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COMPARATIVE STUDIES OF SOME SEMISYNTHETIC K-STROPHANTHINS WITH NATURAL CARDIAC GLYCOSIDES*

HODA TAWFIK†, ALANNA A. L. FOX and KURT GREEFF‡
Institut für Pharmakologie der Universität Düsseldorf, Moorenstraße 5, D-4000 Düsseldorf, Federal Republic of Germany

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Abstract—In this study the pharmacological effects of seven semisynthetic cardenolides have been investigated and compared with those of 11 natural cardiac glycosides. These compounds are of different potency on electrically driven isolated guinea-pig atria. Their concentration response curves showed different slopes, which could be an indication of varying therapeutic range. The pharmacodynamics of these compounds on isolated guinea-pig atria are in good correlation with the data obtained from binding studies on guinea-pig ventricular homogenate as well as that obtained from comparative experiments on Na^+,K^+-ATPase activity inhibition.

In the treatment of heart failure cardiac glycosides are still indispensable. Their use is, however, rendered difficult by their narrow therapeutic range. Cases of intoxication are frequently observed during digitalis therapy (8–25%, [1–3]). Many chemical modifications of cardiac glycosides have been made [4] in order to find new cardenolides with an improved therapeutic ratio. Recently reports have been published about some compounds with a wider therapeutic range: for example actodigin [5–7], which is the β-glucoside of a digitoxigenin isomer and ASI-222 [8–10], which is the 4-amino-4,6-dideoxy-galactoside of digitoxigenin. For these two derivatives, the biological activity seems to be altered due to changes in the sugar moiety. In the case of actodigin Thomas *et al.* [11] suggested that the biological activity of the molecule is increased because of the isomeric lactone influence on the drug receptor interaction, in a way that the sugar portion is directed away from its binding site. For ASI-222 Caldwell and Nash [8, 9] suggest that the amino sugar is responsible for the improvement in the therapeutic ratio. Another digitoxin derivative (3-α-methyl-digitoxigenin-3-β-glucoside, 3-α-MDG) has been investigated in the last few years and has been postulated to possess a wider therapeutic range [12–19].

In our experiments we made comparative studies of the biological activity of some semisynthetic (Figs 1 and 2) and some natural cardenolides. The pharmacodynamic parameters have been tested on isolated electrically driven guinea-pig left atria. The biochemical parameters have been investigated by measuring the inhibition of the Na^+,K^+-ATPase activity and by an indirect binding study on heart homogenate. The inhibition of binding of labelled ligands by unlabelled analogues gives a measure of the affinity of the analogues as their equilibrium dissociation constants (K_D values) [20, 21].

MATERIALS AND METHODS

Chemicals. ^{3}H-ouabain with a specific activity of 14 Ci/mmole was obtained from NEN (Dreieich, FRG), ATP, NADH, phosphoenolpyruvate (PEP) and pyruvate-kinase/lactate-dehydrogenase were obtained from Boehringer–Mannheim GmbH (FRG). Lyophilised beef albumin was supplied by Serva Feinbiochemica (Heidelberg, FRG) and Unisolve scintillation liquid by Werner Zinsser (Frankfurt, FRG). All other chemicals were of analytical grade and were supplied by Merck (Darmstadt, FRG).

Glycosides. Dihydroouabain, digoxigeninmonodigitoxoside, digitoxigeninmonodigitoxoside, k-strophanthin, k-strophanthoside (Boehringer–Mannheim GmbH, FRG); digoxin, digitoxin, g-strophanthin (ouabain) (Merck, Darmstadt, FRG); ^{3}H-ouabain (NEN, Dreieich, FRG); 3-α-methyldigitoxigenin-β-glucoside (3-α-MDG) (Beiersdorf AG, Hamburg, FRG); strophanthidol, convallatoxol, cymarin, convallatoxin, nicotinoyl-k-strophanthidin (NS) Gö 2616, nicotinoyl-k-strophanthidin methoiodide (NSM) Gö 2485, methylconvallatoxin Gö 3333, convallatoxinacetonide (CAc) Gö 2487, convallatoxol-acetonide-borate ester (CAcBE) Gö 2571, butyryl-convallatoxin-acetonide (BCAc) Gö 2800 (Gödecke AG, Freiburg, FRG).

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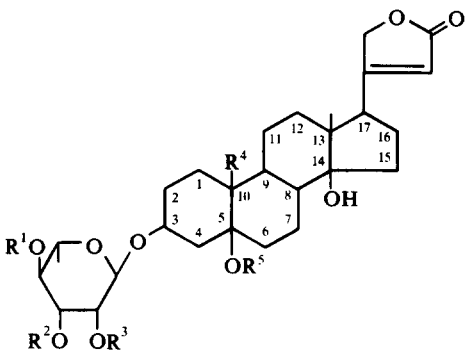
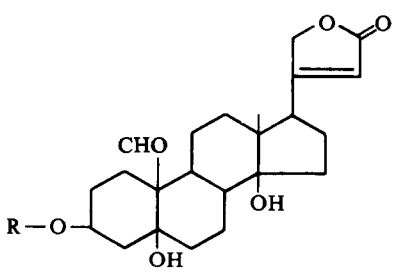
A part of these results has been presented at the meeting of the Deutsche Gesellschaft für Herz- und Kreislaufforschung, H. Tawfik, A. A. L. Fox and K. Greeff, *Zeitschrift für Kardiologie* **72**, *Suppl.* 1, 104 (1983).
† Present address: Cairo University, Faculty of Pharmacy, Department of Pharmacology, el Kasr el Einy Str., Cairo-Egypt.
‡ To whom correspondence should be addressed.



Glycoside	R_1	R_2	R_3	R_4	R_5
Convallatoxin	H	H	H	—CHO	H
Methylconvallatoxin *Gö 3333*	H	$-CH_3$	H	—CHO	H
Convallatoxinacetonide (CAc) *Gö 2487*	H	$-C(CH_3)_2-$		—CHO	H
Butyryl-convallatoxinacetonide (BCAc) *Gö 2800*	$CH_3(CH_2)_2CO-$	$-C(CH_3)_2-$		—CHO	H
Convallatoxolacetonideborate ester (CAcBE) *Gö 2571*	H	$-C(CH_3)_2-$		$-CH_2O-B-$ OH	

Fig. 1. Structural formula of convallatoxin derivatives.

Isolated guinea-pig left atrial preparation. Male guinea-pigs (weight range 300–450 g) were killed by a blow on the back of the neck, the hearts were quickly excised and the atria dissected and fixed on two platinum electrodes. The preparations were incubated in Krebs–Henseleit solution (10 ml) at 31° and aerated with carbogen (95% O_2 and 5% CO_2). The left atria were electrically stimulated by impulses of 1 Hz, 1 ms duration, and a voltage of 2.5-fold the threshold value. The preload applied on the atria was 1 g. Force of contraction was recorded under isometric conditions by inductive force transducer (Statham-UC 2). The tested substance was added to the bath cumulatively according to the WL-6 or WL-12 system of Hackenberg and Bartling [22]. The next dosage was applied when force of contraction had reached a steady state. The results were evaluated as concentration–response curves, the upper limit was the last concentration before the appearance of intoxication (arrhythmia or decrease in contractile force). For the determination of the onset and offset of the effect, a single high concentration of the cardenolide (as listed in Table 3) was applied and the time till steady-state (t_{max}) was measured. Then the tissue was washed six times by overflow and the time until force of contraction reached its original value (t_0) was determined.



k-strophanthidin R = H

Nicotinoyl-k-strophanthidin (NS) *Gö 2616* R =

Nicotinoyl-k-strophanthidin methoiodid (NSM) *Gö 2485* R =

Fig. 2. Structural formula of k-strophanthidin derivatives.

Preparation of Na^+,K^+-ATP*ase from guinea-pig hearts.* The isolated guinea-pig hearts were freed from atria and connective tissue, weighed and minced with scissors. The minced tissues were homogenised in a solution containing 0.25 mole/l sucrose, 1 mmole/l Tris buffer and 1 mmole/l EDTA (pH = 7.5) with an Ultra Turrax for 30 sec. From this homogenate the cardiac Na^+,K^+-ATPase was extracted according to the methods of Pitts and Schwartz [23]. The activity of the Na^+,K^+-ATPase measured by the coupled optical assay according to Pullman *et al.* [24] and Fritz and Hamrick [25] was between 20 and 30 μmoles Pi/(mg protein·hr).

Binding of ^{3}H-*ouabain to ventricle homogenate.* The binding studies were carried out according to Yamamoto *et al.* [26]. Binding experiments were carried out on homogenates as it was thought that this preparation may represent the situation in the intact heart better than investigations carried out on

the purified enzyme. On the other hand to accurately estimate inhibitory potency of the various cardenolides on Na^+,K^+-ATPase activity partial enzyme purification was necessary, as homogenates showed very low catalytic activity. Ventricular tissues were homogenised in a solution of 1 mmole/l EDTA and 10 mmole/l Tris–HCl (pH 7.5) by Ultra Turrax for 30 sec at 4° (30 mg tissue/ml). The homogenate was incubated at 37° with 10 nmoles/l ^{3}H-ouabain in a medium containing 200 mmoles/l NaCl, 5 mmoles/l $MgCl_2$, 5 mmoles/l ATP and 50 mmoles/l Tris–HCl buffer (pH 7.5), in the presence or absence of various concentrations of the unlabelled cardenolides. The length of the incubation period varied according to the time required for equilibrium to be reached between the radioactive ouabain and the respective unlabelled cardenolide. The time to equilibrium was determined in separate binding experiments. Bound and free ^{3}H-ouabain were separated by filtering the aliquot through nitrocellulose filter (type AA, pore size 0.8 μm, Millipore Corp.) as described by Ku *et al.* [27] and the radioactivity was estimated using a liquid scintillation spectrometer. The K_D values of the tested cardenolides were calculated according to the method of Erdmann and Schoner [20] and that for ouabain by Scatchard analysis.

Protein concentration. Determined by the method of Lowry *et al.* [28].

Statistical analysis. Statistical analysis of dose–response curves by logit transformation to determine the slope was performed according to Hafner *et al.* [29]. Results are expressed as $\bar{X} \pm$ S.E.M.

RESULTS

Effects on the force of contraction of guinea-pig left atria

All cardenolides investigated in this study cause an increase in force of contraction of electrically driven guinea-pig left atrial preparations. From the concentration–response curves (Fig. 3) three important parameters were estimated: (a) The potency was determined by the calculation of the EC_{50} [29], which reflects the concentration of cardenolide producing a half maximal increase in force of contraction, (b) the intrinsic activity was measured as the contractile state immediately prior to the onset of arrhythmias or increase in diastolic tension, (c) the slope of the concentration–response curve which may be used as an indication of the therapeutic range.

In Table 1 the cardenolides are arranged according to their potency. CAcBE shows the highest potency in atrial tissue having the lowest EC_{50} value (0.061 μmoles/l) and k-strophanthidin shows the lowest potency (EC_{50} = 18.6 μmoles/l). The difference in the maximal inotropic effects of the cardenolides (intrinsic activity) is not statistically significant, k-strophanthidin, dihydroouabain and NSM produce the highest and digoxigeninmonodigitoxoside and BCAc the lowest maximal inotropic effect (Fig. 3). The slopes in concentration–response curves of the semisynthetic substances NSM and CAcBE are significantly lower than that of the other cardenolides (s_{NSM} = 1.31, s_{CAcBE} = 1.37). The concentration–response curve for dihydroouabain has the highest slope (s = 3.06). In Table 2 the values t_{max} and t_0 of the tested cardenolides are listed representing the onset and offset of the positive inotropic effect. As seen in Fig. 4 the onset and offset times are in good correlation (t_{max} = 0.5, t_0 = +5.8 and r = 0.78) with the exception of digitoxin and CAcBE, which have the highest t_0 values. Excluding these two from the calculation we obtain a coefficient of r = 0.96.

Effect on the Na^+,K^+-ATP*ase activity*

Of the cardenolides so far tested we chose eight to investigate their effects on the Na^+,K^+-ATPase. All of them inhibit the activity of Na^+,K^+-ATPase. As in the experiments with guinea-pig atria, CAcBE and digitoxin showed the highest and NSM and 3-α-MDG the lowest activity from those cardenolides chosen. The concentrations which caused 50% inhibition of the Na^+,K^+-ATPase activity are listed in Table 3.

Binding studies

With the same eight cardenolides we performed binding studies in the presence of ^{3}H-ouabain. The data obtained from the inhibition of ^{3}H-ouabain

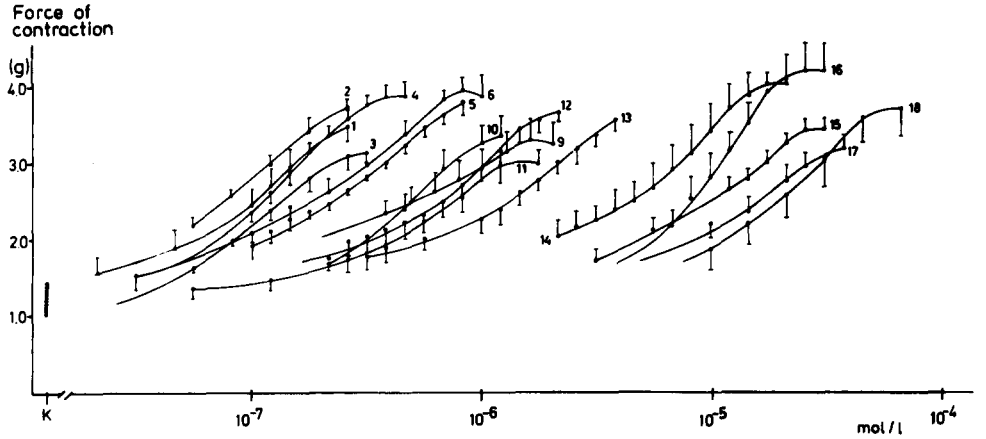


Fig. 3. Concentration response curves of the tested cardenolides on electrically driven isolated guinea-pig atrial preparations (N = 6; $\bar{X} \pm$ S.E.M.; for substances see Table 1).

Table 1. Effects on the force of contraction of electrically driven guinea-pig atria: EC_{50}, relative potency and slope of the concentration–response curve

	EC_{50} (μmoles/l)	Potency related to strophanthidin	Slope s (N = 6)
CAcBE (1)	0.061 ± 0.002	305	1.37 ± 0.05
Digitoxin (2)	0.076 ± 0.002	245	2.03 ± 0.13
Digitoxigeninmonodigitoxoside (3)	0.087 ± 0.002	214	2.32 ± 0.12
Convallatoxin (4)	0.106 ± 0.002	175	2.01 ± 0.07
Methylconvallatoxin (5)	0.197 ± 0.004	94	1.51 ± 0.06
CAc (6)	0.202 ± 0.006	92	1.55 ± 0.08
Ouabain (7)	0.250 ± 0.005	74	2.60 ± 0.14
Digoxin (8)	0.312 ± 0.008	60	1.91 ± 0.08
BCAc (9)	0.347 ± 0.015	54	1.45 ± 0.10
k-Strophanthoside (10)	0.352 ± 0.010	53	2.28 ± 0.11
Digoxigeninmonodigitoxoside (11)	0.535 ± 0.014	35	1.32 ± 0.05
Cymarin (12)	0.566 ± 0.013	33	1.63 ± 0.06
Strophanthidol (13)	1.31 ± 0.28	14	1.57 ± 0.05
NSM (14)	4.37 ± 0.13	4.2	1.31 ± 0.05
3-α-MDG (15)	6.56 ± 0.17	2.8	1.76 ± 0.08
Dihydroouabain (16)	9.51 ± 0.13	1.9	3.06 ± 0.13
NS (17)	10.2 ± 0.42	1.8	1.89 ± 0.15
k-Strophanthidin (18)	18.6 ± 0.49	1.0	1.70 ± 0.09

Table 2. Onset time (t_{max}) and offset time (t_0) of the cardenolide effect. C = single cardenolide concentration used (N = 6; $\bar{X}$ ± S.E.M.)

Glycoside	C (μmoles)	t_{max} (min)	t_0 (min)	t_{max}/t_0
CAcBE (1)	0.261	51 ± 1.4	161 ± 4	0.32
Digitoxin (2)	0.261	114 ± 5	111 ± 9	1.27
Convallatoxin (4)	0.383	30 ± 2.2	59 ± 2.2	0.51
Methylconvallatoxin (5)	0.825	8 ± 0.5	17 ± 1.7	0.47
CAc (6)	0.825	48 ± 0.9	91 ± 7.8	0.53
Ouabain (7)	0.825	30 ± 2	30 ± 3	1.00
Digoxin (8)	1.21	59 ± 2.6	97 ± 12	0.61
k-Strophanthoside (10)	1.21	57 ± 2	104 ± 8	0.55
Cymarin (12)	2.15	23 ± 0.1	45 ± 8.4	0.51
Strophanthidol (13)	3.16	15 ± 1	20 ± 3	0.75
NSM (14)	17.8	7 ± 0.3	8 ± 0.9	0.88
3-α-MDG (15)	21.5	9 ± 0.7	5 ± 1	1.80
Dihydroouabain (16)	26.1	17 ± 1.7	13 ± 1.7	1.31
NS (17)	56.2	40 ± 4.4	57 ± 10	0.70
k-Strophanthidin (18)	68.1	25 ± 1.2	38 ± 9	0.66

Table 3. Tne cardenolide concentrations, which cause a 50% increase in force of contraction (EC_{50}), 50% inhibition in Na^+/K^+-ATPase activity (I_{50}), dissociation constants (K_D) measured indirectly by ^{3}H-ouabain displacement and their ratio to one another (N = 6)

	μmoles/l			Ratio	
Glycoside	EC_{50}	I_{50}	K_D	EC_{50}/I_{50}	EC_{50}/K_D
CAcBE (1)	0.061	0.411	0.023	0.2	2.7
Digitoxin (2)	0.076	0.152	0.023	0.5	3.2
Convallatoxin (4)	0.106	0.637	0.098	0.2	1.1
CAc (6)	0.202	0.895	0.083	0.2	2.4
Ouabain (7)	0.250	0.739	0.088	0.3	2.8
k-Strophanthoside (10)	0.352	0.676	0.122	0.5	2.9
NSM (14)	4.370	7.90	1.07	0.5	4.1
3-α-MDG (15)	6.560	12.3	1.75	0.5	3.8
Mean values				0.4 ± 0.1	2.9 ± 0.3

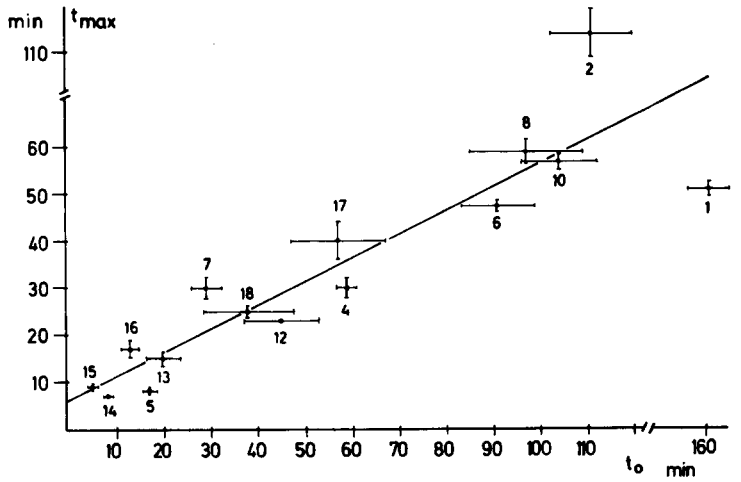


Fig. 4. Correlation between the onset (t_{max}) and offset (t_0) time of the cardenolide effect on guinea-pig left atrial preparation (N = 6; $\bar{X} \pm$ S.E.M.; for substances see Table 1).

binding by unlabelled ligands are used to calculate the K_D for ouabain directly by Scatchard analysis and for the other cardenolides indirectly by the procedure of Erdmann and Schoner [20]. In Table 3 the K_D values of the tested cardenolides, the ratios EC_{50}/I_{50} and EC_{50}/K_D are listed. The three concentrations EC_{50}, K_D and I_{50} are in good correlation to each other as shown in Fig. 5.

DISCUSSION

During the last few years many investigations have been carried out with respect to new cardenolides with optimal therapeutic properties [4, 6, 8, 9]. As Pastelin and Mendez [7] stated, the ideal cardiac glycoside would be a compound that retains the therapeutic pharmacological effects of digitalis and

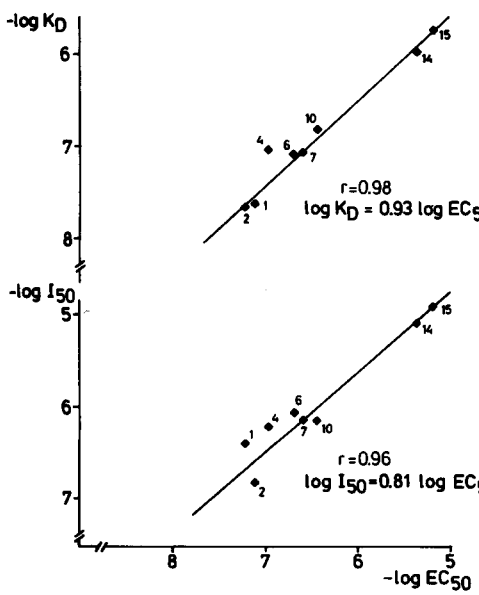


Fig. 5. Correlation between EC_{50}, K_D and I_{50} (for substances see Table 1).

has a large margin of safety and a long duration of action. In the present study we investigated the pharmacodynamic properties of some new semisynthetic k-strophanthidin derivatives compared to other glycosides, their inhibitory effects on the Na^+,K^+-ATPase activity and their binding to ventricular homogenate of guinea-pig hearts. All tested cardenolides show the classical positive inotropic effect of digitalis, but they differ in their potencies in cardiac tissue. In the convallatoxin group the order of potency, measured by EC_{50} was as follows: CAcBE > convallatoxin > methylconvallatoxin > CAc > BCAc. Zorbach and Pietsch [30] postulated that the effect of a glycoside increases with increasing numbers of free hydroxyl groups in the molecule. This postulation is in agreement with our results. A similar sequence of the cardenolides with respect to EC_{50} values is formed when the number of free hydroxyl groups is taken into account (convallatoxin > methylconvallatoxin > CAc > BCAc). CAcBE is an exception having the lowest number of free hydroxyl groups, but still the highest potency compared with the other investigated cardenolides. Like CAc and BCAc, the two hydroxyl groups at C2′ and C3′ in the sugar moiety of CAcBE are substituted by an acetonide group, in addition the two hydroxyl groups at C5 and C19 are esterified with boric acid. This indicates that the free hydroxyl groups in the sugar moiety have a different biological function than that in the aglycone. The esterified C5 and C19 hydroxyl group is the only explanation for the increased affinity of CAcBE. Other authors also reported about the variation of the biological action of cardiac glycosides due to esterification of the aglycone hydroxyl groups with nitric acid [31–33] or with acetic acid [34]. With respect to the structural formula of CAcBE one can propose that in solution the ester linkage at C5 and C19 will be hydrolysed giving an equimolar solution of convallatoxol-acetonide and boric acid. This proposal means that the increased affinity of CAcBE compared with CAc is only due to a catalytic effect of the boric acid in the solution. Therefore we performed the same

experiments with equimolar solutions of convallatoxol/boric acid, CAc/boric acid, k-strophanthoside/boric acid and k-strophanthidol/boric acid [35]. In all cases the concentration–response curves of the various glycosides were superimposable with concentration–response curves of their equimolar solutions with boric acid. This finding excludes the possibility of a catalytic action of boric acid in the action of cardenolides and is proof that esterification at C5 and C19 increases the affinity of the cardenolide to its receptors. The very low intrinsic activity of BCAc, as compared with the other tested glycosides, could be due to the butyryl group at the sugar moiety.

According to Yoda and Hokin [36] and Yoda [37] the sugar moiety has a special binding site on the receptor. The butyryl group probably reduces the binding of the sugar to its site through steric hindrance. NS is a k-strophanthidin derivative in which the C3 hydroxyl group is substituted by a nicotinoyl group. Its quaternary form is NSM. The order of potency here is as follows: NSM > NS > k-strophanthidin. In spite of this it has a higher affinity to atrial tissues, and the onset and offset of its positive inotropic effect is faster than that of the other more lipophilic glycosides. This fact could be evidence for the assumption that the glycoside receptor is located extracellularly. CAcBE and NSM show low slopes in their concentration–response curves if compared with the other glycosides. This could be an indication for a wider therapeutic range. The slope of the concentration–response curve of 3-α-MDG is lower than that of digitoxin as also found by other authors [13]. Large differences however with respect to intrinsic activity of the two glycosides as reported by the above authors could not be verified in our experiments.

The sequence in affinities of the tested cardenolides as found in experiments with guinea-pig left atria is in good correlation with that obtained from binding studies on guinea-pig ventricle homogenate and from the inhibitory effect on guinea-pig cardiac Na^+,K^+-ATPase. The ratio between the three parameters: positive inotropic effect (EC_{50}), inhibition of Na^+,K^+-ATPase activity (I_{50}) and binding to ventricle homogenate (K_D) as given in Table 3 means that the positive inotropic effect appears at a concentration 2.9 times higher than the concentration at which binding occurs. This difference may be due to the absence of K^+ in the binding medium, which would increase the K_D by increasing the concentration of cardenolide needed for half maximal binding. On the other hand the positive inotropic effect occurs at approximately half the concentration needed to inhibit the Na^+,K^+-ATPase. We can conclude from these results that the positive inotropic effect is related to the inhibition of the Na^+,K^+-ATPase but this is not proof of a causal relationship.

Finally NSM and CAcBE seem to be of special interest. Both compounds show low slopes in their concentration–response curves, which might be an indication of a wider therapeutic range. NSM is of interest, being a quaternary compound with a high polarity to which the cell membrane is probably impermeable. It is also probably lacking in central nervous effects and has a fast onset and offset of biological action. CAcBE is the cardenolide with the highest potency in cardiac tissues compared with the other glycosides tested. It has a long duration of action and a wider concentration range in which it is effective. These properties may fulfil the basic requirements for an ideal cardiac glycoside.

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