On the Mechanism of Cardiac Glycoside Action

STIMULATION OF MYOSIN B SUPERPRECIPITATION BY OUABAIN AND DIGOXIN

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ABSTRACT

A study has been made of the effect of ouabain and other cardiac glycosides on a faithful model of contraction, viz. the "superprecipitation" which occurs when ATP is added to a suspension of purified myosin B. Structural changes during superprecipitation were followed by a turbidity method, and rates of ATP utilization with a pH stat. At "optimal" concentrations (ca. $10^{-9}$ M and $10^{-3}$ M) these substances approximately double the structural and the ATP utilization rates of the myosin B system, but at no concentration do they affect the ATPase of myosin A. In the one case studied, cardioactivity of the substance appeared to parallel its effect in our test system.

ADDITIONAL KEY WORDS
- myosin A
- ATP utilization
- actin-myosin junctions

Skou$^1$ and Post et al.$^2$ have shown that the cardiac glycoside, ouabain, affects the "membrane ATPase" of crab nerve and human erythrocyte, respectively. Since these ATPases are thought to be involved in "active" ion transport, it has been natural to conjecture that the inotropic effect of glycosides is exerted via the electrical properties of cardiac cells; this may indeed be so, but it is noteworthy that in the model systems the cardiac glycoside concentration is of the order of $10^{-5}$ M, while in reinforcing papillary muscle contraction it often acts at $10^{-8}$ M (Hoffman, personal communication).

In this paper it will be shown that ouabain and digoxin affect the "in vitro contractile system" (superprecipitation of myosin B) at concentrations as low as $10^{-11}$ M. Hints that cardiac glycosides affect contractile proteins directly have emerged in the past (see, for example, Bowen$^3$ and Robb and Mallow$^4$), but lack of a sufficiently sensitive method has delayed definition of a direct effect. In this work the structural aspect of superprecipitation was followed by the turbidity method of Ebashi,$^6$ as modified by Yasui and Watanabe,$^6$ and the fast ATP utilization accompanying superprecipitation was followed in a pH stat. Structural change and ATP utilization seem to be "coupled," and both are sensitive to cardiac glycosides. The test material is a fine, sonicated suspension of thrice-purified myosin B; the possibility of contamination with cellular debris or with particulate relaxing factor is remote.

The fact that cardiac glycosides potentiate the ATP response of myosin B does not mean that they exert their physiological effect on the contractile system, any more than the fact that cardiac glycosides affect membrane ATPase means that their physiological effect is exerted on membranes. A restrained statement at this juncture is that cardiac glycosides seem to affect at least two systems of energy-transducing ATPases.

Materials and Methods

The bulk of this work was done with crystalline ouabain (Nutritional Biochemicals); when in a
polar solvent this material moved as a single spot in thin-layer chromatography; contamination with Ca was less than 3 parts per million and with Mg less than 2 parts per million using the method of atomic absorption. Digital was supplied by Burroughs-Wellcome, and strophantidinonic acid was kindly donated by Dr. Maximilian Ehrenstein. Naja naja venom (lyophilized was obtained from Ross Allen’s Reptile Institute, Silver Springs, Florida.

Disodium dichydrogen ATP, Tris (tris-hydroxy-methyl aminomethane) and repurified maleic acid were obtained from Sigma Chemical Co. Inorganic salts were J. T. Baker, reagent grade. Double-distilled (the second time from a glass still) water was used throughout.

The preparation and characterization of myosin A has been recently described by us. Brei for the extraction of myosin B was made from (1) (Waring) blended beef hearts, chilled on excision, dissected free of connective tissue and fat, and used within 2 hr of sacrifice, or (2) (Latapie) minced bled-rabbit dorsal muscles immediately chilled, dissected free of fascia and fat, and used within 30 min of sacrifice. Breis were extracted with 20-22 hr at 0°C in "Weber-Edsall solution" (0.60 M KCl, 0.04 M KHCO3, and 0.01 M K2CO3). Following extraction, preparations were centrifuged at 8,000 g (this and subsequent centrifugations were performed in a Spinco Model L-2 preparative ultracentrifuge with rotor temperature of 0°C, kept cool at all times, except that the final solution was centrifuged at 46,000 g for 30 min at 8,000 g. The supernatant was discarded and the precipitate was collected and weighed, and enough neutralized concentrated KCl solution was added to make [KCl] = 0.60 M and thereby redissolve the precipitate. The solution was now centrifuged at 48,000 g for 30 min, employing centrifuge tubes with nylon caps. The central portions of the supernatants were collected. Starting with dilution to [KCl] = 0.25 M, the foregoing procedure was repeated two more times, except that the final solution was centrifuged for a full hour. The physical nature of the myosin B suspension obtained on dilution to low ionic strength depends on the schedule of dilution. Except when otherwise specified, the following was done at 0°C, using prechilled containers. Stock myosin B solution (usually 1%) was diluted by addition of neutral 0.60 M KCl, first to 0.5%, then to 0.1%. Then the myosin B was precipitated by adding 0.06 M KCl and 0.02 M Tris-maleate (pH 7.0); in this operation the concentrations of protein, KCl, and Tris-maleate fall, respectively, to 0.02%, 0.17 M and 0.016 M (ionic strength, ca. 0.185 M). The resulting suspension was allowed to stand overnight. Of this suspension, 5 ml was pipetted (after the pipette had been rinsed several times with suspension) into a beaker, and the system was allowed to warm to 25°C in a constant temperature bath (ca. 15 min), was sonicated for 10 sec (at 20 Kps in a Brownwill Sonicator), and the contents were poured gently into a Zeiss 2 cm × 2 cm quartz cuvette. Then 5 ml of 25°C solvent (0.06 M KCl, 0.02 M Tris-maleate, pH 7.0), or of 25°C solvent containing twice the desired cardiac glycoside concentration were pipetted and mixed with a gently turning magnetic stirrer for a constant length of time. In this final solution, [KCl] = 0.12 M, [Tris] = 0.018 M and ionic strength is ca. 0.13 M. The drug thus interacted with the protein for 90-120 sec; much longer incubations gave the same result. Superprecipitation was initiated by adding 250 μl of the desired ATP concentration. Again, the stirrer frequency was reproduced with a Variac and maintained throughout the measurements. The apparent optical density at 550 μm of the superprecipitating suspension was compared with the same suspension prior to ATP addition. The difference between these two readings (when these are converted to optical density units) is taken as the "extent" of structural change, and the rate at which extent varies with time will often be called the "structural" rate to distinguish it from the rate of ATP hydrolysis. The contents of the cuvette were held at 25°C in the constant temperature jacket (equipped also with stirrer) of a Zeiss PMQ II spectrophotometer. The output of the spectrophotometer was applied to a Zeiss log converter and, finally, was linearly displayed in OD units on a Minneapolis-Honeywell recorder (t1/2 = 0.05 sec; accuracy, 0.005 OD units).

Measurements of ATP utilization or of ATPase activity were sometimes made with our variant of the Fiske-Subbarow orthophosphate assay, employing SnCl2 as the reducing agent. Rates were calculated from the zero order phase of the reaction (first 200 sec) by drawing a line through 3 or 4 points. In such cases the conditions for the assay were exactly those in which superprecipitation was observed. More often ATPase activity was recorded in a Radiometer pH stat, again in 0.12 M KCl, pH 7.0, 25°C, except that the Tris was omitted. In separate measurements it was ascertained that rates of proton production multiplied by 1.59 equaled rates of ATP hydrolysis. In pH stat measurements, stirring and electrode artifacts subsided in less than 20 sec; normally the linear phase of superprecipitation lasted for at least 150 sec.
Results

The conditions of our assay are such that 0.1 mM ATP "saturates" the myosin B system; i.e., at this or higher concentrations the final extent and the rate of superprecipitation and the ATPase activity are maximal and independent of the ATP concentration. As did Rainford et al. we found concentrations of 0.025 or 0.05 mM ATP to be "undersaturating"; as in their work also, we found indications that, for a given preparation, the sensitivity to ATP varies with the age of the preparation.

In this work we studied the effects of both ouabain and digoxin, on both skeletal (14 different preparations) and cardiac (4 different preparations) myosin B, but the system ouabain-skeletal myosin B was studied most extensively. Nonetheless, the data permit us to say that the systems ouabain-cardiac, digoxin-skeletal, and digoxin-cardiac were studied enough to ensure that the basic findings on ouabain-skeletal apply as well to them. Early on, we examined for the possibility that progressive purification of myosin B progressively eliminated sensitivity to ouabain, and also for the possibility that increased length of incubation of myosin B with cardiac glycoside leads to increased effect; neither phenomenon was found.

EFFECT OF OUABAIN ON SUPERPRECIPITATION PARAMETERS AT DIFFERENT CONCENTRATIONS OF ATP

In a typical record of superprecipitation extent following ATP addition, the extent first rises rapidly and then more slowly approaches a horizontal asymptote; in such cases the initial "structural rate" is easily measured from the plot. Occasionally, the plot has a slight sigmoid character; then we have taken the maximum rate (at the inflection) to represent the structural rate. If the [ATP] is saturating, then an "optimal" (see below) concentration of cardiac glycoside approximately doubles the structural rate, but has little or no effect on the asymptotic, or final, value of the extent (Fig. 1). On the other hand, if [ATP] is chosen to be undersaturating, both rate and final extent are elevated (Fig. 2). Such results with ouabain are very reproducible on every preparation tested. Analogous results are obtainable with digoxin or strophanthidinic acid with saturating [ATP], but the other glycosides were not studied with undersaturating [ATP].

THE INFLUENCE OF OUABAIN CONCENTRATION ON STRUCTURAL EFFECTS

In Figure 3 we show results which are very typical of ouabain when this substance is added to systems in which [ATP] is undersaturating (when saturating [ATP] is employed, optima of effect on rate are obtained at the same concentration of ouabain, but then of course effects on final extent cannot be ob-
The effect of ouabain on the rate and extent of suprercipitation of skeletal myosin B with undersaturating [ATP]. Actual recorder tracing. A, $10^{-11}$ M ouabain. B, control. Myosin B 0.01%, 0.12 M KCl, 0.018 M iris maleate, pH 7.0, ATP, 0.05 mm, 25°C. Full vertical scale, 0.5 OD units. Smallest horizontal division, 25 sec.

served). However, it must be said here that while all preparations show two optima, the cardiac glycoside concentrations at which these optima occur vary with age of a preparation, and from one preparation to another, but not within a single stock suspension; thus, sometimes optima may occur at $10^{-8}$M and $10^{-4}$ M, and in another instance at $10^{-11}$M and $10^{-6}$ M. Most frequently the low concentration optimum occurs at $10^{-9}$ M with either ouabain or digoxin, and the high concentration optimum with ouabain occurs at $10^{-5}$ M (insolubility of digoxin precludes definition of a high concentration optimum, but at $10^{-6}$ M digoxin effect is increasing with concentration).

**THE INFLUENCE OF OUABAIN CONCENTRATION ON RATE OF ATP UTILIZATION**

For reasons to be discussed, we studied the effect of ouabain at various concentrations on two rates of ATP utilization. In order to define precise rates, saturating [ATP] was used. For various concentrations of ouabain, Figure 4, curve I, shows the dependence of the fast ATPase of myosin B (the activity concurrent with structural events of suprercipitation); curve II shows the dependence of the ATPase of myosin A dissolved in precisely the same solvent. For reference, structural rate (curve IV) and extent (curve III) measurements, made at the same hour on the same myosin B preparation, are also shown. Clearly, ouabain has no effect on the activity of myosin A. Also it has no effect on the ATPase of myosin B observed after suprercipitation is over. Moreover, it is evident that in concentrations between $10^{-18}$ M and $10^{-7}$ M, ouabain affects the fast myosin B ATP utilization in much the same way that it affects the structural parameters. Between $10^{-6}$ M and $10^{-3}$ M the structural parameters often decreased while the ATP utilization rate increased.

**OTHER SYSTEMS AND OTHER CARDIAC GLYCOSIDES**

Figure 5 shows the effect of digoxin on the "structural rate" of cardiac myosin B. At the same molarity range, digoxin has roughly the same effect as ouabain. On the other hand, strophanthidinic acid must be 100 times more
concentrated in order to elicit the same effect as ouabain (Fig. 6).

**POSSIBLE COMPLICATIONS BY SALTS**

Because superprecipitation under our conditions is very sensitive to Mg\(^{2+}\) we studied the effect of Mg\(^{2+}\) addition (10\(^{-6}\) M MgCl\(_2\)) and subtraction (10\(^{-4}\) M EDTA) on experiments such as shown in Figure 3. In both instances, at all ouabain concentrations, the ouabain effect was additive with the salt effect. A concentration of 10\(^{-5}\) M CaCl\(_2\), thought to be capable of inactivating particulate relaxing factor, was also additive with the ouabain effect.

**POSSIBLE COMPLICATIONS BY PARTICULATE "RELAXING FACTOR"**

It is well known that particulate relaxing factor is destroyed by phospholipase. To test whether the ouabain effect might be merely an inhibition of membranous factor, we pre-incubated the myosin B preparation with phospholipases before testing with ouabain.

We studied phospholipase A (unheated snake venom); this enzyme was very active (94 units mg\(^{-1}\) hr\(^{-1}\), pH 7.0, 25°C, no Ca\(^{2+}\) added) toward lecithin when in superprecipitation solvent. The incubations were carried out in superprecipitation solvent for 40 min at 25°C. The weight concentration of phospholipase was about equal to that of myosin B. In all experiments the ouabain effect per se was unchanged following the incubations. The rate and extent of superprecipitation in the absence as well as in the presence of ouabain were reduced by these high concentrations of phospholipase, but this reduction was independent of incubation time and therefore not due to enzymatic action of the phospholipase.

**DISPERSION OF DATA**

In the present state of knowledge, the degree to which myosin is directly bonded to F-actin, and the degree to which the myosin B floe is dispersed are essentially uncontrolled.
Effect of increasing ouabain concentration on: III, the extent of superprecipitation (Supptn.) of myosin B (Δ—Δ); I) rate of utilization of ATP by myosin B (●—●); IV, rate of superprecipitation of myosin B (×—×); and II, myosin A ATPase (○—○). Superprecipitation data, 0.01% myosin B, 0.12 M KCl, 0.018 M tris maleate, pH 7.0, 0.05 M ATP. ATP utilization by myosin B (pH stat), 0.01% myosin B, 0.12 M KCl, 0.1 M ATP, 5 M KOH as titrant, pH 7.0. Myosin A ATPase, 0.02% myosin A, 0.12 M KCl, 0.2 M ATP, 5 M KOH as titrant, pH 7.0. Myosin A ATPase data, mean of three assays; all other data, mean of two assays. All experiments done at 25°C.

Additionally (see Discussion), myosin (A) not directly bonded to actin contributes substantially to ATP hydrolysis (for example, the magnitude of the effect which a given cardiac glycoside concentration has on the rate of ATP utilization can be reduced to zero by experimental addition of myosin (A)), but relatively little to optical changes on ATP addition. From these circumstances arises a pronounced variability among measurements (of either structural or enzymatic parameters) made on different preparations, or on the same preparation at different ages. Our more detailed conclusions are based on more reproducible results (for example, those displayed in Figs. 3, 4, and 5) obtained in special paired experiments (see below). However legitimate, this is a form of data selection, so it is necessary to show that also by using all recent data (382 experiments on superprecipitation rate, 162 experiments on ATP utilization rate, on a total of 5 separate myosin B preparations) obtained in this laboratory, the major conclusion of this paper is firmly upheld. On the one-dimensional graph of Figure 7 are shown all the responses (as percent deviations from the mean of several controls) ever obtained to ouabain, regardless of concentration. If ouabain had no effect, then so large a number of rate measurements would tend to a distribution symmetrical about the origin; however, the overwhelming majority of points deviate positively; that is, ouabain accelerates both the rate of structural change and the rate at which ATP is hydrolyzed during the structural change. With our present technique it is not possible to measure optical density and proton release on the very same aliquot of floc, hence to make a strict pairing of the two measurements.
The effect of different concentrations of digoxin on the rate of superprecipitation of cardiac myosin B using saturating [ATP]. 0.01% myosin B, 0.12 M KCl, 0.018 M tris maleate, pH 7.0, 1 mM ATP, 25°C.

Comparison of the effect of ouabain (●) and strophanthidinic acid (○—○) on the rate of superprecipitation of skeletal myosin B using saturating [ATP]. 0.01% myosin B, 0.12 M KCl, 0.018 M tris maleate, pH 7.0, 1 mM ATP, 25°C.

In many instances, however, it was possible for two operators to draw aliquots and make the two measurements at very nearly the same time (circumstances of Fig. 4). Still more frequently it was possible for a single operator to make one type of measurement in the...
Discussion

Our investigation shows that, at two distinct concentrations, ouabain and digoxin nearly double the rate at which suspensions of purified myosin B undergo "in vitro contraction," i.e., superprecipitation. One of these concentrations is as low as or lower than the concentration of cardiac glycoside which will just show an inotropic effect on living heart muscle (Hoffman, personal communication).

A second inference from our work is that ouabain seems to act at myosin-actin "contacts." The underlying reasoning was set forth by Rainford et al. in another context: It is supposed that "myosin B" is really two ATP-utilizing systems operating in parallel—one of these systems is a substantial number of myosin A molecules not directly bound to actin (existence of this can be shown by ultracentrifugal analysis); this component undergoes no structural changes, is K⁺-activated, and catalyzes the hydrolysis of ATP relatively slowly both during and long after superprecipitation is complete. The other system arises somehow at myosin-actin contacts, its hydrolysis of ATP is tightly coupled with the structural change detectable as "superprecipitation," it is Mg²⁺-activated, and it hydrolyzes ATP rapidly only during superprecipitation. It can be said that for this structure-coupled hydrolysis there are two substrates, ATP and non-transformed structural protein elements; thus the reaction ends when either ATP is exhausted or when all the structural elements are transformed. With the foregoing hypothesis the behavior with concentration of

Outer axes: the effect of ouabain, regardless of concentration, on superprecipitation rate (382 experiments) or ATP utilization rate (162 experiments). Central, two-dimensional graph: points representing experiments in which the superprecipitation and ATP utilization rate measurements could be temporarily paired (87 paired experiments).

morning and the other (on another floe) in the afternoon, employing in both cases the same sequence of controls and cardiac glyco-
an agent which affects only the structure-coupled system is predictable. For Rainford et al., the agent was hydrostatic pressure, and it caused inhibition; here it is ouabain, and it causes activation. If ATP is presented to the systems in unlimited amount, the observed extent of superprecipitation will be governed by the amount of non-transformed protein. Addition of an activator like ouabain will increase the rate of the structural change, but the extent will be the same because it is still protein limited. When ATP is presented in limited quantity, it will be parcelled out to the two enzymes according to their zero-order rate constants; the reactions will stop when ATP is exhausted, and the observed extent will depend on the fraction of the ATP which the structure-coupled system won in the competition. Under these conditions, addition of an activator like ouabain gives the structure-coupled system an advantage, i.e., aided by ouabain, it will win a larger fraction of the ATP and the observed extent will therefore be larger, not only the observed rate. It is in terms of the foregoing that we explain why ouabain (in an effective concentration) increases rate (ATPase or structural) only if [ATP] is saturating, and both rate and extent if [ATP] is undersaturating; also, why ouabain does not affect the ATPase of myosin A, or the ATPase which continues long after superprecipitation is over. In these terms, the "concentration" of the "structure-coupled system" is not defined alone by the concentration of, say, myosin or actin, but depends markedly on the manner in which the actin and myosin are co-precipitated in the course of preparing the myosin B suspension; furthermore, the "concentration" is considerably less than the molar concentration of the lesser component (myosin A).

Finally, more of a suggestion than a conclusion, is that, in the one case tried, there was a correlation between the effectiveness of two cardiac glycosides (ouabain and strophanthidinic acid) in their ability to kill cats and in our in vitro tests, and it is conceivable that superprecipitation could be used for assay or screening.

While we consider the data which we have cited reliable, and the interpretations which we have offered reasonable, we would be remiss to slur over some obvious difficulties. It is already perplexing that the effect of the cardiac glycosides passes through an "optimum," indicating the existence of an inhibitory factor which we do not understand; to make matters worse, there are actually two optima, even though we seem to be working with a pure cardiac glycoside. Because a cardiac glycoside inhibition of "membrane ATPase" has been established, because a hydrophobic steroid molecule might be expected to interact with lipids, because the optimal concentrations are erratic, and because the concentration of "receptor substance" in our system would have to be about 1/100 of the concentration of myosin, one must seriously consider the hypothesis that the cardiac glycoside is neutralizing a contamination of particulate relaxing factor. Against this hypothesis we have the experimental observations that the cardiac glycoside effect is unaffected by treating the myosin B with either Ca$^{2+}$ or phospholipases; also we can argue that the concentration of "actin-myosin contacts" can very well be 1/100 the concentration of myosin, and that it is bound to be erratic because it is almost impossible to co-precipitate two proteins reproducibly.

As already remarked, the argument that cardiac glycosides act physiologically on membranes is no better than the argument that these substances act in the region of thick-thin filament interaction (at this stage both are weak arguments). Indeed, that the contractile system is affected by physiologically plausible (i.e., low enough) concentrations while membranes aren't, and that tritiated digitoxin locates in the A-band (see ref. 13), are facts which undermine the "membrane theory." To be sure, in a 1961 symposium Repke$^{12}$ reported a 30-50% activation of "cardiac muscle membrane ATPase" by 10$^{-9}$ M to 10$^{-8}$ M k-strophanthoside, but activation of relaxing factor is not equivalent to activation of contraction—indeed it is just the reverse. Also, in a recent communication, Fozzard
and Smith report a correlation of tritium-labeled cardiac glycoside with properties usually ascribed to membrane fragments—easy sedimentation and "Na-K ATPase"; because of this correlation they appear to surrender their original hypothesis, and accept more the views of Sonnenblick et al., who feel that the tritium label is in the sarcoplasmic reticulum. This later report of Fozzard and Smith gives no details concerning ultracentrifugal fractionation, but we may remark that in a sucrose density gradient it is essentially impossible to separate thick and thin filaments from other cellular debris, and that activation by mixtures of K+ and Na+ would normally not discriminate between the ATPase of relaxing factor and that of, say, myosin.

Addendum

After the present work was presented in abstracted lectures at the Universities of Tokyo and Washington, and at the College of Physicians and Surgeons of Columbia University, Dr. Arnold Katz (Fed. Proc. 25: 643, 1966) reported on work ostensibly designed to examine the main conclusions of this paper. Dr. Katz confirmed the insensitivity of myosin A ATPase to ouabain, but was otherwise led to conclusions very divergent from ours. In order to facilitate authentic examination and extension of our present work we list useful precautions culled from our experience. (1) Factors affecting the degree of direct actin-myosin bonding are certain to be important. Choice of system may be such a factor; for example, a "reconstituted" actomyosin system is mechanically weaker (as a thread) and gives a much smaller percent turbidity change on ATP addition than the myosin B system used here. (2) Because of the intrinsic variability in both types of measurement it is highly desirable to record OD and H+ liberation (ATP hydrolysis) continuously, but even so, the measurements are unavoidably erratic, so that a very large number of data have to be gathered to establish a conclusion. (3) The ATPase of myosin which is not directly bonded to actin is insensitive to ouabain. Accordingly, care must be taken to minimize the contribution of this component. For example, if a system contains much Mg++ (e.g. mM) superprecipitation is preceded by an extremely long "clearing phase," during which ATP is hydrolyzed by unbound, thus insensitive, myosin; likewise after superprecipitation is over, the persisting myosin A ATPase is insensitive; it is only the high ATP utilization rate actually accompanying superprecipitation which is activated by ouabain and digoxin. (4) Other general features and useful precautions pertaining to the techniques used here will be found in references 6, 8, and 16.

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