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Research Article

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The Influence of Ouabain and Alpha Angelica Lactone on Calcium Metabolism of Dog Cardiac Microsomes

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ABSTRACT The influence of ouabain and alpha angelica lactone on ^{45}calcium accumulation in cardiac microsomes was studied. Calcium binding (accumulation in the absence of excess oxalate or phosphate) was augmented by both ouabain and alpha angelica lactone in the presence of adenosine triphosphate (ATP) but unaffected in its absence. Calcium turnover (defined as the change in $^{45}Ca^{++}$ bound to the microsomes after the specific activity is changed) was studied to determine if the augmented bound pool was freely exchangeable at equilibrium. Ouabain and alpha angelica lactone augmented calcium turnover in both the presence and absence of ATP. Calcium-stimulated ATPase was increased by both agents.

It is proposed that these two unsaturated lactones, with known cardiotonic activity, may exert their effects by providing an increased contraction-dependent calcium pool to be released upon systolic depolarization.

INTRODUCTION

The role of sarcoplasmic reticulum in the control of intracellular calcium concentration has assumed a central place in the theory of the mechanism of myocardial excitation-contraction coupling (1–4). According to this theory, sarcoplasmic reticulum releases Ca^{++} in response to the excitation which initiates muscular contraction. Relaxation is thought to be caused by the rebinding of Ca^{++} by the sarcoplasmic reticulum. The evidence for this concept has been derived mainly from in vitro studies on the microsomal fraction thought to represent the sarcoplasmic reticulum. In these studies, sarcoplasmic reticulum has been found to accumulate $^{45}CaCl_2$ (2–6) and to release it in response to activation, although the precise mechanism and stimulus for release is not clear (1). Moreover, it has been shown that frog skeletal muscle, which is fixed during relaxation, accumulates $^{45}Ca^{++}$ in the area of the transverse tubular system (7).

The in vitro "uptake" of calcium by the microsomal fraction has been measured in the presence of oxalate or phosphate which augments accumulation, possibly by precipitating the calcium inside the microsomal vesicles. This process was found to require adenosine triphosphate (ATP) and to be capable of accumulating quantities of calcium many times more than that necessary to lower the concentration below the threshold level for actomyosin superprecipitation (4, 6, 8).

The applicability of this "uptake" system was open to question because of (*a*) the added oxalate or phosphate and (*b*) the slow time course of the accumulation. Katz has defined calcium "binding" as the accumulation of $^{45}Ca^{++}$ in the absence of oxalate or phosphate (8). The time course of this binding is too rapid for measurement since it reaches near equilibrium with the media in less than 15 sec (8, 9) and is relatively constant after 5 min (9). Although "binding" accumulates much less Ca^{++} than "uptake," the amount "bound" has been calculated to be sufficient to lower the calcium ion concentration to levels below the threshold for activation of actomyosin in vitro (8). Therefore, this is the system used for study in this communication.

Because both ouabain and calcium increase myocardial contractility of the cardiac muscle, the influence of ouabain on calcium metabolism has been studied in many systems. For example, Lullman and Holland (10), using isolated, perfused guinea pig myocardium found that digitalis increased the rate of turnover of labeled calcium without altering myocardial calcium concentration. Investigations of the influence of ouabain on the

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calcium "uptake" system, however, have yielded conflicting results (11–13).

This communication presents studies on the effects of ouabain on the calcium binding system of a dog myocardial microsomal fraction thought to represent principally sarcoplasmic reticulum. The data show that ouabain and a nonsteroidal unsaturated lactone, 3-pentene-1,4-olide (alpha angelica lactone), with positive inotropic activity (14–16), do increase the binding of Ca^{++} by this fraction, increase its turnover at equilibrium, and augment the "calcium-stimulated ATPase."

METHODS

The microsomal fraction was isolated and purified from hearts of mongrel dogs immediately after pentobarbital anesthesia by the method of Katz and Repke (8) by use of a sucrose gradient of equal parts of 20 and 35% sucrose for the final purification step. In addition, alpha tocopherol in a concentration of 0.2 mmole/liter was used in all isolation media. The purified preparation was stored at 4°C and was used within 3 days. Protein concentration was measured by the biuret method. All experiments were done with cellulose vessels and polypropylene syringes to avoid calcium binding to glassware.

To measure calcium binding, 0.5–1.0 mg (protein) of microsomes was incubated in a 3 ml volume at 25°C. The basic reaction mixture contained 0.12 M KCl, 5 mM MgATP, 10 mM histidine buffer, pH 7.0, with a $^{45}CaCl_2$ concentration of 2.5×10^{-5} mole/liter containing about 25,000 cpm/mμmole. Samples were extruded through a Swinney adapter containing a Millipore filter (0.45 μ, pore diameter), and aliquots of the filtrate were counted in Triton X-100 : toluene (1 : 2 v/v) with 2,5 diphenyloxazole (PPO), 4 g and 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP), 0.1 g/liter in a liquid scintillation spectrometer. Individual unfiltered blanks were pipetted for each sample, and the amount bound was calculated by using the difference between the filtered and unfiltered samples. Solutions containing no microsomes demonstrated no binding. Ouabain and alpha angelica lactone were added in the concentrations described in the Results and tables. In experiments done in the absence of ATP, 5 mM $MgCl_2$ was substituted for MgATP. No sodium was added to any of the reaction mixtures.

Two methods were used to assay calcium turnover. In the first method, microsomes were incubated with $^{45}CaCl_2$ as previously described for 5 min. Filtered and unfiltered aliquots were taken, and 0.15 ml of 10^{-3} M unlabeled $CaCl_2$, a sufficient quantity to triple the calcium concentration, was added to the 3.0 ml incubation mixture. Samples were then filtered after 30 sec, 1 min, and 2 min to measure the change in $^{45}Ca^{++}$ binding. The values for this binding change were corrected for the new specific activity of $^{45}Ca^{++}$, and values were expressed in millimicromoles of Ca^{++} exchanged per mg of protein.

The second method was designed to measure Ca^{++} turnover without changing the Ca^{++} concentration. The microsomes were incubated with nonradioactive $CaCl_2$ at a concentration in the reaction mixture of 2.5×10^{-5} mole/liter for 5 min. At 5 min, 0.2 ml of $^{45}CaCl_2$ at a concentration of 2.5×10^{-5} mole/liter, the same concentration as in the preincubation mixture, was added, and filtered samples were taken as before. In these experiments, ouabain was added either before or after the initial incubation with similar qualitative results in each case. Some experiments were done in absence of ATP. Values given represented the $^{45}Ca^{++}$ bound to microsomes in exchange for the nonradioactive Ca^{++}.

ATPase activity was measured in the presence of 4 mM MgATP, 8 mM histidine buffer, 0.12 M KCl, and 0.1 mg/ml of microsomal protein at 25°C. Incubation of 5-ml volumes was done in the presence and in the absence of 10^{-4} M $CaCl_2$. Controls were compared in the presence of either 10^{-6} M ouabain or 10^{-3} M alpha angelica lactone. Samples were taken after 10, 20, and 30 min and extruded through a Swinnex adapter containing a Millipore filter (0.45 μ) and then iced. Inorganic phosphate (P_i) concentration was measured by the method of Wahler and Wollenberger as adapted by Post and Sen (17). "Calcium-stimulated ATPase" was calculated by subtracting the released phosphate values in the absence of calcium from those values in the presence of calcium at each sample time. Results were expressed in micromoles of of P_i per milligram of protein per minute.

Mitochondrial contamination of the microsomal preparations was assessed by electron microscopy and by assay of glutamic dehydrogenase activity. No intact mitochondria were visible in OsO_4-fixed preparations. Glutamic dehydrogenase activity (18) of the microsomal fraction was less than 1% of that of a preparation of dog heart mitochondria of similar protein concentration.

Endogenous microsomal calcium was measured by sedimenting the final product from the sucrose gradient at 368,000 *g* for 30 min. The sediment was then ashed in an acid-washed nickel crucible and resuspended in a 5% HCl, 1% lanthanum solution. The calcium level was determined in an atomic absorption spectrometer (Perkin-Elmer Corp., Norwalk, Conn.). The endogenous Ca^{++} concentration was 4–5 mμmoles/mg of protein, a value similar to that found by Weber (4).

Alpha angelica lactone was kindly supplied by Dr. Edward Cook of the Research Triangle Institute, Durham, N. C. and was 95% pure with 5% contamination of the 2-pentene-1,4-olide (beta angelica lactone) as measured by gas chromatography. The ouabain (Nutritional Biochemicals Corporation, Cleveland, Ohio) was found to be pure by using thin-layer chromatography with silica G and a solvent system containing benzene : 95% ethanol (7 : 3, v : v) (19). The R_f was 0.09 for ouabain. All reagents were found to be free of significant Ca^{++} by atomic absorption spectroscopy. The ATP used was the dipotassium salt and was free of measurable Na^+ by flame photometry.

All experiments were analyzed using paired *t* test for data from a given day's experiment with the same membrane preparation.

RESULTS

Calcium binding. The effect of ouabain on radioactive calcium binding by the microsomal fraction is shown in Table I. Ouabain augmented the amount of calcium bound only in the presence of ATP. As Katz and Repke have shown (20), the stimulatory effect of ATP on calcium binding is decreased in a low potassium media. In a low potassium media (0.01 M KCl) there was less calcium bound to the microsomal fraction in the presence of ATP than in the basic media (0.12 M KCl). However, the stimulatory effect of ouabain on calcium binding was also seen in the low potassium media (cf. Table I). In the absence of ATP, ouabain had no effect on calcium binding at either concentration of

TABLE I

Effect of Ouabain on Calcium Binding

	0.12 M KCl, 5 mM ATP	0.01 M KCl, 5 mM ATP	0.12 M KCl, 0 ATP	0.01 M KCl, 0 ATP
	n = 14	n = 4	n = 6	n = 4
10^{-6}M Ouabain	19.7 ±4.7	13.9 ±2.2	4.9 ±1.2	6.4 ±0.2
Control	14.8 ±2.7	11.5 ±2.8	5.0 ±1.4	6.2 ±0.3
P	<0.01	<0.01	>0.5	>0.5

Dog cardiac microsomes (0.2 mg of protein per cc) were incubated at pH 7.0 in 10 mM histidine buffer, 5 mM Mg^{++}, 2.5×10^{-6}M $^{45}CaCl_2$, and ATP and KCl as shown. Incubation was carried out for 5 min at 25°C with a total volume of 3 cc. The reaction was terminated by expulsion through a Millipore filter (0.45 μ). Results expressed in millimicromoles of ^{45}Ca per mg of protein ± standard deviation.

potassium. These data also confirm those of Katz and Repke (20) that in the absence of ATP, calcium binding by the microsomal fraction is greater in the low potassium media.

The effect of varying concentration of ouabain on calcium binding are shown in Table II. Ouabain augmented calcium binding to a significant degree at concentrations of 10^{-5} and 10^{-6} mole/liter ($P < 0.01$). The binding at 10^{-7} and 10^{-4} mole/liter were not significantly greater than control values.

Alpha angelica lactone had effects similar to that of ouabain on the system (Table III). At a concentration of 10^{-3} mole-liter, calcium binding was increased in the presence of ATP ($P < 0.01$) but not affected in its absence ($P > 0.5$).

Calcium turnover. Calcium turnover (measured by the addition of nonradioactive calcium) is shown in Fig. 1. Nonradioactive calcium was added at zero time to bring the calcium concentration to three times its former value. Calcium turnover was defined as the difference between $^{45}Ca^{++}$ binding before the addition of nonradioactive calcium and binding at each of the intervals afterward. Fig. 1, therefore, represents the decrease in $^{45}Ca^{++}$ binding after decreasing the specific activity by increasing the concentration of nonradioactive

TABLE II

Effect of Varying Ouabain Concentrations on Calcium Binding

Ouabain concentrations	mμmoles Ca/mg protein ±SD
Control	15.0 ±1.5
10^{-7}M Ouabain	18.8 ±2.6
10^{-6}M Ouabain	26.1 ±2.6
10^{-5}M Ouabain	20.3 ±4.0
10^{-4}M Ouabain	17.7 ±3.2

The incubation mixture was the same as in Table I except 5 mM ATP and 0.12 M KCl were used and ouabain added as shown. Incubation time and binding determination were as described in Table I.

TABLE III

A Comparison of Effects of Ouabain and Alpha Angelica Lactone on Calcium Binding

	5 mM ATP	0 mM ATP
	n = 8	n = 8
	mμmoles/mg protein ±SD	
Control	16.8 ±3.4	6.4 ±0.2
10^{-6}M Ouabain	19.2 ±1.8	6.2 ±0.3
10^{-3}M Lactone	23.1 ±3.2	6.3 ±0.4

Each reaction mixture and binding determination were as described in Table I except ATP, ouabain, and alpha angelica lactone were varied as shown.

calcium. This decrease in $^{45}Ca^{++}$ binding was termed "turnover" because, as is shown below, it represents an exchange of the previously bound radioactive calcium for nonradioactive calcium introduced into the mixture. Both ouabain and alpha angelica lactone increased the calcium turnover as measured by this method. Because this method necessitated the changing of the calcium ion concentration, it was not a reliable index of exchange at equilibrium. To obtain values which would reflect equilibrium conditions, the agents were tested in an experiment in which calcium concentrations were not changed (addition of a dilute radioactive label after incubation with nonradioactive calcium).

In Figs. 2–4, calcium turnover was measured by adding a radioactive calcium label in the same concentration as the preincubation mixture to microsomes which had been preincubated with nonradioactive calcium for 5 min (a time found in our binding studies to be sufficient for equilibration). In this case, labeled calcium ions replace bound nonradioactive ions, and the result is

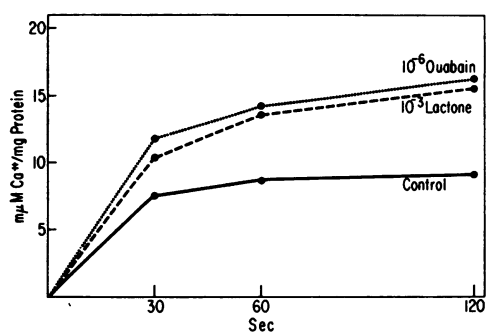

FIGURE 1 Calcium turnover measured by dilution of $^{45}Ca^{++}$ with unlabeled Ca^{++}. Microsomes (0.2 mg of protein per cc) were incubated in the presence of 5 mM adenosine triphosphate (ATP), 10 mM histidine, 0.12 M KCl, and 2.5×10^{-5} M $^{45}CaCl_2$ for 5 min at 25°C. At time zero 150 mμmoles of $CaCl_2$ are added, and the change in bound $^{45}Ca^{++}$ is measured at intervals shown. Abbreviations: lactone, alpha angelica lactone.

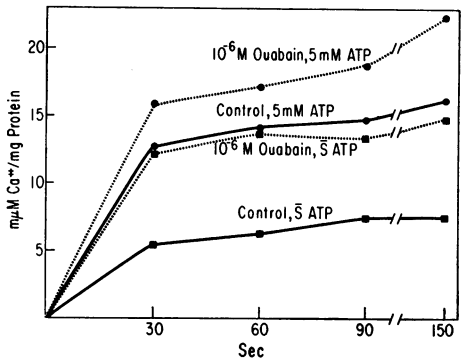

FIGURE 2 Calcium turnover at equilibrium in the presence and absence of ATP. Microsomes (0.2 mg of protein per cc) were incubated in the presence of 10 mM histidine, 0.12 M KCl, and 2.5×10^{-6} nonradioactive $CaCl_2$ for 5 min at 25°C. At zero time 1 μc of $^{45}CaCl_2$ in a concentration of 2.5×10^{-5} mole/liter is added, and samples are taken at the intervals shown.

$^{45}Ca^{++}$ binding in exchange for nonradioactive Ca^{++} or "turnover." An increase in Ca^{++} binding upon addition of $^{45}Ca^{++}$ is unlikely because since calcium concentration is unchanged, equilibrium is not disturbed. In Fig. 2 are shown the effects of ouabain on the calcium turnover at equilibrium. Ouabain increases calcium turnover in a similar manner to the previously described experiments. Unlike the binding studies, ouabain also increases turnover in the absence of ATP (Fig. 2). Experiments in low potassium concentration demonstrated similar results.

The effect of varying the ouabain concentration is shown in Fig. 3. These data show the greatest calcium turnover in the presence of 10^{-5} M ouabain. In addition, a small but consistent increase could be demonstrated at an ouabain concentration of 10^{-7} mole/liter.

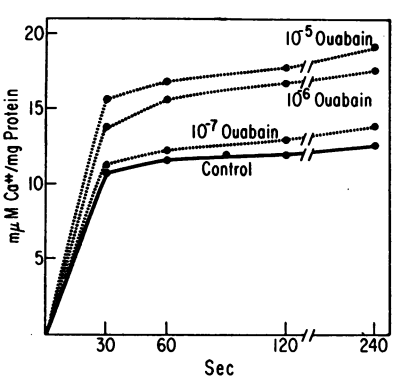

FIGURE 3 Calcium turnover at equilibrium in the presence of varying ouabain concentrations. Microsomes (0.2 mg of protein per cc) were incubated in the presence of 5 mM MgATP, 10 mM histidine, 0.12 M KCl, and 2.5×10^{-5} nonradioactive $CaCl_2$ for 5 min with ouabain concentrations as shown. Method as in Fig. 2.

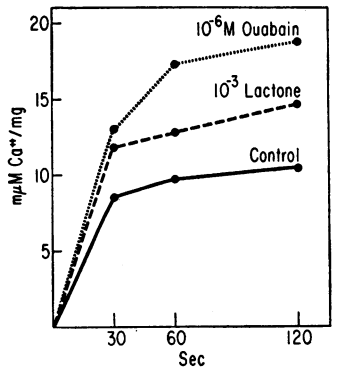

FIGURE 4 Calcium turnover at equilibrium in the presence of ouabain and alpha angelica lactone. Method as in Fig. 3, ouabain and alpha angelica lactone as shown.

Alpha angelica lactone (10^{-3} mole/liter) increased calcium turnover at equilibrium in a similar manner to ouabain (Fig. 4). Because of limited solubility of the lactone higher concentrations could not be tested. A concentration of 10^{-4} mole/liter produced no change in calcium turnover.

ATPase effects. The fact that ouabain exerted its effects on binding only in the presence of ATP suggested that at least part of the observed effects were related to ATP utilization. Both ouabain (10^{-6} mole/liter) and alpha angelica lactone (10^{-3} mole/liter) depressed the ATPase activity by 25% in the presence of Mg^{++} (4 mmoles/liter) and K^+ (0.12 mole/liter) only (Fig. 5).

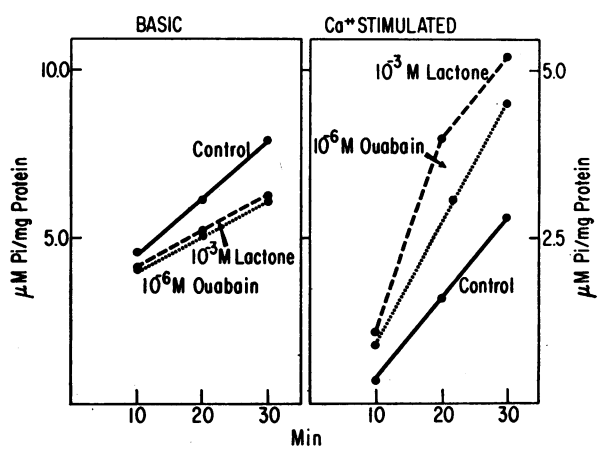

FIGURE 5 Effect of ouabain and alpha angelica lactone on phosphate released from ATP in the presence and absence of calcium. Microsomes (0.1 mg/cc) were incubated at 25°C in the presence of 4 mM MgATP, 8 mM histidine, 0.12 M KCl, in the presence and absence of 10^{-4} M $CaCl_2$ with ouabain and alpha angelica lactone as shown. Samples were taken at 10, 20, and 30 min. Left panel shows released inorganic phosphorus (P_i) in absence of Ca^{++}; right panel is additional P_i released in the presence of Ca^{++}. (Curves fail to go through the ordinate at zero because blanks were not subtracted.)

The addition of $CaCl_2$ stimulated phosphate release in all systems. Fig. 5 demonstrates this "calcium-stimulated" phosphate release to be significantly greater in the presence of ouabain or alpha angelica lactone. The increase was consistently 40–50% with 10^{-6}M ouabain and almost 100% with 10^{-3}M alpha angelica lactone.

DISCUSSION

The data in this communication demonstrate that ouabain and alpha angelica lactone, two agents with known positive inotropic activity (14–16), increase calcium binding and turnover in the cardiac microsomal fraction. Furthermore, calcium binding was increased by these agents only in the presence of ATP, a finding which demonstrates an influence on an ATP-dependent mechanism for calcium binding. However, ouabain and alpha angelica lactone were found to increase the turnover of calcium at equilibrium in the absence of ATP as well as in its presence. Consonant with the augmented ATP-dependent calcium metabolism, ouabain and alpha angelica lactone also increased the calcium stimulated ATPase. This finding suggests a relationship of these parameters in intact microsomal fractions, although they are dissociable in damaged microsomes which may reduce Ca^{++} uptake without a concomitant reduction in Ca^{++}-stimulated ATPase (21).

Previous work on the effects of ouabain on calcium metabolism in cardiac microsomes has yielded conflicting results (11–13). The difference in these results reflects, in part, differences in methods of isolation and of purification of the preparation (5, 6, 11, 13). In addition, in most of the previous studies, the effects of ouabain have been measured in the presence of oxalate or excessive P_i (5, 6, 11–13) both of which, by continually precipitating accumulated calcium, maintain a constant calcium ion concentration within the vesicle. The effect of ouabain on a purified preparation in the absence of anion which causes calcium precipitation within the microsomes has not been previously reported. To our knowledge, the effect of alpha angelica lactone on cardiac microsomes has not been previously investigated. Krespi, Fozzard, and Sleator reported inhibition by ouabain of basic microsomal ATPase in the presence of K^+ and Mg^{++} (22).

Ouabain and alpha angelica lactone appear to affect at least two processes of Ca^{++} accumulation by these microsomes, one ATP-dependent and the other not. Hauser and Dawson (23) have demonstrated that calcium is selectively bound in the absence of ATP to artificially prepared unimolecular films of purified lipids. They found that binding was roughly proportional to the nucleophilic acid phospholipid content of the membranes and suggested this as a mechanism of biologic membranes. In addition, Martinosi, Donley, and Halpin demonstrated that removal of the phospholipids from skeletal muscle sarcoplasmic reticulum destroyed their Ca^{++}-accumulating activity (21).

The distribution of the ATP-dependent Ca^{++} pool(s) cannot be characterized by this study, and it can only be said that a major part of this pool(s) is freely and rapidly exchangeable with the external media. Carvalho and Leo (24) point out a number of mechanisms by which this ATP-dependent Ca^{++} accumulation and exchange may occur. This study does not permit a choice among them.

The agents described exert effects both in the presence and absence of ATP. In the absence of ATP, they increase the turnover of Ca^{++} but do not change the total steady-state Ca^{++} binding. If nucleophilic residues account for the non-ATP–dependent binding, it might be postulated that ouabain and alpha angelica lactone may act on a membrane with a fixed number (the amount of nucleophilic residues) of available binding sites by altering the turnover on these sites.

The effect of ouabain and alpha angelica lactone on the ATP-dependent Ca^{++} accumulation is complex. Whether they increase the Ca^{++} available to a less accessible binding pool or merely increase the intravesicular unbound calcium concentration is speculative. Both effects may be operative and interdependent (24). At any rate, the effect on any "binding sites" or "binding constants" probably represents a summation of several effects.

The increased exchange of the ATP-dependent Ca^{++} pool demonstrates that ATP-dependent Ca^{++} transport is augmented continually and not just upon the initial preequilibrium exposure to Ca^{++}. Further evidence for this is the augmented Ca^{++}-stimulated ATPase at 10, 20, and 30 min. Increased Ca^{++} accumulation in this system might well be visualized as an altered steady state with continued rapid exchange. Because of the rate of calcium exchange is so rapid, we have no measurement of its initial rate constants. The 30-sec samples taken represent an integrated value for total calcium exchanged during that interval. The augmentation of total calcium exchange is roughly equal to the augmentation of calcium binding and indicates to us only that the enlarged pool is also freely exchangeable. In absence of initial rate constants, we cannot ascertain whether exchange rate on an individual site is augmented, although the data in the absence of ATP suggests this possibility for at least the non-ATP–dependent site. Likewise, in absence of more knowledge of pool distributions and initial rate constants, the mechanism of augmentation of calcium flux cannot be well defined. These agents may act primarily on the transport enzyme or on the membrane itself.

That ouabain and alpha angelica lactone increase Ca^{++} binding is consonant with the data of Ueba and Chidsey

who found that microsomal Ca^{++} was increased in perfused rabbit hearts after addition of ouabain to the perfusate (25). In addition, it would explain the findings of Lullman and Holland of an increased calcium turnover in perfused guinea pig myocardium in the presence of ouabain (10). Indeed, Gouvier and Holland (26) suggested a "contraction-dependent" calcium pool which was increased by ouabain (26). This pool might well be the increased calcium bound to sarcoplasmic reticulum which, upon electrical activation, would yield an increased calcium concentration in the myofilament during systole.

The presence of an unsaturated lactone on most active glycosides led to this investigation of the effect of alpha angelica lactone. This compound has been demonstrated to have positive inotropic activity in frog hearts (14–16), and Giarman found it to be 1/600th as active as ouabain in its cardiotonic effect (14). The finding in the work reported here that alpha angelica lactone has similar effects to those of ouabain on microsomal calcium metabolism further suggests that these effects may correlate with their positive inotropic activity.

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