Digitalis: new actions for an old drug

J. Andrew Wasserstrom1,2,3 and Gary L. Aistrup2

Departments of 1Medicine and of 2Molecular Pharmacology and Medicinal Chemistry, and 3the Feinberg Cardiovascular Research Institute, Northwestern University Feinberg School of Medicine, Chicago, Illinois

Wasserstrom, J. Andrew, and Gary L. Aistrup. Digitalis: new actions for an old drug. Am J Physiol Heart Circ Physiol 289: H1781–H1793, 2005; doi:10.1152/ajpheart.00707.2004.—The mechanisms by which digitalis causes its therapeutic and toxic actions have been studied for nearly a half century, revealing a great deal about cardiac cell regulation of intracellular ions via the Na-K-ATPase (NKA) and how it is altered by cardiac glycosides. However, recent observations suggest that digitalis may have additional effects on cardiac cell function in both the short and long term that include intracellular effects, interactions with specific NKA isoforms in different cellular locations, effects on intracellular (including nuclear) signaling, and long-term regulation of intracellular ionic balances through circulating ouabain-like compounds. The purpose of this review is to examine the current status of a number of the newest and most interesting developments in the study of digitalis with a particular focus on cardiac function, although we will also discuss some of the new advances in other relevant cardiovascular effects. This new information has important implications for both our understanding of ionic regulation in normal and diseased hearts as well as for potential avenues for the development of future therapeutic interventions for the treatment of heart failure.

Cardiac glycosides; ouabain; digoxin; sarcoplasmic reticulum; ouabain-like compound; sodium-potassium adenosinetriphosphatase; excitation-contraction coupling; mitogen-activated protein kinase

THE MECHANISMS of action of digitalis have been under extensive investigation for nearly 50 years, yielding one of the most specific mechanisms thus far defined for any agent so extensively used. Its ability to bind to and inhibit the Na-K-ATPase (NKA) has been well established, as has the resulting increase in cellular [Ca2+] responsible for its positive inotropic action and its toxicity as well. There are several excellent reviews on this subject that detail our current understanding of the binding interactions and physiological outcomes of Na+ pump inhibition (66, 69). Its importance was recently acknowledged by the awarding of the Nobel Prize in chemistry to Jens Christian Skou in 1997 in recognition of his discovery of the NKA in the crab nerve and its implications for both basic cell physiology and for clinical practice. The importance of this work resides in our understanding of the physiological regulation of ion distribution across the cardiac cell membrane, of disease processes that alter this vitally important balance, and of clinical application of this knowledge, including how digitalis produces its therapeutic actions. However, recent observations suggest that additional actions of glycosides may modulate a number of other cell-signaling processes, with a potential for both short- and long-term changes in the way digitalis might affect cardiac function.

The purpose of this review is to reconsider some of the old observations about glycosides that have not been adequately explained in terms of this theory as well as to summarize new information suggesting that other mechanisms may also be at play during acute and chronic treatment with digitalis. These old and new findings about this old drug have yet to be reconciled with our current understanding of its pharmacological mechanism involving a singular action via NKA inhibition. In addition, this review is directed at discussing the cardiovascular, and in particular, the cardiac actions of glycosides. Although there have been a number of observations of serious interest in the peripheral and central nervous system, a discussion of these actions must be summarized by others and is outside the scope of the current work.

CELLULAR MECHANISM FOR POSITIVE INOTROPIC EFFECTS OF DIGITALIS

Na+ Pump Lag Hypothesis

The “Na+ pump lag” theory (64, 134) was proposed to explain the physiological mechanism underlying the positive inotropic actions of digitalis and suggested that all cardiac glycosides bind specifically to and inhibit the sarcolemmal NKA. Once glycoside has bound to and inhibited the enzyme, intracellular Na+ (Na+) increases in proportion to the new balance between influx and diminished extrusion, such that the accumulation of Na+ causes a secondary increase in free intracellular Ca2+ concentration ([Ca2+]i) via Na/Ca exchange

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(NCX). The resulting increase in sarcoplasmic reticulum (SR) Ca\(^{2+}\) uptake is responsible for the positive inotropic (therapeutic) action. The toxic (arrhythmogenic) effects occur when cytoplasmic Ca\(^{2+}\) increases to levels exceeding SR storage capacity (61, 78). In Ca\(^{2+}\) overload, SR storage capacity is exceeded so that several oscillations of the release–reuptake cycle are required to reestablish the Ca\(^{2+}\) equilibrium between cytoplasm and SR. These transient increases in Ca\(^{2+}\) activate a transient inward (depolarizing) current, which is primarily forward-mode NCX current (I\(_{\text{NCX}}\)) and is responsible for generation of delayed afterdepolarizations (DADs). DADs may achieve threshold under the appropriate physiological conditions and give rise to extrasystoles and sustained ventricular arrhythmias in vitro and, possibly, in vivo (32). These manifestations of toxicity are suppressed by reducing SR Ca\(^{2+}\) release by Ca\(^{2+}\) chelation or exposure to ryanodine, supporting the idea that intracellular Ca\(^{2+}\) oscillations provide the driving force behind DADs and triggered arrhythmias.

**Role of NCX in Effects of Digitalis**

Because the exchanger is electrogenic (12, 24, 93, 133), depolarization brings membrane voltage positive to the equilibrium potential of the exchanger (E\(_{\text{NCX}}\)) early during the cardiac cycle. Ca\(^{2+}\) influx can then occur as the direct result of this voltage relationship between transmembrane potential and E\(_{\text{NCX}}\). When intracellular Na\(^+\) is increased during Na\(^+\) pump inhibition, Ca\(^{2+}\) efflux is diminished even at resting potential where forward-mode exchange would ordinarily remove the Ca\(^{2+}\) that entered during systole (7). In addition, Ca\(^{2+}\) influx via the exchanger in the reverse mode at potentials positive to E\(_{\text{NCX}}\) is actually promoted by the same increase in Na\(^+\) resulting from pump inhibition thus causing positive inotropy and the development of SR Ca\(^{2+}\) overload toxicity (68, 106). Consequently, cardiotonic steroids that cause both an increase in Na\(^+\) and membrane depolarization secondary to Na\(^+\) pump inhibition, such as ouabain and digoxin, respond with an increase in force as a result of changes in E\(_{\text{NCX}}\). Presumably, toxicity develops and a negative inotropic effect occurs as the capacity of SR Ca\(^{2+}\) storage is exceeded following excessive pump inhibition and Na\(^+\) accumulation, leading to DADs and arrhythmias.

The importance of the NCX contribution to the increase in intracellular Ca\(^{2+}\) underlying these cellular effects is underscored in several particularly compelling recent reports. Reuter et al. (105) demonstrated a central role for NCX in increasing intracellular Ca\(^{2+}\) transients in cardiac myotubes because its absence in a mouse knockout was associated with the loss of response to ouabain. These results suggest that the positive inotropic effect of glycoside requires an active exchanger in this carefully controlled model system. However, the ultrastructural and physiological basis for excitation-contraction coupling is not well defined in this in vitro system; therefore, it is not clear how these results apply to a fully formed mammalian heart.

Another recent report has proposed an interesting extension of the role of NCX in the actions of glycosides. Satoh et al. (108) found that KB-R7943, an inhibitor of reverse-mode NCX, selectively reduced automaticity due to Ca\(^{2+}\) overload with little change in the inotropic effect of strophanthidin in rat ventricular myocytes. They suggested that NKA inhibition produces Na\(^+\) accumulation that first diminishes Ca\(^{2+}\) removal by the exchanger, causing positive inotropy, but is then responsible for net Ca\(^{2+}\) influx via the exchanger, leading to Ca\(^{2+}\) overload and arrhythmias. These observations come as we are just beginning to appreciate the fact that the exchanger is upregulated in several disease models of heart failure in rabbits (71), humans (40), and rats (132). Thus the increased sensitivity to glycoside toxicity (and possibly inotropy) might be exaggerated in cardiac hypertrophy and failure partly as a result of alterations in NCX expression and activity, with a resulting enhancement in susceptibility to arrhythmias in already sick patients.

These studies strongly suggest an important role for the exchanger in both the positive inotropic and toxic effects of digitalis. However, a singular action via NKA inhibition and activation of NCX precludes the possibility that different glycosides might possess different cellular actions and toxic-to-therapeutic ratios, because it requires a fixed relationship among intracellular Na\(^+\) concentration ([Na\(^+\)]\(_i\)), [Ca\(^{2+}\)]\(_i\)), inotropy, and toxicity. Profound differences are known to exist between agents, however, in toxic-to-therapeutic ratios in vivo (2, 84) and in action potential configuration in vitro (43, 56, 106, 123, 130, 131). For example, Karagueuzian and Katzung (60) and Ruch et al. (106) reported a wide variety of effects on resting potential, action potential characteristics, inotropy, and the development of toxicity for a series of different glycosides. These results are difficult to reconcile with a single site and mechanism of action in the heart. However, it is important to recognize that the variety in results may be at least partly the result of differences in experimental conditions, including tissue types (Purkinje fibers vs. ventricular myocardium), endocardial versus epicardial recordings (whose differences were not recognized at the time), isolated myocytes from unspexitified ventricular regions, species (rat, mouse, dog, human, cat, guinea pig, and more), different external [K\(^+\)], and so forth. Temperature is an extremely important variable because the Na\(^+\) pump is highly sensitive to temperature such that much or, in some cases, most NKA activity is blocked at room temperature, although the extent of the resulting pump inhibition is also species dependent (79). The result is that cell shortening at room temperature is probably already near maximal, leaving very little inotropic potential remaining to be developed by glycoside before toxicity occurred. The lack of standardized recording conditions is understandable given the fact that these experiments were performed in many labs around the world beginning in the 1960s, but it certainly complicates our ability to compare results obtained throughout this intervening period.

The data shown in Fig. 1 provide an example of the discrepancies found between agents even under near-optimal experimental conditions. Figure 1A shows recordings of Na\(^+\) pump current (21) in cat ventricular myocytes using patch-clamp methods. Steady-state current was recorded in the absence and then in the presence of cumulatively increasing concentrations of dihydroouabain. Outward current was reduced with increased drug as expected until the maximal effect of the drug was obtained at a saturating concentration (1 mM) of ouabain, presumably representing complete pump inhibition. The same approach was then repeated for two analogs, ouabain and ouabagenin.

The concentration-effect curve data from all three drugs are summarized in Fig. 1B. All three agents blocked the pump current (I\(_p\)) and did so with different potencies. The effects of
Inotropic and toxic concentrations were taken from Ref. 106. However, ratios of IC50 for I_p to effective concentration at 50% of maximal inotropic effect (EC50, Table 1, column B) are different (ouabagenin < ouabain < dihydroouabain, A/B), demonstrating variation in the relationship between pump inhibition and inotropy. When the extent of pump inhibition is calculated at EC50 (column C), the differences between agents become even more apparent; dihydroouabain produced only 7% I_p inhibition at the concentration causing the inotropic EC50, whereas ouabagenin caused threefold greater I_p inhibition at the same inotropic level. Ouabain was tested between the other two agents in both instances.

When I_p inhibition was calculated (Table 1, column E) at the lowest toxic concentration of each drug (column D), all three agents caused similar amounts of pump inhibition when toxicity occurred (ouabagenin = ouabain = dihydroouabain). Thus, in contrast to inotropy, there appears to be a single mechanism underlying toxicity for all three agents, possibly involving Na⁺ pump inhibition. When the ratio was calculated for pump inhibition at toxicity to that at the inotropic EC50 (Table 1, F/D) as an index of toxic-to-therapeutic ratio, there is once again a wide disparity between agents (ouabagenin < ouabain < dihydroouabain), reflecting the fact that the level of pump inhibition underlying inotropy varies dramatically, whereas that for toxicity does not.

These data suggest that other mechanisms are involved in the toxic and/or inotropic effects of different ouabain analogs. The importance of these particular observations is that they represent one of the few attempts to correlate Na⁺ pump inhibition to inotropic and toxic effects under nearly identical conditions; a single glycoside-sensitive species was studied at a physiological temperature with normal K⁺ and Na⁺ gradients. Thus, when all the experimental limitations are minimized in an attempt to correlate glycoside effects with pump inhibition, the results raise doubt that the differences between agents can be easily explained by a simple mechanistic scheme.

Evidence for Additional Cellular Actions of Digitalis

Intracellular actions of digitalis are not such an unlikely prospect given the fact that different glycosides have been shown to cross the sarcolemma and accumulate inside the cell (15, 16, 22, 23). Besch and Watanabe (8) found evidence that positive inotropic effects of glycoside persisted in the whole heart after Na⁺ influx is largely abolished by Na⁺ channel blockade (with tetrodotoxin) or inactivation (by elevating [K⁺] to 22 mM), suggesting a separation of intracellular Na⁺ accumulation from inotropic effects. Direct iontophoretic injection of ouabain and digoxin into isolated bovine ventricular cells increased cell shortening, even in Na⁺-free solutions and in the presence of extracellular digoxin FAB antibodies, which excluded an action on the NKA (55). More recently, Wasser-

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<th>Table 1. Inotropic and toxic effects of ouabain analogs as a function of pump current inhibition</th>
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OUA, ouabain; DHO, dihydroouabain; OBG, ouabagenin; I_p, pump current. Inotropic and toxic concentrations were taken from Ref. 106.
strom’s group (95) reported that patch-clamped myocytes under Na-free conditions showed increased Ca\(^{2+}\) transient magnitude and a positive inotropic effect in a glycoside-sensitive species (cat) but not in an insensitive species (rat). As a result of these observations made over the last nearly half century, it may still be necessary to consider that Na\(^+\) accumulation may not be the only mechanism responsible for all of the cardiac actions of digitalis.

**Direct Intracellular Action of Glycosides on SR Ca\(^{2+}\) Release Channel**

One potential locus of intracellular action was suggested by the specific binding of glycosides to SR fractions (22, 23) and increased \(^{45}\)Ca\(^{2+}\) release from cardiac SR by ouabain (35, 36). The latter authors subsequently identified a 31.5-kDa protein in the cat heart that is involved in excitation-contraction coupling, serves as a putative high-affinity, digitalis-binding site, and is located in the junctional SR in the vicinity of the dyadic junction (34, 35). Its localization in the vicinity of the ryanodine receptors (RyRs) may indicate an association with RyR protein and/or function in this critical space.

We also reported a direct SR action by several glycosides to increase single channel open probability (P\(_o\)) of canine crude cardiac RyRs inserted into artificial planar lipid bilayers (104). Subsequently, McGarry and Williams (83) confirmed that digoxin (1–3 nM) increased P\(_o\) by increasing the number of openings but did not increase open time except at high drug concentrations (30–100 nM). Skeletal RyR activity was unaffected by even micromolar concentrations. The effect of digoxin was most likely the result of a sensitization of the channel to Ca\(^{2+}\) as evidenced by a leftward shift of the relationship between [Ca\(^{2+}\)] and P\(_o\). These authors subsequently reported direct \(^{3}H\) digoxin binding to SR vesicles, with both high [inhibitory constant (K\(_i\) = 10 nM] and low (K\(_i\) = 3.5 \(\mu\)M) affinity binding (82).

Wasserström’s laboratory (107) has also recently extended the observation that glycoside activation of cardiac RyRs occurs at extremely low concentrations (EC\(_{50}\) = approximately 0.2 nM), which would not be unreasonable given normal clinical blood concentrations of digoxin (1–2 nM). In addition, activation requires a well-loaded SR, suggesting that this mechanism might work in conjunction with the pump lag theory by serving to amplify SR Ca\(^{2+}\) release as intracellular Na and Ca\(^{2+}\) loads increase. Finally, channel activation does not occur in the purified RyR, suggesting that it may not be the RyR itself that serves as the binding site for glycoside but that ancillary proteins associated with RyRs [possibly including that recently identified by Fujino et al. (35)] might bind the drug and regulate RyR activity in its native environment. This idea was to some extent reinforced by observations that, under Na\(^+\)-free conditions, there is an increase in an apparent SR Ca\(^{2+}\) leak induced by glycosides as measured in the form of Ca\(^{2+}\) sparks and waves in both intact and permeabilized cat cardiac myocytes (94). These observations were interpreted to suggest that a putative high-affinity glycoside binding site may involve a protein or proteins that exist as part of a complex with the RyR, thus providing the ability to fine-tune and amplify Ca\(^{2+}\) release from the SR.

The contribution of a putative direct effect of digitalis on RyR function is shown in Fig. 2. Figure 2 shows NKA sites on the sarcolemma that are inhibited after digitalis binding, causing the expected increase in [Na\(^{+}\)] and [Ca\(^{2+}\)]. The binding of glycoside to RyRs with a resulting increase in Ca\(^{2+}\) sensitivity is independent from the interaction between the NKA and its binding site for digitalis but serves to amplify release of the increased SR Ca\(^{2+}\) load imposed as a result of Na\(^+\) pump inhibition.

**ROLE OF NKA ISOFORMS IN POSITIVE INOTROPY**

There are several \(\alpha\) - and \(\beta\) -subunits that make up what we used to think of as “the” Na\(^+\) pump in the heart. The functional Na\(^+\) pump is composed of a heterodimeric combination of \(\alpha\) - and \(\beta\) -subunits. The \(\alpha\)-subunit is the catalytic entity and contains cation, ATP, and glycoside binding sites (70). The smaller \(\beta\)-subunit is glycosylated and is thought to be primarily involved in membrane insertion and proper assembly of the functional enzyme and is present only as the \(\beta_1\)-isoform in hearts (41, 116).

The three primary \(\alpha\)-subunits in the heart have different sensitivities to glycoside binding (97) and are distributed differentially in the hearts of different species. For example, the human heart expresses \(\alpha_1\)-, \(\alpha_2\)-, and \(\alpha_3\)-isoforms, all of which share nanomolar sensitivity to inhibition by glycosides (91), whereas the rat ventricle expresses primarily a low-affinity \(\alpha_1\)-isoform with some \(\alpha_2\)-isoform (74, 128). O’Brien et al. (97) reported that three \(\alpha\)-isoforms in the rat have dissociation constants of 5 \(\mu\)M, 115 nM, and 1.6 nM, respectively. The dog heart has mostly \(\alpha_1\)- and \(\alpha_3\)-isoforms, whereas \(\alpha_1\)-isoform predominates in sheep and guinea pigs (120), despite the fact that all three species are glycoside sensitive. It should be noted, however, that there is evidence that both a glycoside-sensitive and -insensitive isoform exist in the guinea pig that may correlate with the \(\alpha_2\)- and \(\alpha_1\)-isoforms found in rats, respectively (37, 38, 87). The fact that these isoforms are conserved across species has improved our understanding of their individual role in ion regulation in cardiac cells but has made it difficult to understand how different species might have different sensitivities to glycoside even though they share similar \(\alpha\)-isoform distribution.

**Glycoside Sensitivity of \(\alpha\)-Isoforms**

The association between NKA isoform inhibition and inotropy is fairly close in the glycoside-sensitive species, providing strong support for the Na\(^+\) pump lag theory. It has been more difficult, however, to understand how the theory works when the same isoforms are expressed in the less-sensitive species, such as the rat. One possible explanation for the difference in glycoside sensitivity between species was based on preferential expression of glycoside-sensitive and -insensitive isoforms in the different species. Thus the prevalence of a glycoside-resistant form of the \(\alpha_1\)-isoform in the rat [>75%, (74)] may be responsible for a lower sensitivity to the positive inotropic and toxic effects of glycosides than the so-called sensitive species. However, we would expect that the relatively glycoside-sensitive \(\alpha_2\)-isoform (the remaining 25% NKA protein expressed in the rat ventricle) would be affected at lower concentrations to a sufficient extent to allow a positive inotropic effect. Both low and high concentration positive inotropic effects have in fact been reported in rats (3), although it has been difficult to relate these physiological effects to direct
binding studies because of micromolar (not nanomolar) inotropic sensitivity compared with the binding data. A more likely explanation may lie in a study of specific amino acid substitutions that identified sites on the extracellular portion of the \( \alpha_{1l} \)-isoform that affect glycoside binding. When the two border residues in the sheep \( \alpha_{1l} \)-isoform were mutated to those in the rat (Gln111Arg and Asn122Asp), the resulting protein exhibited the reduced glycoside sensitivity characteristic of the rat heart isoform (100). Other substitutions in this same extracellular region of the sheep \( \alpha_{1l} \)-isoform at Asp121 (101), as well as within the first membrane spanning segment (112), also imparted glycoside resistance to the protein, suggesting the importance of this region in influencing the interaction between enzyme and drug. These observations demonstrate that amino acid substitutions at the first extracellular loop between M1 and M2 of \( \alpha_{1l} \)-isoform have enormous effects on glycoside binding, thus explaining why the \( \alpha_{1l} \)-isoform in rat heart has such low sensitivity to glycoside binding compared with sensitive species. Thus glycoside sensitivity of different species occurs most likely as the result of differential distribution and expression of the specific glycoside-sensitive and -insensitive \( \alpha \)-isoforms.

Fig. 2. Summary of positive inotropic and intracellular signaling effects of cardiotonic steroids. Left: summary of interactions between Na\(^+\)/K\(^+\)-ATPase (NKA) inhibition and a putative effect of ryanodine receptor (RyR) activation to produce positive inotropic effects of digitalis. Glycoside binding to NKA inhibits its Na\(^+\)/K\(^+\) pumping function. Result is increased internal [Na\(^+\)] and therefore [Ca\(^{2+}\)] as a result of a net influx of Ca\(^{2+}\) via the Na/Ca exchange (NCX) when the Na\(^+\) gradient is reduced. In addition, glycoside may bind to and increase activity of RyRs when sarcoplasmic reticulum (SR) luminal [Ca\(^{2+}\)] is elevated, thus amplifying SR Ca\(^{2+}\) release at higher SR Ca\(^{2+}\) load. Right: cell signaling pathways induced by digitalis binding to and inhibition of NKA. Binding of glycoside to and possibly pump inhibition may also initiate sarcoma kinase-epidermal growth factor receptor (SRC-EGFR)-mediated signal transduction-MAPK, and/or phospholipase C-\( \gamma \) (PLC)-protein kinase C (PKC) cascades. NKA is then depicted as residing both on the external sarcolemma and on the T-tubular membrane, possibly located in but probably not restricted to, the caveolae. AP-1, transcription activator protein 1; MAPK (ERKI/2), mitogen-activated protein kinase (extracellular-signal regulated kinases 1 and 2); NF-\( \kappa \)B, nuclear factor-\( \kappa \)B; RAF, renin-angiotensin factor kinase; MEK, mitogen-activated ERK-activating kinase; RAS, renin-angiotensin system (GTPase); SHC, Src homology collagen-like protein; SOS, Son of Sevenless protein; SRC, sarcoma kinase; VGCC, voltage-gated Ca\(^{2+}\) channel; SERCA, sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase; ROS, reactive oxygen species.
addition, however, it is equally important to recognize that even small differences in sequence may have important effects not only on drug sensitivity for any given isoform but also on other regulatory processes that affect their function in vivo. This latter point becomes all the more important when considering glycoside activity that occurs as a result of changes in protein expression and function in different pathophysiological conditions.

Alterations in α-Isoform Expression in Disease

Not only are there species differences for glycoside binding to the α-subunits in the heart, but there is also evidence that there are important differences in isoform expression in disease. Thus it seems likely that increased glycoside sensitivity in heart failure compared with the normal human heart may be the result of the decreased total expression of NKA protein (116, 118) that occurs primarily as a decrease in expression of α1-isoform (38% decrease) and α3-isoform (30% decrease) but not in α2-isoform. Interestingly, there is a different pattern in the right atrium where α1-isoform and α2-isoform are reduced with little change in α3-isoform (92). These observations are probably related to the fact that overall NKA expression is decreased in heart failure, with estimates ranging from ~30% to 50% decrease in nondigitalized patients (80, 129). These findings also raise the possibility that one of the homeostatic mechanisms invoked to compensate for heart failure may be diminished NKA expression and activity, which in itself should raise [Ca^{2+}], and restore contractility to at least partially compensate for reduced cardiac output. In addition, a reduced expression of NKA in heart failure would mean that the so-called “pump reserve” (the amount of NKA activity that must be blocked before changes occur in cellular Na\(^+\) and K\(^+\) concentrations) is reduced or abolished so that small decreases in NKA activity are now capable of producing larger changes in ionotropy. This increase in sensitivity to glycosides and higher resting Na\(^+\) in congestive heart failure (CHF) have both in fact been reported (115, 118) and strongly suggest that the reduction in NKA activity with increasing severity of CHF has important influences both on resting Na\(^+\) and in sensitivity to the effects of glycoside. Furthermore, the increased glycoside sensitivity occurs not because of changes in drug sensitivity of specific isoforms but rather as a result of impaired ability of the myocardium to keep the rise in Na\(^+\)], from accelerating with disease severity. In addition, there are changes in isoform expression in CHF that are related to a reversion to a fetal or neonatal type as is common in this disease. Thus hypertrophy induced by abdominal aortic constriction in rats caused an increase in expression of the glyco- side-sensitive (presumably α3) isoform similar to that found in newborns (14). Extensive expression of the α3-isoform in heart failure was subsequently confirmed (28), supporting the idea that ventricular remodeling is associated with expression of genes that are characteristically more active early in development (see Ref. 86 for review). Clinically, it is important to note that the hemodynamic benefits of glycosides have been reported to be directly related to the severity of CHF (42), underscoring the importance of altered NKA expression, pump reserve, and the sensitivity to glycoside effects in CHF.

The fact that there may be differences in α-isoform expression in heart failure may have some additional influences on sensitivity to digitalis beyond those major issues described above. In the human ventricle, there are subtle differences in isoform sensitivity to glycosides based on both binding affinities and kinetics of the drug-receptor interaction. More importantly, the enzyme turnover rate for α1β1 is about twice that for α3β1, so that any equivalent reductions in α1 and α3 in heart failure would have a greater impact on any contribution of α1 to cell function than α3 (17, 91). Consequently, any differences in isoform distribution and localization and/or selective functions might be altered in disease, making these variables all the more sensitive to glycosides.

Theory of Differential Localization of NKA α-Isoforms in the Heart

Another possible explanation has recently been offered for the differences in glycoside response in different tissues and species in terms of preferential localization of certain α-isoforms that might provide a specific microenvironment responsible for the cellular actions of glycosides. Blaustein et al. (11) proposed that the regional expression of specific NKA α-isoforms might explain some of the discrepancies observed with glycoside actions between species and under different experimental conditions. Figure 2 incorporates this scheme of localized NKA expression in the vicinity of the SR. This proposal suggested that only certain isoforms were associated with Na\(^+\) accumulation that could lead to local Ca\(^{2+}\) increases, whereas other isoforms were not. The general scheme in Fig. 2 shows all isoforms in this location to reflect our uncertainty about the role of specific isoforms in various species that might participate in this mechanism. However, it is the glycoside-sensitive α3-isoform (present in larger mammals but not rat hearts) in the original proposal that was suggested to be colocalized with the NCX in the vicinity of the SR in normal smooth muscle so that only local changes in Na\(^+\) and therefore Ca\(^{2+}\) occurred with a result of local Ca\(^{2+}\) increases in terminal SR at the dyadic junction. In addition, there is evidence of colocalization of NKA and NCX near calsequestrin-containing structures (presumably associated with the SR) in rat smooth muscle (90). This observation provides a link between NKA and NCX functions and SR Ca\(^{2+}\) release in this tissue. The implication might then be that there is no need for global increases in Na\(^+\), because local microenvironments were able to increase SR Ca\(^{2+}\) stores only where they were needed to produce a positive inotropic effect. In contrast, another isoform (α1) was more generally distributed and had a lower sensitivity to glycoside, thus its inhibition caused a more general rise in Na\(^+\) and Ca\(^{2+}\) and was responsible for Ca\(^{2+}\) overload toxicity. There is some structural and biochemical evidence for this hypothesis in rat arterial myocytes, where the α3-isoform shows a distinct distribution pattern similar to NCX on the sarcotubules (58, 59). Also, the inotropic effects of ouabain in this cell type occur in the absence of measurable changes in Na\(^+\) (6), suggesting a diffusion-limited system with a privileged relationship between these two transport systems.

This controversial mechanism could only work if there is selective exclusion of NCX from the immediate vicinity of a particular NKA isoform with diffusion barriers that prevent ionic balances from being achieved in the region immediately surrounding that isoform. There is evidence that NCX is concentrated in rat heart T tubules (90, 117, 125, 139), which
would place this transporter in proximity to any NKA that might also be located near SR release units. In contrast, there is little evidence for diffusion barriers involving NKA isoforms in the subsarcolemmal space in the heart. McDonough et al. (81) used isoform-specific antibodies to demonstrate that anti-α₁ labeling was found primarily in rat cardiac T tubules and less on the sarcolemma, whereas anti-α₂ and anti-β₁ labels were found both throughout the T tubules and the entire sarcolemma. Thomas et al. (125) have recently reported uniform α₁-antibody labeling in both the T system and surface membrane, similar to that reported for α₂ by McDonough et al. (81). These studies agree that both α₁- and α₂-isoforms are expressed at the dyadic junction in the T tubules, where any restricted diffusion and privileged relationship between NKA and junctional SR uptake and release might take place. In the guinea pig ventricle, an anti-α-antibody (indicating α₁ labeling) labeled both peripheral sarcolemma and T tubules. These data demonstrate quite clearly that Na⁺ pumps of both types are located in the T tubules and that α₁ distribution may be patchy along the cell surface, seemingly the opposite of that required for the privileged access idea to work in heart. These observations make it unlikely that any proposed functional unit complex of (α₂)NKA-NCX-RyR-junctional SR might exist in the rat heart that provides a preferential diffusion-isolated source for the positive inotropic effects of glycosides. However, information in other species will be essential if this idea is to be considered seriously as a viable mechanism in larger mammals.

A subsequent study by James et al. (57) supported this notion by demonstrating that the partial knockout of the α₁-isoform through genetic reduction in the mouse heart resulted in a positive inotropic effect that was the exclusive result of inhibition of the remaining α₂-isoform. They even went so far as to suggest that there was a separation of function between isoforms with α₁ responsible for toxicity and α₂ for inotropy. Moreover, this group then developed a mouse model in which only a glycoside-insensitive α₂-isoform was expressed in heart (20). The resulting lack of inotropic stimulation was interpreted as suggesting the exclusive reliance of the positive inotropic effect on the α₂ NKA.

These conclusions must be considered, however, in light of a number of other well-established observations about glycoside actions. First, a role for RyR activation in glycoside actions is based on both increased SR Ca²⁺ load resulting from NKA inhibition (or an additional source) and the fact that rodent RyR2 appears to have a greatly reduced sensitivity to glycoside actions than larger mammals, including dogs and humans. Thus amplification of a positive inotropic effect resulting initially from NKA inhibition would not be expected in its absence.

Second, the notion that there is a clear functional separation between the α₁- and α₂-isoforms (57) is difficult to reconcile with the physiology underlying inotropic and toxic effects of digitalis. Under conditions in which external [Ca²⁺] is either normal or reduced, numerous studies have shown that there are two inotropic responses (high and low affinity) in rodents that have been reported by Lingrel’s group and by others to represent the inotropic responses to inhibition of the two rodent α-isoforms (3, for review see Ref. 113). It is extremely difficult to understand how NKA inhibition can be inotropic in one instance and toxic in another, even if different isoforms are involved and are located in different sites in the cell membrane; the increase in internal [Ca²⁺] will necessarily produce positive inotropy before Ca²⁺ overload no matter where in the cell the increase in Ca²⁺ originates. Thus it is very difficult to accept at this point that a spatial separation of α-isoforms could account for distinct physiological roles even with quite different sensitivities to glycoside inhibition of pump activity.

These experimental issues must be resolved before any definitive statement can be made about an exclusive role for α₂ alone in the inotropic effects of glycosides. However, in the meantime, this interesting and important issue remains an area of active research and is especially important because of reports that NKA expression is suppressed by 42% in heart failure (118) and that relative isoform expression is altered in various forms of heart disease, including CHF (114, 116).

CARDIAC GLYCOSIDES AS ENDOGENOUS REGULATORS OF CARDIOVASCULAR FUNCTION

In the last few years, a great deal of effort has been focused on endogenous factors that might regulate the NKA, especially in hypertension (for recent review, see Ref. 111). The notion that endogenous glycoside regulators existed in mammals was first suggested in 1953 (121) at about the same time that glycosides were found to bind with high affinity to and selectively inhibit the plasmalemmal NKA (109). The search was then initiated for natriuretic factors or hormones that might be involved in the regulation of plasma salt concentrations, blood pressure, and blood volume (18; for reviews, see Refs. 10 and 19). These efforts have yielded some extraordinary findings about circulating factors that might be involved in NKA regulation in normal as well as under pathophysiological conditions. Initial reports found glycoside-like compounds similar to ouabain in mammalian blood that bound to and inhibited the NKA (9, 47, 49). The first such agent identified was reported to be a novel steroidal isomer of ouabain (48), which was surprising because it had previously only been associated with plant and not animal origins. It is still not certain whether this factor is ouabain or an analog, thus the term often used to describe the active factor is ouabain-like compound. Endogenous ouabain is now thought to be synthesized in the adrenal cortex under the regulation of hormonal stimulation possibly involving the sympathetic nervous system (65). Circulating levels of ouabain were shown to be elevated 30%–45% among Caucasians with essential hypertension (50), supporting the idea that NKA inhibition in the cardiovascular system would produce hypertension. There is also evidence that elevated levels of circulating ouabain-like compound are present in patients with heart failure, suggesting a clinical role for this endogenous regulatory system either as contributor to or effect of CHF (44). A specific glycoside binding protein has also been identified that may be involved in the transport and regulation of circulating glycosides in both bovine and human plasma (5, 63).

Additional surprises came as other cardiotonic steroids were found in human plasma that more closely resemble toad venoms. The bufadienolide proscllariadin A was identified in bovine adrenal gland in addition to ouabain (110), and other bufadienolides were found to be elevated in hypertensive patients (119). A marinobufogenin (MBG)-like factor was reported to be present, in addition to elevated ouabain levels, in

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women with preeclampsia (73) and to be released with elevated salt load in rats (27). However, a recent study in patients with CHF found that MBG, a fairly selective inhibitor of the \( \alpha_1 \) NKA isofrom, correlated with levels of atrial natriuretic peptide and systolic diameter and inversely with ejection fraction, whereas the ouabain-like factor level was most closely associated with blood pressure (33). These data suggest that there may be multiple cardiotonic steroids, with quite varied chemistry, involved in endogenous regulation and that they do not necessarily share specific actions.

Given that there are several \( \alpha \)-isoforms of the NKA in the human heart, it is important to bear in mind that this endogenous regulation might also be isoform specific, which may have a bearing on the development of the disease. This fact may contribute to the observations that digitoxin appears to be able to counteract the hypertensive effects of ouabain, and an inverse relationship exists between the potency of NKA inhibition and induction of hypertension for different ouabain analogs (75). In addition, an ouabain-like compound analog from patients with renal failure inhibited the NKA in the kidney, skeletal muscle, and brain, caused high-affinity binding to all three isoforms of the \( \alpha \)-subunit, but had a greater affinity for \( \alpha_2 \)-isofrom in rat (122). Another such compound originating in the hypothalians was found to have a high-binding affinity for the low-affinity (presumably \( \alpha_1 \)) isofrom of the NKA and to show elevated tissue levels in a strain of hypertensive rats (30). In another study that examined differential drug sensitivities in different membrane fractions of the aorta, ouabain exhibited greater activity in the fraction derived from nerve endings (\( \alpha_3 \)-isofrom), whereas MBG was more active in the sarcolemma (\( \alpha_1 \)-isofrom) (25). This result suggests that, in contrast to ouabain, MBG exhibits a greater affinity for the ouabain-resistant (renal) \( \alpha_1 \)-subunit of NKA. More recently, these investigators also reported that MBG is elevated in salt-sensitive hypertensive rats, and because of its extremely high affinity for the renal isoform compared with ouabain (26), they suggest that it may serve as a natriuretic mechanism in hypertension and that isoform expression shifts during the development of hypertrophy and again in the heart failure that develops in these animals (28).

However, there are several important observations that argue against a role for ouabain-like compounds as true natriuretic hormones. First, as mentioned above, the relationship between its ability to induce hypertension is inversely related to its ability as an NKA inhibitor (75). More importantly, ouabain-like compound not only failed to rise in patients in response to salt load but was in fact elevated only by either acute and chronic salt restriction (76), raising the prospect that these agents serve as part of the adaptive mechanisms to sodium depletion than as a natriuretic hormone. It appears more likely that MBG may act in this role because levels of bufadienolides have been shown to increase in hypertension (29, 98).

Clearly, multiple mechanisms must be involved in the responses to this endogenous regulatory system that may or may not involve multiple \( \alpha \)-isoforms of the NKA and their differential sensitivities to the different circulating cardiotonic steroids and may, in fact, include mechanisms completely unrelated to any interaction with the NKA.

These new observations indicate that our understanding of glycoside actions in the heart and cardiovascular system is far from complete. If NKA regulation is accomplished, at least, in part, through these particular circulating factors, then they almost certainly also serve as endogenous regulators of other aspects of the cardiovascular system in both normal and pathophysiological conditions. It is also important to note the fact that several studies have reported that extremely low concentrations of cardiotonic steroids alter function in the cardiovascular system that may affect blood pressure regulation and cardiac function. These include effects on vascular smooth muscle cells (1), kidney (27), and the central nervous system (67). In addition, there also may be effects of extremely low (picomolar) concentrations of bufadienolides to suppress T cells (124). These studies indicate that there may be extensive effects on numerous organ systems, including those that affect cardiovascular function and those that may not, by very low concentrations of these endogenous circulating factors.

Before this system can be fully accepted as a major endogenous regulator of blood pressure and NKA activity, however, it is extremely important to acknowledge certain problems with this system that have not yet been addressed fully. Aside from the lingering question about the true molecular identity of ouabain-like compounds, the most important of these is the low quantities of these agents that have thus far been detected and identified as putative circulating factors. It remains to be determined whether or not these substances are present in sufficient quantities in vivo to participate in normal or pathophysiological regulation of cardiovascular function (111).

**GLYCOSIDE ACTIVATION OF INTRACELLULAR SIGNALING**

Much of the initial work suggesting a role for glycosides in signal transduction came from studies in rat cardiomyocytes initially aimed at elucidating cell proliferation/growth/hypertrophy. These studies indicated that ouabain, at concentrations known to partially inhibit NKA in rat embryonic cardiac myocytes (10–100 \( \mu \)M), increased transcription of the hypertrophy-regulated genes \( c-fos \) and \( c-jun \), and the NKA \( \alpha_3 \)-isofrom gene in a \( \text{Ca}^{2+} \)-, protein kinase C-, Ca-calmodulin kinase-, and mitogen-activated protein kinase-dependent manner (53, 54, 99). A barrage of follow-up studies led to a much more intricate and expanded scenario of ouabain-induced NKA signaling, including activation of epidermal growth factor receptor (EGFR) that initiates the ERK1/2 MAPK cascade, reactive oxygen species generation (13), and myocyte proliferation/growth/hypertrophy (45, 46, 62, 72, 88, 126, 138). Recent findings have further demonstrated that doses of ouabain that initiate ERK1/2 activation also produce positive inotropy in both rat and guinea pig hearts (89) and that ouabain-induced increases in \([\text{Ca}^{2+}]_i\) via activation of ERK1/2, partial inhibition of the NKA pumping, and ouabain-induced generation of reactive oxygen species via opening of mitochondrial ATP-sensitive potassium channels may all contribute to this ouabain-mediated positive inotropy (127).

These studies have led to the development of a signaling scheme (89, 135–137) in which two pools of NKA are postulated to exist in rat cardiomyocytes (Fig. 2). The first is the glycoside-sensitive \( \alpha_2 \)-isofrom NKA pool of which the ion-pumping functions are inhibited by ouabain in the classical pump lag scheme. This pool of NKA may be localized preferentially in the T tubules and may or may not be located in caveolae in these structures. The second involves the glycoside-insensitive \( \alpha_1 \) NKA-sensitive pool, possibly located in...
caveolae on the sarcolemma. The signal transduction functions are initiated by ouabain binding that activates EGFR by a NKA-Src protein-protein interaction. This, in turn, activates the PKC-MAPK cascades that, in conjunction with increased \([\text{Ca}^{2+}]\), activates ERK1/2 and modulates gene activity. Thus it was suggested that ouabain modulation of cardiomyocyte contractility and hypertrophy/growth entails a cooperative relationship between NKA signal-transducing and ion-pumping functions (89, 135, 137).

There are still issues that remain to be clarified in this scheme. First, it has thus far been difficult to definitively separate a stimulation of NKA signaling from changes in intracellular ion (\(\text{Na}^{+}, \text{K}^{+}, \text{Ca}^{2+}\)) concentrations. Second, this scheme relies on proximal protein-protein interactions for its realization, which is indeed fostered by recent findings indicating that components of the signaling pathways that link NKA to ERK1/2 and \([\text{Ca}^{2+}]\), modulation are organized within caveolae microdomains (Fig. 2) in rat cardiomyocytes (102). It is important to point out, however, that this scheme may require that glycoside-sensitive NKA isoforms (\(\alpha_2, \alpha_3\), and possibly \(\alpha_1\)) preferentially colocalize with the NCX and communicate with endoplasmic and/or SR in a restricted space microenvironment, i.e., in caveolae on T tubules. In contrast, glycoside-insensitive NKA isoforms (\(\alpha_1\)) preferentially colocalize with Src/EGFR/phospholipase C-\(\gamma\), i.e., in caveolae on peripheral sarcolemma. Thus it is highly uncertain whether the location and the specific isoform required to support the proposed of NKA signaling are consistent with this idea in its current form. However, one of the implications of this work is that NKA signaling may be specifically attached to the \(\alpha_1\) NKA isoform (in addition to its ion-pumping function), whereas the \(\alpha_2\) and \(\alpha_3\) NKA isoforms may have primarily ion-pumping functions that may also serve to modulate \(\alpha_1\) NKA signaling indirectly.

It is also important to note that some very interesting data have been presented recently that may be pertinent to the questions of both intracellular signaling and endogenous regulation and may also resolve the long-established but curious observation that low concentrations of glycosides are known to stimulate NKA (37, 39, and see Refs. 77 and 96 for reviews; also, see the data points at \(1 \times 10^{-7}\) M for all glycosides in Fig. 1). Low concentrations of glycosides activate exclusively the glycoside-sensitive isoforms of NKA in virtually all species tested, including the guinea pig, dog, and human ventricle (39). This effect to stimulate NKA activity may in fact be the physiological action of low concentrations of cardiotonic steroids applied exogenously as well as those that may occur endogenously. Thus it may in fact be that the resulting decreases in both \(\text{Na}^{+}\) and \(\text{Ca}^{2+}\) are involved in responses to disease states that induce release of endogenous regulators rather than the typical consequences expected from pump inhibition. In addition, these anomalous changes in ion concentrations might be involved in intracellular regulation rather than those expected to occur at higher (exogenously administered) glycoside concentrations. Although speculative at this point, a role for pump activation at low concentrations of cardiotonic steroids may in the future serve to resolve these seemingly unrelated physiological actions.

The idea that cardiotonic steroids may affect intracellular signaling has met with considerable skepticism (51) particularly in relation to the cardiovascular system, because nearly all of the early evidence for glycoside-induced NKA signal transduction in cardiac tissue came from studies utilizing rat cardiomyocytes and requiring extremely high drug concentrations. This raises the question as to whether such glycoside action is significant in human hearts, because all human NKA isoforms exhibit high glycoside sensitivity (129). The more recent findings that NKA signaling in guinea pig hearts and in human and canine vascular smooth muscle cells is induced by much lower concentrations of ouabain, similar to therapeutic concentrations in the former and near concentrations of putative endogenous glycoside in the latter, seem to indicate that this glycoside action does not depend on species or a particular NKA glycoside sensitivity. Thus there may be grounds for claiming that NKA-mediated signaling represents the physiological and/or pathophysiological action(s) of endogenous glycosides.

Finally, it is worth noting that, in addition to its effects in the heart, studies in renal cells have shown that ouabain-induced slow \(\text{Ca}^{2+}\) oscillations occur as a result of an interaction between NKA and inositol 1,4,5-trisphosphate [Ins(1,4,5)P\(_3\)] receptors in a cellular microdomain (4, 85). Binding of ouabain to NKA activated Ins(1,4,5)P\(_3\) receptors directly, without the need for Ins(1,4,5)P\(_3\) generation, caused \(\text{Ca}^{2+}\) oscillations that were abolished by disruption of the cytoskeleton and truncation of the \(\alpha_1\)-subunit. In contrast to cardiac cell signaling, pump inhibition with low [K\(^+\)] did not produce \(\text{Ca}^{2+}\) oscillations in renal cells. These studies have been interpreted to suggest that there is a close spatial and functional interaction between the NKA and Ins(1,4,5)P\(_3\) receptors in which the former serves to translate ouabain binding to the NKA into intracellular \(\text{Ca}^{2+}\) signaling (which in turn activates certain transcription factors) via direct Ins(1,4,5)P\(_3\) receptor activation. It is not yet clear how a role in renal signaling may relate to the heart, especially because different \(\alpha\)-subunit isoforms are involved and Ins(1,4,5)P\(_3\) actions differ in the two tissue types. However, these observations provide support for the general notion that ouabain may have important effects aside from pump inhibition that might affect cellular signaling in different sites in the cardiovascular system.

**DOES DIGITALIS MATTER ANY MORE?**

Despite waning use of glycosides in recent years in the United States, the high incidence of toxicity associated with digoxin, and its replacement with angiotensin-converting enzyme inhibitors, recent clinical trials [Prospective Randomized Study of Ventricular Failure and the Efficacy of Digoxin (PROVED) trial, Randomized Assessment of Digoxin on Inhibitors of the Angiotensin Converting Enzyme (RADIANCE) trial, and in particular, the Digitalis Investigation Group trial (DIG) trial] have indicated that digoxin is in fact quite effective in reducing cardiovascular hospitalizations (52). It is also still used very extensively worldwide. In addition, heart failure survival and hospitalization rates are improved with digoxin, especially compared with patients withdrawn from the drug. The success of digoxin in these trials supports consideration of its continued use in some patients for reasons of quality of life, even if there is no overall improvement in survival.

With this in mind, there may also be a future role for effective, less toxic positive inotropic agents in the future treatment of CHF. Given both old and new evidence for multiple cellular actions, it may in fact be possible to develop
new agents that act at the cellular level to produce positive inotropy but with a reduced incidence of toxicity. The actions of such agents may or may not involve a glycoside molecule or any of the cellular and subcellular mechanisms related to glycoside actions. Whether or not any such molecules can be used to target function of specific α-isomers of the NKA to produce an improvement in cardiac function in CHF is unknown at present but could also provide a rationale for development of new pharmacological therapies.

Perhaps equally important is to answer the more general question of the existence and role of endogenous regulators of Na⁺ pump function in normal physiology as well as in hypertension and CHF. Evidence continues to accumulate that circulating glycosides may either contribute to cardiovascular development of new pharmacological therapies.

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**GRANTS**

This work was supported by National Heart, Lung, and Blood Institute Grant HL-30724 (to J. A. Wasserstrom) and National Institute of Alcohol Abuse and Alcoholism Grant AA-01915 (to J. A. Wasserstrom) and AA-10969 (to G. L. Astrup).

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