OF Na^+/K^+-ATPase

KURT R.H. REPKE,* RUDOLF MEGGES and JÜRGEN WEILAND

Max Delbrück Center of Molecular Medicine, Robert-Rössle-Straße 10, D-13122 Berlin-Buch, Germany

(Received 9 September 1996)

Keywords: Na^+/K^+-ATPase; isoforms differentiation; inhibitors/inotropes of various types.

INTRODUCTION

The need for new inotrop cardiac drugs (inotropes) follows from the prevalence of congestive heart failure in the developed countries, and from the fact that the cardiac drugs currently available are clearly inadequate to restore health or even to minimize discomfort and disability.[1] In following up the targeted strategy in the search for ideal cardiac inotropes [2–4], we test here the ability of three types of compounds with perhydrophenanthrene nucleus to differentially interact with isoforms of Na^+/K^+-ATPase. This integral membrane protein, ubiquitous in the cells of higher eukaryotes, has been recognized to be the receptor of digitalis-like acting inotropes[2].

1. The cardenolide glycoside ouabain (**1**) (see Figure 1) has almost exclusively been applied for the analysis of the modalities of interaction between digitalis compounds and Na^+/K^+-ATPase.[5] Bufadienolide glycosides such as rhamnosyl-bufalin (**2**) have not yet been considered. They can attain tenfold

*Correspondence.

Abbreviations: Na^+/K^+-ATPase, Na^+/K^+–transporting adenosine triphosphate phosphorylase (EC 3.6.1.37); digitalis, generic name of steroids inhibiting Na^+/K^+-ATPase by intercalation in the digitalis binding cleft.

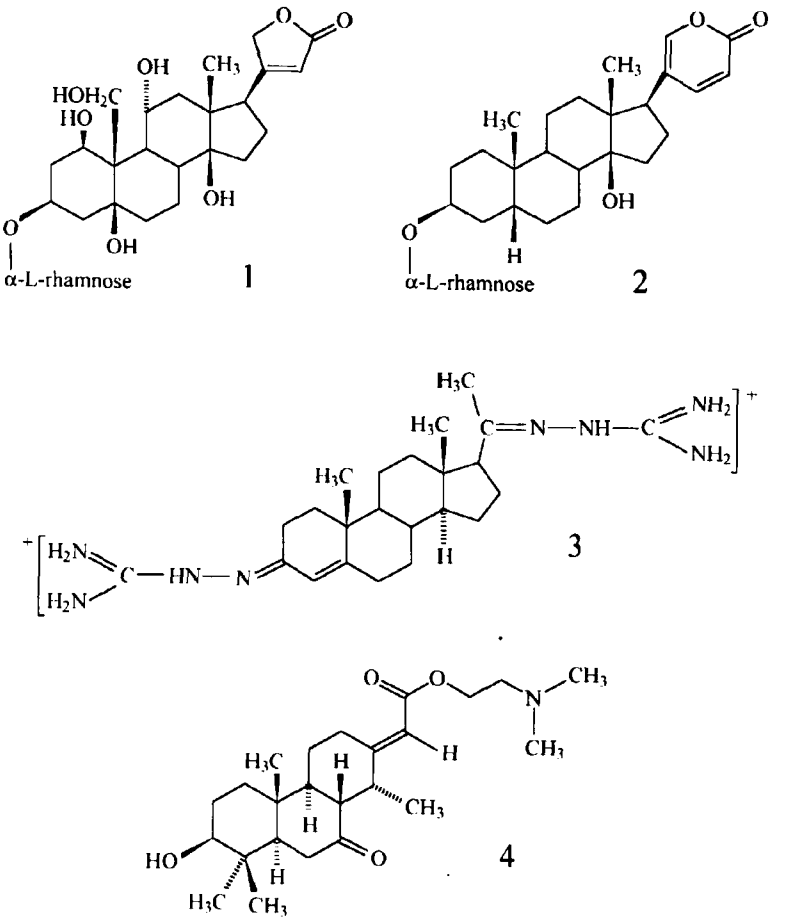

FIGURE 1 Structural formulae of ouabain (**1**), rhamnosyl-bufalin (**2**), progesterone-bisguanylhydrazone (**3**) and cassaine (**4**).

higher potencies than ouabain which may preclude the ability to differentiate between isozymes of different digitalis sensitivity.

2. The bisguanylhydrazones of prednisolone and progesterone (**3**) attracted much interest in the 1960s as possible therapeutic replacements for digitalis.[6] They produce a digitalis-like positive-inotropic effect which is closely related to inhibition of both Na^+/K^+–transport and Na^+/K^+-ATPase activity.[7] As reviewed in 1996[8] the bisguanylhydrazones show marked differences in

potency between species, although they do not show high and low affinities for Na^+/K^+-ATPase isoforms. Interestingly, they exclude ouabain binding, possibly indicating competition for binding to the digitalis receptor site. Actually, various steroids of a hormone type have been shown to operate through a digitalis-like submolecular mechanism, i.e., competent occupancy of the digitalis recognition cleft.[3,9]

3. The alkaloids from the Erythrophleum species such as cassaine (**4**) do not seem to satisfy any of the structural requirements thought to be associated with digitalis-like activity since their perhydrophenanthrene skeleton does not carry either a lactone or a sugar side chain. Nevertheless, cassaine appears to inhibit Na^+/K^+-ATPase by occupying the digitalis binding site.[9,10] Consistent with this hypothesis is that the enzymes from dog, guinea-pig and rat heart showed differing sensitivities to cassaine paralleling their differing sensitivities to ouabain.[11] Remarkably, already in 1971 the perhydrophenanthrene nucleus of the Erythrophleum alkaloids has been thought worthy of consideration as a starting point for the partial-synthesis of new inotropes.[12]

The purpose of the present study has been to clarify whether these diverse structures can be reconciled in terms of a single model for the digitalis binding receptor site. This should allow us to draw constructive information in the search for new inotropes.

MATERIALS AND METHODS

Inhibitors Used in This Study

Ouabain (**1**) and cassaine (**4**), and progesterone-bisguanylhydrazone (**3**) were gifts from Bayer (Leverkusen) and Merck (Darmstadt), respectively. Rhamnosyl-bufalin (**2**) was prepared according to established procedures.[13]

Preparation and Determination of Na^+/K^+-ATPase

Homogenates of rat sciatic nerve were used as enzyme source as described by others.[14] The portion of ATPase activity suppressed by 3 mM ouabain was taken as Na^+/K^+-ATPase activity, on the average amounting to 11 μmol h^{-1} mg $protein^{-1}$. This accounted mostly for half of the total ATPase activity. The presence of such a high percentage of ouabain-resistant ATPase activity accounts for the high degree of scattering found in the inhibition parameters (cf. Table I). The determination of Na^+/K^+-ATPase activity was performed as described earlier.[15]

TABLE I The effect of certain inotropes on the isoforms of Na^+/K^+-ATPase in rat sciatic nerve.

Compound	*Inhibitor, systematic (trivial) name*	*No. of expts.*	*High affinity isozyme traced*[a] *(%)*	IC_{50}[b] *(± S.D.) μM*	IC_{50}[c] *(± S.D.) μM*
1	3β-Rhamnosyloxy-1β,5,11α, 14,19-pentahydroxy-5β,14β -card-20(22)-enolide (ouabain)	$\eta = 5$	36 ± 9	0.21 ± 0.13	120 ± 59
2	3β-Rhamnosyloxy-14-hydroxy-5β,14β-bufa-20,22-dienolide (rhamnosyl-bufalin)	$\eta = 6$	38 ± 7	0.015 ± 0.009	1.56 ± 0.53
3	Pregn-4-ene-3,20-bisguanyl-hydrazone (progesterone-bisguanylhydrazone)[d]	$\eta = 2$	not traceable	8.7 ± 1.9	
4	Cassaic acid dimethyl-aminoethyl ester (cassaine)	$\eta = 3$	29 ± 5	0.78 ± 0.45	127 ± 30

[a]Computed by the algorithm introduced for the simulation of dose-response curves with two independent receptors.[16] [b,c]Inhibition constant of the complexes with the high-affinity and low-affinity isozyme, respectively. [d]Hill coefficient n = 1.7.

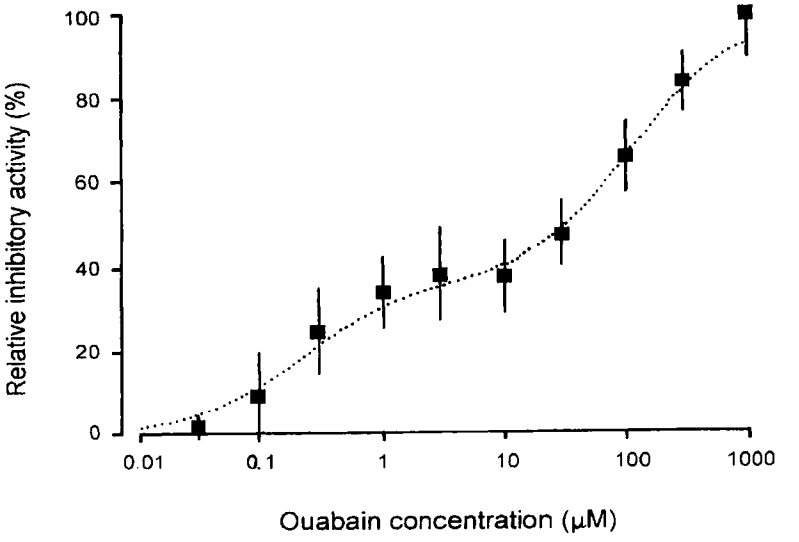

FIGURE 2 Simulation of the curve describing the relation between varying concentrations of ouabain (**1**) and inhibitory action on two isoforms of Na^+/K^+-ATPase from rat sciatic nerve (■···■) by applying the algorithm for two independent receptors.[16] The results of the quantitative analysis of such biphasic curves, obtained with three inhibitors (**1**, **2**, **4**) is shown in Table I.

TABLE II Comparison of the IC_{50} (μM) ouabain values reported for rat $\alpha 1$ and $\alpha 2$ isozymes with those found for rat sciatic nerve.

$\alpha 1$	$\alpha 2$	*Reference*
40	0.1	Berrebi-Bertrand *et al.*[17]
48 ± 3.6	0.098	O'Brien *et al.*[18]
98 ± 9	0.15 ± 0.002	Blanco *et al.*[19]
120 ± 59	0.21 ± 0.13	This paper

Determination of Inhibitory Steroid Potency

This was done in principle as reported previously.[15] The parameters presented in Table I were computed by assuming the presence of two isoforms of Na^+/K^+-ATPase and applying the algorithm introduced by others for the simulation of dose-response curves with two independent receptors.[16]

RESULTS AND DISCUSSION

The occurrence of the three isoforms of Na^+/K^+-ATPase with high and low ouabain affinity in rat brain membranes has been described[17] such that their presence in rat sciatic nerve (Figure 2 and Table I) is not surprising. The comparison of the IC_{50} values for oubain determined here with those reported for the rat isozymes[17–19] (Table II) suggests that the enzyme species of low and high ouabain affinity correspond with the $\alpha 1$- and $\alpha 2$-catalytic subunits of Na^+/K^+-ATPase, respectively. The possible admixture of a small percentage of the $\alpha 3$-isozyme cannot be traced by the applied procedure.

Rhamnosyl-bufalin (**2**), although developing an inhibitory activity 10- to 100-fold higher than ouabain (**1**), does nevertheless sense the two isoenzymes (cf. Table I). Their common structural feature, the cycloperhydrophenanthrene nucleus, appears to mediate the isoform distinction.

Although built from a similar nucleus, progesterone-bis-guanylhydrazone (**3**) does not show high and low affinities for the α-subunit isoforms (cf. Table I). Moreover, (**3**) inhibits the ouabain-resistant Mg^{2+}-ATPase which is a notorious contaminant of Na^+/K^+-ATPase preparations. In addition, (**3**) shows a Hill coefficient near 2, whereas n lies for (**1**) and (**2**) near 1 or below. Furthermore, (**3**) does not promote the phosphorylation of Na^+/K^+-ATPase from orthophosphate which otherwise digitalis-like acting inhibitors characteristically do.[9] Taken together, the findings exclude the penetration of (**3**) into the digitalis intercalating matrix as a

mechanism of its inhibitory interaction with Na^+/K^+-ATPase. In conclusion, the guanylhydrazones of 3,20-diketosteroids appear to be unsuitable as models for the design of novel inotropes.

Most remarkably, the Erythrophleum alkaloid cassaine (**4**) differentiates between the α1- and α2-forms as well as ouabain does. Their common structural moiety >C=CH-C=O could mediate H-bridge binding to the -SH group of cysteine-104 in the digitalis intercalating matrix,[20] and could hence help to explain the similarity of their potency in inhibiting the low- and high-affinity isoforms of Na^+/K^+-ATPase (cf. Table I) . In conclusion, the perhydrophenanthrene skeleton could well be considered as a precursor structure for the synthesis of new inotropes.

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