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COMMENTARY

CARDIAC GLYCOSIDES

NEW/OLD IDEAS ABOUT OLD DRUGS

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The cardiac glycosides are positive inotropic agents still widely used in the treatment of congestive heart failure. They continue to elicit controversy as to their mode of action ever since Withering wrote his *Account on Foxglove* some two centuries ago [1].

The currently postulated mechanism, known as the "pump lag hypothesis" [2], formulated the primary sequence of events as follows: Cardiac glycosides bind with absolute specificity and a high affinity to specific receptors located on the external surface of the sarcolemma as part of the α subunit of the Na^+,K^+-ATPase. The sodium/potassium pump becomes partially inhibited, Na^+ efflux is inhibited, and intracellular Na^+ is retained. This, in turn, alters the activity of the Na^+/Ca^{2+} exchanger causing a transient rise in intracellular Ca^{2+} by enhancing Ca^{2+} influx and/or inhibiting Ca^{2+} efflux or both. The increased availability of Ca^{2+} augments contractility and hence positive inotropy. Therapeutic doses of digitalis may not necessarily cause apparent elevated diastolic levels of $[Ca^{2+}]_i$ or only to a small extent. It is the increase in the size of the sarcoplasmic reticulum stores that provides Ca^{2+} for contraction during systole [3]. However, toxic doses of the drugs cause appreciable elevation in diastolic and systolic levels of $[Ca^{2+}]_i$ contributing to arrhythmia. It was suggested that arrhythmogenic effects of digitalis (i.e. toxicity) represent an extension of the same mechanism responsible for positive inotropy. This so-called "unified mechanism" is thought to account for the lack of dissociation between inotropic and toxic effects of digitalis [4].

In essence this mechanism requires elevation of intracellular levels of Na^+. Partial inhibition of the sodium pump by cardiac glycosides, by removal of extracellular K^+ or other manipulations that inhibit the pump, could yield higher $[Na^+]_i$. Other alternatives have also been proposed. For example, the Na^+/H^+ exchange process contributes to the uptake of Na^+ in quiescent chick heart myocytes. This mechanism may participate in the control of $[Ca^{2+}]_i$ and, therefore, of contractility. Several groups have reported an increase in $[Ca^{2+}]_i$ induced by digitalis due to a lowering in pH which leads via the Na^+/H^+ exchanger to influx of Na^+ [5, 6].

Although the "pump lag hypothesis" has the widest support, evidence has accumulated suggesting that the mechanism of action of cardiac glycosides may be more complex. Several groups have observed that the same low doses of cardiac glycosides that augment force of muscles' contraction may stimulate rather than inhibit the sodium pump [7, 8]. Others have shown an increased force of contraction but decreased total Na^+ content of atrial muscles [9, 10]. None of these results supports the hypothesis that the only inotropic action of digitalis is to cause inhibition of the sodium pump. These and similar observations led Noble [11] and others [12] to propose a pluralistic view which suggests more than one mechanism for inotropy. If indeed this is so, one is inclined to speculate that inotropy and toxicity of digitalis drugs may not necessarily represent one and the same mechanism.

A recent molecular biological approach included the purification of the Na^+,K^+-ATPase, determination of its primary structure, cloning and sequencing its cDNA, and deducing the amino acid sequence of the α and β subunits. Progress was made to establish the exact location of the digitalis binding site(s) along the peptide sequence [13, 14].

In the present account, I wish to raise some questions and offer experimental approaches which address problems relevant to the mode of action of digitalis.

The Model of Cultured Cardiac Myocytes from Postnatal Rats

Spontaneously contracting cultured cardiomyocytes from postnatal rat hearts offer certain advantages in studying mechanistic aspects of cardiac glycosides in the heart for the following reasons [15, 16].

(1) Myocytes represent a much simpler though adequate model.

(2) They are sensitive to digitalis and possess two binding sites, i.e. two isoforms of the Na^+,K^+-ATPase α subunit (i.e. α and $\alpha+$), with high and low affinities for ouabain.

(3) They respond to therapeutic doses of digitalis with increased amplitudes and beating frequencies, representing inotropic effects.

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(4) Active transport of Na^+ and K^+ is affected by digitalis in a concentration-dependent manner.

(5) The cultures are practically free of non-muscle cells and nervous fibers; the cells are devoid of diffusion barriers or large extracellular spaces allowing easy access to the surface location of the digitalis receptor. These factors complicate the interpretation of intact cardiac tissues.

However, not all the consequences of digitalis action can be studied with this model. Basically the following parameters, related to the mechanism of cardiac glycosides at the cellular level, can be dealt with:

(A) Rates of binding and release of labeled cardiac glycosides and appropriate constants.

(B) Effects on the transport of cations, measured as influx of Rb^+ or other fluxes.

(C) Contractile responses of myocytes to inotropic or toxic doses of these drugs.

(D) Induced changes in the concentrations of free cytosolic Ca^{2+} ($[Ca^{2+}]_i$).

Modifications of Structure and Properties of Ouabain

In addition to cultured monocytes, the effects of different cardiac glycosides on intact cardiac tissues are discussed in relation to some of the above parameters and also to show their effects on contractility. A derivative of ouabain (i.e. oxidized ouabain "ox-ouabain") proved to be very useful in the attempts to re-evaluate the mechanism and to examine the possibilities of improving pharmacologic properties such as increased contractility of myocytes and wider therapeutic indices.

Ouabain is composed of a steroid nucleus, ouabagenin, and a sugar, rhamnose, attached to the steroid via a glycosidic bond. Selective cleavage between C-2′ and C-3′ of the rhamnose by specific and mild $NaIO_4$ oxidation required the presence of KH_2PO_4 and acidic conditions. The derivative was thoroughly analyzed by ^{1}H-NMR spectroscopy before and after $NaIO_4$ oxidation. The steroid nucleus remained intact.*

Both compounds reacted similarly with the antibody raised against ouabain and, therefore, can be determined quantitatively by radioimmunoassay.†

In this respect it should be noticed that a number of cardiac glycosides, obtained either from natural sources or prepared/modified chemically, were tested for their inotropic effects using guinea pig atria or ventricles [17–25]. Inotropy was measured as ΔF_{75}, i.e. concentrations of digitalis drugs required to augment contractility by 75%. The values were compared to that of digitoxigenin whose ΔF_{75} value was arbitarily set to 1. On certain occasions inotropy was correlated also with the following values (Table 1):

(a) Half-life time ($T_{1/2}$) of onset and of offset of contractility.

(b) K_D values of interaction of the drugs with their receptors.

About fifteen compounds were divided into three major categories according to their pharmacological "potency" and affinity (K_D) relative to digitoxigenin (given again the arbitary value of 1):

Group A: Compounds with a relative "high" potency (values $\geqslant 2$) and high affinity [low K_D ($\geqslant 2$)].

Group B: Compounds with a relative "intermediary" potency (about 2) and K_D (about 2).

Group C: Compounds with a relative "low" potency ($\leqslant 2$) and affinity [high K_D ($\leqslant 2$)].

It was found that compounds of groups A and B also have longer $T_{1/2}$ values of onset and of offset of contractility. In practical terms, this means that the time required to reach 50% of maximal stable inotropic effects (onset) or the time required to return to half way of control levels after washout of the drug (offset) is longer than 10 min.

Compounds of group C have "low" potency and low affinities (high K_D values). Their $T_{1/2}$ values were short ($\leqslant$10 min) [17–24].

In addition, two other groups of compounds also were examined (Table 1):

Group D represents compounds derived from ouabain by certain modifications and are represented by oxidized ouabain (ox-ouabain). As shown later, this compound displays rapid binding kinetics, increased inotropy and a wider range of "positive inotropic" response vs dose compared to ouabain. Compounds of *Group E* have photoaffinity residues which label specifically and covalently (i.e. irreversibly) the α subunit of Na^+,K^+-ATPase. These compounds serve as models for the formation of stable, covalent digitalis–receptor complexes [25–28]. All compounds of the other groups form reversible complexes with the receptors, of weaker or stronger stabilities.

Contractility of Myocytes

Ouabain and ox-ouabain induce changes in the amplitude of systolic cell motion (ASM), beating frequencies, and the position of maximal relaxation (MR) of cultured rat myocytes. "Positive inotropic" effects are manifested by an increase in ASM without an increase in the position of ΔMR. There is also a tendency to reduce beating frequency. Toxic effects cause a decrease in ASM, increased beating frequencies, and elevation in the position of MR [29, 30]. Clear concentration-dependent distinctions were observed in the "positive inotropic" and toxic effects of ouabain and ox-ouabain. Ox-ouabain started to show inotropic effects at 5×10^{-8} M which extended to 5×10^{-6} M, whereas ouabain induced inotropic changes between 10^{-7} and 5×10^{-7} M (*therapeutic range*). Higher concentrations of both compounds were toxic. These results agree with the narrow therapeutic index of ouabain and of other classic digitalis preparations but emphasize the expanded therapeutic index of ox-ouabain, i.e. 2 orders of magnitude compared to 1/2 for ouabain. This difference between ouabain and ox-ouabain seems to originate from the differences in the turnover rates of binding to, and release from, the receptor. In other words, the residence times of ox-ouabain at the receptor site are much shorter than

* Yanuka Y, Hallaq H and Heller M, unpublished observations.

† Heller M and Wurzburger R, unpublished observations.

Table 1. Potency and Affinity of Cardiac Glycosides

Compound	Relative Potency*	Relative Affinity*
Group A (high potency):		
Digitoxigen-α-L-thevetoside	27	15
Gomphoside	23	—
Digitoxigen-β-L-rhamnoside	22	—
Digitoxigenin-β-D-digitoxose	15	—
19-Formyl-gomphoside (calactin)	14	—
Group B (intermediary potency):		
Digitoxin	8.8	9.2
Ouabain	4.8	2.5
Digitoxigenin-β-D-glucoside	2.8	4.0
Digoxin	2.1	—
Group C (low potency):		
Uzarigenin	1.2	0.2
Digitoxigenin	1.0	1.0
19-OH-Uzarigeninin-β-D-6-deoxy alloside (frugoside)	0.88	
Uzarigenin glucoside	0.44	0.21
3α-Methyl-digitoxigenin glucoside	0.14	0.24
Strophanthidin	0.11	
Group D: Modified cardiac glycosides at the sugar moiety only: $NaIO_4$ oxidized ouabain, digoxin, digitoxin		
Group E: Photoaffinity-labeled cardiac glycosides		
At the sugar moiety:		At the C-17 side chain:
Nitroazidophenyl (NAP) ouabain or strophanthidin		24-Azidophenyl-
p-Nitrophenyltriazene (NPT) ouabain		Digitoxigenin-β-D-
Aryl diazonium (ABD) ouabain		Digitoxose

* Values are relative to those for digitoxigenin: Potency (i.e. Δ_{75}) and affinity (i.e. K_D) were measured in guinea pig atria. Digitoxigenin as reference basis of 1: relative potency, 1.4×10^{-6} M; relative affinity: 3.1×10^{-7} M.

those of ouabain. The affinity of binding has apparently no influence (cf section on Kinetics of Binding and Release. . .).

Furthermore, contractility measurements (i.e. dp/dt) and maximal inotropic effects in intact hearts from normal cats perfused *in situ* with ouabain or ox-ouabain also support an apparent expanded therapeutic index for ox-ouabain.* Similarly, Lüllmann and his coworkers [12, 31–33] perfused cardiac glycosides into isolated left ventricles from guinea pig hearts and demonstrated that the contractile responses were both concentration and time dependent. The equilibrium binding of classic cardiac glycosides is a relatively slow process. The complexes between receptor and drug are quite stable, having half-life time values of 5–20 min, and their washout is also slow.

However, Lüllmann *et al.* made a number of observations which may also suggest a dissociation between inotropy and toxicity using digitoxin and 3α-methyl-digitoxigenin-glucoside (MDG) as follows:

(1) At 10^{-6} M, digitoxin caused in the cardiac tissues a maximal of 100% increase in contractile response within 10–20 min. At 2×10^{-7} M, contractility started to decline after 60 min, toxic signs appeared, and the muscle underwent contracture. Muscle tissues exposed for 10, 20 or 60 min to 10^{-5} M MDG caused the same contractile response.

(2) When equilibrium is reached, the inotropic concentrations of *digitoxin* augment contractility only by 40%; these concentrations are very close to the concentrations which cause toxic signs, i.e. 2–3×10^{-6} M. On the other hand, similar inotropic effects were measured with MDG at concentrations which are far removed from toxicity. The effects of digitoxin were washed out very slowly, whereas those of MDG were rapidly reversible.

(3) An interesting comparison can be made with regard to inhibition of the Na^+,K^+-pump. Fifty per cent inhibition of the pump by digitoxin caused only 50% increased contractility but an increase of 200% with MDG!

It seems that different cardiac glycosides elicit different quantitative contractile and toxic responses which are dependent on concentrations, time and reversibility of binding [12, 31–33].

Stimulation of Transport of Cations

Low concentrations of cardiac glycosides have been reported to *stimulate* the sodium pump rather than inhibit it, thus lowering $[Na^+]_i$ concentrations. This, in turn, should *decrease* intracellular Ca^{2+} via Na^+/Ca^{2+} exchange and should, therefore, *reduce*

* Arad M and Heller M, unpublished observations.

contractility [34]. It is possible, however, that mechanisms other than the sodium pump respond to the low concentrations of cardiac glycosides.

Indeed, ouabain and ox-ouabain in the nanomolar range, which is usually found in the plasma of patients maintained on digitalis therapy, *stimulated in cultured myocytes* the *total* rates of $^{86}Rb^+$ uptake by 15 and 25% respectively. This stimulation was observed in cells with intracellular physiological ionic concentrations and in cells that had been loaded with Na^+ following an exposure to a K^+-free medium. The stimulated influx of $^{86}Rb^+$ was abolished completely by 10^{-4} M bumetanide which is a loop diuretic. Although total influx rates were stimulated by not more than 25%, the *bumetanide-sensitive $^{86}Rb^+$ influx component increased up to three or four times*. Similarly, nanomolar concentrations of ox-ouabain stimulated even more the total uptake of $^{86}Rb^+$ (by more than 30–40%). Only much higher concentrations of ouabain or ox-ouabain (i.e millimolar concentrations) inhibited $^{86}Rb^+$ influx in myocytes [35]. These results tend to rule out the Na^+,K^+-pump as the target for stimulation by nanomolar concentrations of ouabain. On the other hand bumetanide and other loop diuretics are known inhibitors of the Na^+,K^+,Cl^- Co-transporter [36, 37]. Therefore, it seems more likely that ouabain at 10^{-8} M concentrations stimulated the bumetanide-sensitive co-transporter rather than the Na^+,K^+-pump. Rb^+ (or K^+) influx is accompanied by Na^+ influx via the co-transporter [36–38]. Therefore, it is assumed that increased rates of bumetanide-sensitive $^{86}Rb^+$ influx are accompanied by increased rates of (bumetanide-sensitive) Na^+ influx, leading to transient elevations of $[Na^+]_i$ levels and hence to transient elevations of $[Ca^{2+})_i$ as proposed earlier.

Kinetics of Binding and Release of the Cardiac Glycosides from Intact Myocytes

The interaction of ox-ouabain with myocytes is much faster than that of ouabain, namely the association rate constant (k_a) and the dissociation rate constant (k_d) are greater. Both compounds, however, had very similar *dissociation constants* (K_D).* High- and low-affinity binding sites had been detected for both compounds. The heterogeneity of digitalis receptors and their role in digitalis inotropy and toxicity are important issues and have received considerable attention [39–44]. These data agree well with previous results and with the recent description of the two isoforms of the α subunit of Na^+,K^+-ATPase in newborn and adult rat hearts [45, 46].

The results show that ouabain and its oxidized derivative bind to the two types of receptors with similar high and low affinities but the turnover rate of ox-ouabain interaction (binding and release) is much faster than that of ouabain. Faster turnover rates of ox-ouabain binding means here that ox-ouabain, which has higher k_a and k_d values compared to those of ouabain, binds to and dissociates from the receptors more times per minute than ouabain does. Since both k_a and k_d are higher, their ratio which yields the dissociation constant K_D is in this case the same as that for ouabain. This implies that the time which ox-ouabain occupies the receptors is shorter. The binding of ox-ouabain to the digitalis receptors seems to be more of a dynamic process and the effects of ox-ouabain are, therefore, rapidly reversible [47]. This may explain the decreased toxicity of the oxidation derivative compared to ouabain. On the other hand, ouabain binds to the same sites in a more static manner or occupies them for longer periods. It forms more stable complexes with the receptors which extend the inhibition of the pumping unit.

A support to this concept comes also from studies done by Lüllmann and his coworkers. They had compared the association and dissociation rates of labeled ouabain and MDG to sarcolemmal membranes obtained from bovine hearts. They measured the time required for these two compounds, under proper conditions, to reach 50% occupancy and 50% inhibition of the Na^+,K^+-ATPase. Binding equilibrium of MDG was reached within 90 min, whereas for ouabain, 180 min was required. Dissociation rates were measured by adding either 10^{-7} M ouabain or 3×10^{-5} M MDG to the respective receptor–digitalis complexes. The MDG–receptor complexes dissociated much faster (i.e. with shorter $T_{1/2}$) than those of ouabain. These shorter half-life times allow faster turnover rates and reversibility of binding [12, 31–33].

The results also support the contention that compounds which form less stable complexes with receptors may perhaps be better inotropes and display less toxicity.

Levels of Free Calcium in Myocytes

The ultimate inotropic and toxic effects of ouabain and ox-ouabain are strongly determined by their influence on the levels of $[Ca^{2+}]_i$. Quin-2 and fura-2 were used to compare the effects of these compounds on the time-averaged levels of cytosolic calcium. Unfortunately, the resolution did not allow the determination of beat to beat levels of calcium. Quin-2 has been criticized as a probe for intracellular calcium. However, the effect of ouabain on contractility of quin-2 loaded cells was the same as on unloaded cells [30]. Furthermore, comparative results obtained with ouabain and ox-ouabain allowed the drawing of a number of interesting conclusions [48]. Loading myocytes with quin-2 had no effect on their spontaneous contractions. The measured values of cytosolic calcium were similar to the published data obtained with quin-2 or fura-2 [30].

Addition of ouabain (5×10^{-8} to 5×10^{-6} M) caused a transient increase in $[Ca^{2+}]_i$, reaching a peak 30 sec after adding the drug to the cultured myocytes ("initial phase"), followed by slow oscillations for about 10 min ("secondary phase") and establishment of a new steady state at higher levels of $[Ca^{2+}]_i$ ("steady state phase"). Concentrations of ouabain between 1 and 5×10^{-7} M caused an increase in the amplitude of ASM, whereas above 1×10^{-6} M they caused a decrease in ASM, increased beating frequencies and an upward shift of the baseline, i.e. impaired relaxations. Both ouabain and ox-ouabain showed a qualitatively similar picture with one exception: during the initial phase, the

* Hallaq H, Heller M and Eilam Y, manuscript submitted for publication.

concentrations of $[Ca^{2+}]_i$ were different in response to the two compounds. Furthermore, during the initial phase, a higher concentration of ox-ouabain induced smaller elevations in the levels of $[Ca^{2+}]_i$ than a comparable concentration of ouabain. It would seem, therefore, that ouabain toxicity could originate from increased levels of $[Ca^{2+}]_i$ during this initial phase.

How much is the effect of these compounds influenced by the composition of the external medium?

(1) In a *calcium-free* medium, ouabain failed to cause an initial increase of $[Ca^{2+}]_i$ in myocytes. On the contrary, both ouabain and ox-ouabain caused a transient *decrease* 30 sec after adding the drug, which lasted for 30 sec and then returned to almost initial values. These fluctuations were repeated at least three consecutive times.

(2) In a *low potassium* medium ($[K^+]_0 = 10^{-6}$ M), the Na^+,K^+-pump is inhibited and, therefore, the initial levels of $[Ca^{2+}]_i$ are high (almost twice compared to those in physiological medium). Ouabain (5×10^{-7} M) did not increase background Ca^{2+} but actually caused again only a transient *decrease* in $[Ca^{2+}]_i$ and subsequently a stabilized steady state at a lower level. The low external K^+ caused a positive inotropic effect, which was augmented by ouabain. The combination of low potassium and ouabain rendered the cells more sensitive to additional concentrations of ouabain (a well established phenomenon), e.g. narrowed further the therapeutic width. The low $[K^+]_0$ is known to support better binding of cardiac glycosides to sarcolemma.

It is rather difficult to accommodate these results and channel them into one coherent mechanistic picture describing inotropic effects of ouabain. It does suggest, however, that more than one possibility exists for ouabain and other cardiac glycosides to exert an inotropic effect, at least for the conditions studied in cultured myocytes.

Sarcolemmal Na^+,K^+-*ATPase and* Ca^{2+},Mg^{2+}-*ATPase*

In addition to studies done with tissues or cultured myocytes, various groups have reported that nanomolar concentrations of ouabain stimulated Na^+,K^+-ATPase activity in partially purified preparations of the enzyme [7, 49]. We have confirmed and extended this observation in partially purified enzyme from cat kidney medulla or postnatal rat heart membranes. At 2 and 5×10^{-8} M, ouabain and ox-ouabain, respectively, stimulated, by 20–50%, the activity of the sarcolemmal Na^+,K^+-ATPase. Higher concentrations inhibited the activity with IC_{50} values of 5 and 2×10^{-5} M respectively.

Lüllmann and his coworkers have reported that impermeable inside out cardiac sarcolemmal vesicles, only if prepared in the presence of 2×10^{-7} M Ca^{2+}, from guinea pig or cat hearts that had been perfused with inotropic or toxic doses of ouabain, displayed high and low rates of Ca^{2+} transport respectively. Based on these data, Lüllmann *et al.* proposed that the calmodulin-dependent calcium pump of cardiac sarcolemma might be involved and could be affected by low or high concentrations of cardiac glycosides [31, 33]. This hypothesis was challenged using a simpler model of human erythrocyte membranes [50]. These membranes contain sodium and calcium pumps but lack the sodium/calcium exchanger. The membrane vesicles were depleted with respect to calmodulin. Ouabain caused a biphasic response. Nanomolar concentrations stimulated Na^+,K^+-ATPase and inhibited "basal" Ca^{2+},Mg^{2+}-ATPase activities. At higher concentrations the reverse was true. However, in the presence of Ca^{2+}, calmodulin stimulated the "basal" activity several fold and nanomolar concentrations of ouabain further increased this stimulation.

The transport data in cardiac membrane vesicles and enzymic activities in erythrocyte membrane could suggest the following: At therapeutic concentrations (i.e. nM), ouabain binds to the high-affinity sites, affects the Na^+,K^+-ATPase activity, and indirectly inhibits the "basal" calcium pump. When the cellular levels of free Ca^{2+} are lower than 10^{-6} M, this inhibition may cause retention of Ca^{2+}. As the levels of $[Ca^{2+}]_i$ increase above 10^{-6} M, calmodulin binds to the calcium pump, increasing its pumping rates which are further stimulated by nanomolar concentrations of ouabain. As the pumping rates of Ca^{2+} increase, the $[Ca^{2+}]_i$ levels decrease and the complexes between calmodulin and calcium pump dissociate, lowering the pump functions to "basal" levels. This cycle may be additional to the existing processes.

Conclusions

(1) Distinction can be made in cultured myocytes between sites for positive inotropy and for toxicity: inotropy seems to be associated with the Na^+,K^+,Cl^-–Co-transporter and toxicity with the Na^+,K^+-ATPase.

(2) The effects of ouabain and ox-ouabain on the changes of intracellular, free Ca^{2+} in cultured myocytes point to possible multiple mechanisms for these compounds.

(3) Cardiac glycosides which display fast turnover rates of interactions with the myocytes (i.e. bind and dissociate rapidly, thus forming less stable complexes with the receptors) may prove to be less toxic and be better positive inotropes compared to classic digitalis which tends to form more stable complexes with the sodium pump.

(4) The sarcolemmal (both calmodulin-dependent and -independent) calcium pump is affected in a bimodal manner by cardiac glycosides and may participate in their positive inotropic effects.

(5) How many of the effects of nanomolar concentrations of ouabain on Rb^+ influx are related to the effects on the Na^+,K^+-ATPase and Ca^{2+},Mg^{2+}-ATPase activities in isolated membranes remains an open question.

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