Contraction Dependency of the Positive Inotropic Action of Cardiac Glycosides

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ABSTRACT

The concept that the positive inotropic effect of cardiac glycosides is dependent on the contractile state of the myocardium was tested. Isolated left atria of rabbits, when driven electrically at 15, 30, 60 and 120/min (at 30, 33, or 37°C), responded to ouabain (1 μg/ml) in proportion to the number of contractions and not to the time of exposure or frequency of drive. Contraction-related effects were proportional to concentration of ouabain, acetylstrophanthidin, and digoxin. A contraction-related effect of ouabain was observed in anesthetized dogs with surgical A-V block, ventricular electrical drive and right ventricular contractile force recording. Quiescent rabbit atria exposed to ouabain for 30 min at 30°C and then washed in ouabain-free medium, showed minimal positive inotropic effect when stimulation was resumed. Toxic concentration of ouabain (5 μg/ml) in contact with quiescent atria for 30 min and then washed out, on resumption of stimulation had an immediate positive inotropic (but no toxic) effect which diminished progressively, in contrast to the rapid development of toxic effects (ectopic contractions and contracture) in atria exposed to this dose of ouabain during repetitive contractions. It is concluded that a major part of the positive inotropic effect of cardiac glycosides is contraction dependent.

ADDITIONAL KEY WORDS ouabain acetylstrophanthidin digoxin frequency-force relationships rabbit atria dog heart

The major contemporary theories of the mechanism of the positive inotropic effect of cardiac glycosides designate the site of action at one of three places, the cell membrane, the contractile proteins, or the excitation-contraction coupling system (e.g., sarcotubular system). Although not necessarily expressed, there is in each theory the implied assumption that the reaction between the glycoside and the cellular receptor is a time-dependent one in which conventional kinetic expressions might apply. It is the purpose of this paper to present experimental evidence that the positive inotropic action may, to a large extent, be contraction dependent and not time dependent.

Weizsäcker (1, 2) in 1913 related the number of contractions of the frog heart to the action of K-strophanthoside. Since then, a number of investigators have alluded to a contraction dependency in the action of glycosides. Wilbrandt et al. (3) found the positive inotropic effect of K-strophanthoside to be proportional to the number of contractions of the electrically driven frog heart. Lévi (4) found no demonstrable effect of cardiac glycosides on isolated frog hearts provided the drug was restricted to quiescent hearts. In 1958 Sanyal and Saunders (5) postulated that the effect of ouabain on guinea pig ventricle strips occurs “only in contracting myocardium…”

I (6) have previously described experiments that demonstrate a direct correlation between the positive inotropic effect of ouabain on isolated rabbit atria and the number of contractions from the time of exposure to the glycoside. I also showed that exposure of a quiescent atrium to a low concentration...
of ouabain gave little detectable effect if the drug was removed from the bath before initiating contractions. This paper presents more detailed experiments, the results of which support the hypothesis that the positive inotropic action of cardiac glycosides is largely a contraction-dependent one.

**Methods**

**IN VITRO EXPERIMENTS**

The left atria of young albino rabbits were removed and placed in a 100-ml bath containing a bicarbonate-buffered medium of the following composition: NaCl, 120 mM; KCl, 5.6 mM; CaCl$_2$, 2.2 mM; MgCl$_2$, 2.1 mM; NaHCO$_3$, 25 mM; dextrose, 10 mM (7). The temperature was maintained at 30 ±0.1°C in most experiments. In two series, the temperature of the bath was adjusted to 33°C in one and 37°C in the other. The atria were stimulated at supramaximal voltages through bipolar stainless steel or platinum electrodes with a Grass square-wave stimulator. Isometric contractile force was recorded on a Grass Polygraph via a Grass FT-03 force-displacement transducer. A resting tension of 1 g was maintained throughout each experiment. The atria were driven at a frequency of 60 or 120/min for an initial period of 30 to 45 min for equilibration.

Two main types of experiments were performed on isolated atria. In one, each atrium was stimulated continuously at one of four frequencies (15, 30, 60, or 120/min). When the lower frequencies were used, an additional period of 10 to 15 min of equilibration at the given frequency was allowed. A cardiac glycoside was then added to the bath. Changes in contractile force in response to the glycoside were measured from each record and were related to the time and the number of contractions after the addition of the glycoside. The number of atria used is given in the legends to Figures 1, 2, and 3.

The second type of experiment on rabbit atria was designed to demonstrate the requirement of contractile activity for the positive inotropic effect and to assess the contraction dependency of the binding of the glycoside indirectly. Binding is here defined solely in terms of the ease with which a physiological effect is reversed or arrested by exchanging the bathing fluid for fresh, glycoside-free fluid.

For these experiments, in which the bath fluid was changed occasionally or frequently in the course of several hours, a more consistent test of glycoside action than the increase in contractile force was found in the alteration of the pattern of change of contractile force induced by abrupt reduction of the frequency of stimulation from 120 to 12/min for a period of approximately 2 min. This change in frequency—termed the frequency-reduction test and identical to the decay of the positive inotropic effect of activation as defined by Blinks and Koch-Weser (8)—resulted in an initial augmented contraction with a subsequent characteristic decay to a new, low level of contractile force. Details of the analysis of the decay pattern are described in Results. Periods of quiescence of varying lengths were obtained by turning the stimulator off. Details of the use of quiescent periods are also described in Results. The numbers of atria used in experiments of this type are given in legends of Figures 6 through 9.

The glycosides were dissolved in ethanol (10% for ouabain and 50% for the others, except for the analysis of dose-response relationships when ouabain was also dissolved in 50% ethanol). Ouabain, acetylsyringothidin, and digoxin were used.

**IN VIVO EXPERIMENTS**

Dogs were anesthetized with a pentobarbital-barbital mixture (15 and 220 mg/kg, respectively, iv). Bilateral vagotomy and midsternal thoracotomy were performed. Right ventricular cardiac contractile force was recorded on a Grass Polygraph with a strain-gauge arch, and femoral arterial blood pressure was recorded with a Statham pressure transducer. Atrioventricular block was produced by crushing the bundle of His with an encircling ligature during temporary arrest of venous inflow. The heart was then driven at a constant rate with a stimulator through a small, bipolar platinum electrode sutured to the epicardial surface of the right ventricle. After contractile force and blood pressure had become stable, ouabain was injected intravenously at a constant rate (56 μg/kg over a 2-min period). Changes of contractile force were measured and expressed as a percent of maximum change. The results from 18 dogs were suitable for analysis—5 each at frequencies of 90, 150, and 240/min and 3 at 54/min.

**Results**

**CONTRACTION DEPENDENCY OF POSITIVE INOTROPIC EFFECT OF CARDIAC GLYCOSIDES**

A direct relationship between the development of the positive inotropic effect of ouabain and the number of contractions of the heart muscle was observed both in vitro and in vivo. Figure 1 demonstrates the influence of frequency of stimulation of isolated rabbit left atria on the effect of a standard dose of ouabain on force of contraction at 30°C. Transformation of the abscessas from

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The positive inotropic effect of ouabain on isolated, electrically driven left atria of rabbits as a function of time and of number of contractions at 30°C. Ouabain, 1 µg/ml final concentration, was added at zero time. Upper graphs depict isometric contractile force in arbitrary units; lower graphs, effect as percent of maximum change. Graphs on left show time course of effect and graphs on right show same data as a function of number of contractions after addition of ouabain. Each curve represents mean data from five atria.

The change in contractile force of each experiment (as percent of maximal change) was plotted on probit-log paper as a function of the number of contractions. The number of contractions that ensued from the time of administration of ouabain to the point of 50% of maximal effect was estimated graphically for each of the four frequencies of stimulation and is designated as the C-50. The results of the experiments described above (30°C) and those of two additional series conducted at 33°C and 37°C are shown.

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Positive inotropic action of ouabain as a function of the frequency of stimulation in isolated left atria of rabbits. Ordinate = the number of contractions required to reach 50% of maximum effect at frequencies of stimulation shown on abscissa. Each point is a mean (± se) of data from five atria each at 30°, 33°, and at 37°C. The points for 30° were taken from the data in Figure 1.

Contraction-dependent positive inotropic effect of ouabain, digoxin and acetylstrophanthidin as a function of concentration. Isolated, left atria of rabbits (30°C) stimulated at 120/min. Ordinate = number of contractions from addition of drug to 50% of maximum increase in contractile force. Abscissa = micromolar concentration of drugs. Each point is mean (± se) of data from at least three atria, except for ouabain, 5 μM, which is based on one experiment.

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Contraction-dependent positive inotropic effect of ouabain in dogs. Right ventricular contractile force in anesthetized, vagotomized, open-chest dogs with surgical A-V block. Rates were established by stimulation through electrodes on right ventricle. Ouabain, 56 μg/kg, injected intravenously over 2-min period. Effective zero time was determined by extrapolation of each response curve back to time axis. Results shown as percent of maximum change on a time base (left) and cumulative-contractions base (right). Each curve represents mean results in 3 to 5 dogs.
ventricular rate was controlled by electrical stimulation. The relationship between contractile force and number of contractions was similar to, but less distinct than, that in rabbit atria (Fig. 4).

"BINDING" OF OUABAIN

Experiments were designed to assess the relationship of contractions and binding of ouabain. "Binding" is used here to mean slow reversibility (persistence) of the effect of ouabain in vitro in spite of thorough washing of the muscle with ouabain-free medium.

For these experiments, we used the decay in contractile force that occurs when the frequency of stimulation is abruptly reduced. Figure 5A shows a typical experiment in which the left atrium of a rabbit was driven at 120/min. Abrupt reduction of stimulus frequency to 12/min produced an initial augmentation of contraction followed by rapid decay toward a low steady state. Resumption of rapid stimulation restored the previous contractile force. The decay pattern of this "frequency-reduction" test is highly consistent for hours, even when the level of contractile force declines. Furthermore, ouabain and

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other glycosides alter this frequency-reduction response in a characteristic pattern as shown in Figure 5A.

The time course of action of ouabain can be depicted by plotting the slope of the decay of force in the frequency-reduction test as a function of time. Figure 5B shows the method of analysis in which the first slow-frequency contraction is plotted as 100% and each succeeding slow frequency contraction as a percent of the first. For normal heart muscle, a pattern shown in curve 1 of Figure 5B is observed. Ouabain alters the shape of the curve in a progressive way, as shown from curve 1 to curve 5 in Figure 5B. An empirical relationship was found between the slope of the steepest descending part of each curve and the time after administration of ouabain, as seen in Figure 5C. This relationship was found to be consistent from one experiment to another and served as a reliable expression of the time course of the development of this effect of ouabain.

Figures 6 and 7 show two uses of this relationship. Figure 6 depicts a series of experiments in which ouabain was added to the bath and then washed out at various times. It was predicted that the reaction would be arrested by washing with fresh fluid if the unreacted ouabain was unbound. Figure 6 shows that the reaction, in fact, was arrested and also shows the beginning of slow recovery. This experiment can be interpreted to mean that each contraction

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**Figure 6**

Arrest of effect of ouabain at various stages by washout. Curve of solid circles represents mean results of data from 9 to 23 atria using change of slope of frequency-reduction test as an index of ouabain effect. Each curve of open circles represents mean of data from three atria in which ouabain was quickly washed out of bath at arrows—at 1, 8, 15, 21, and 36 min. Basic driving frequency of isolated atria, 120/min, with frequency reduction tests at 12/min. Ouabain, 1 μg/ml final concentration. Temperature, 30°C.

**Figure 7**

Comparison of the effects of ouabain on isolated rabbit atria with and without pre-exposure during quiescent period. Each curve represents mean responses (± SE) of six atria as time course of change of slope of frequency-reduction test. Atria (30°C) were driven at 120/min with transient reductions to 12/min. The stimulator was turned off for 20 min. The pre-exposure to the quiescent atria did not increase the effect of ouabain.
Relative lack of effect of ouabain on an isolated left atrium of a rabbit (30°C) when exposure was restricted to noncontracting tissue. Each point represents slope of brief frequency-reduction test. Ouabain was first added to quiescent atrium and washed out 30 min later before resuming stimulation. Note slight reduction in slope. Ouabain was then added to contracting atrium with rapid decrease in slope. Four other experiments showed similar results.

Facilitated the binding of some of the free ouabain to a specific (receptor) site. The unreacted ouabain that remained free in the bath at any given time was readily removed by exchange of fluid. The greater the number of contractions, the greater the binding and the effect of ouabain.

Figure 7 shows data allowing a similar conclusion. A 15-min quiescent period was used in two groups of atria (stimulator turned off with no evidence of contractile activity). In one group, ouabain was added to the bath at the beginning of the quiescent period; in the other, the glycoside was added at the time electrical stimulation was resumed. The mean curves showing the development of effect, as expressed by decrease in slope of frequency reduction test, were no different. If ouabain can bind to, and react with, the "glycoside receptor" independently of the state of muscle contraction, one would expect the exposure to ouabain during quiescence to have produced a greater effect than in the other instance. Instead, these data suggest that reaction occurs only when the muscle is contracting.

Figures 8 and 9 demonstrate two additional types of experiments in which long quiescent periods are used. Figure 8 shows the results of one experiment in which ouabain was added to the bath for 30 min, during which the muscle was quiescent. The bath fluid was then exchanged six times, and the stimulator was then turned on. There was a slight decrease in the slope of the frequency-reduction test. In contrast, exposure of the contracting atrium to ouabain produced a characteristic and marked effect. This figure shows one of five experiments, all of which gave comparable results.

Figure 9 shows the effect of a "toxic" concentration of ouabain in a typical experiment. Two left atria were used simultaneously in two baths. In one (upper tracing), ouabain was added to a quiescent atrium for 30 min and then washed out. On resumption of stimulation, a marked effect of ouabain was noted within 3 min, but the effect gradually dimin-

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No drug

Ouabain 5.0 μg/ml for 30 minutes

Wash x 6

Stim on

Stim off

Stim on + 3 min

+ 10 min

+ 17 min

+ 73 min

FIGURE 9

Effect of toxic concentration of ouabain on isolated atria when exposure was restricted to non-contracting tissue. Two atria (30°C) in separate baths were simultaneously driven at 120/min with 12/min frequency reductions. Atrium shown in upper tracings was exposed to ouabain during a period of no stimulation. Ouabain was washed out before stimulation was resumed. Atrium shown in lower tracings was exposed to ouabain simultaneously with resumption of stimulation after a 40-min period of no stimulation. Note rapid development of toxic effect with contracture in lower atrium compared with nontoxic effect on upper atrium. Note partial reversal of effect in upper tracing. Two other experiments using two atria each showed similar results.

Discussion

These results are compatible with the hypothesis that the positive inotropic effect of cardiac glycosides in mammalian heart muscle is primarily a contraction-dependent one in which a slowly dissociable reaction between the glycoside and myocardial receptor occurs with each contraction, the result of which is the augmentation of contractile force in proportion to the number of contractions. Since my first report (6), several other workers have brought forward additional supporting data. Holland (9) described an experiment in which the time required to reach a maximum positive inotropic effect was inversely related to the stimulus frequency, but the number of contractions between the administration of ouabain and the maximum increase in contractile force was approximately the same at all stimulus frequencies. He also showed that exposure of quiescent myocardial tissue to ouabain for 1 hour produced no greater effect when stimulation was resumed than did exposure to ouabain at the end of a 1-hour rest period. Similarly, Lock (10) has found that the positive inotropic effect of ouabain on an isolated, electrically stimulated auricle from a hen was no greater if the auricle had been exposed to ouabain for a 10-min quiescent period before stimulation than if ouabain was added directly to the contracting muscle.
Furthermore, Lock showed that ouabain exposed to a quiescent auricle for 10 min produced no effect if washed out prior to resumption of stimulation, an experiment similar in concept to the one shown in Figure 9. Frommer et al. (11) observed that prolonged, paired stimulation of dog heart in vivo during infusion of ouabain resulted in premature ventricular fibrillation and suggested that the greater number of depolarizations during paired stimulation may result in greater entry of the glycoside into the myocardium than would occur with unpaired stimulation.

In contrast, Vincenzi (12) recently published experiments which in part do not agree with my results. He used isolated left atria of guinea pigs driven at frequencies varying from 0.2 to 190/min at a temperature of 35°C. He concluded that the rate of onset of the positive inotropic action is nearly independent of myocardial activity, except at beat intervals less than 3 sec (frequencies greater than 20/min). Vincenzi states that the beat dependency that I and others have found may be due to the lower temperature at which I worked (30° compared to his 35°) and not to a difference in species. However, my experiments on rabbit atria at 33° and 37° and on the intact dog maintained at a temperature close to 37° (Fig. 4) suggest that this is not the case. The clearly different results (mine versus Vincenzi’s) indicate the need for further experiments at wider ranges of frequencies and temperatures. We are currently evaluating the influence of frequency and temperature on the effects of ouabain on contractile activity in both rabbit and guinea pig atria.

Two recent reports have dealt with the relationship between contractile activity of heart muscle and uptake of 3H-digoxin. Okita et al. (13) claim that guinea pig atria exposed to the labeled glycoside for 30 min while contracting took up no more label than did quiescent atria exposed to digoxin for the same time. They also report that the half-time for disappearance of the labeled drug was 2.5 times shorter than that for disappearance of the positive inotropic effect following removal of the drug from the bath. Kuschinsky et al. (14) found that contracting guinea pig atria took up 3H-digoxin more rapidly than did quiescent atria, but after 3 hours the maximum uptake by both types of atria did not differ. Also the rate of initial uptake was the same for atria driven at 30/min as for those driven at 180/min. In evaluating these results, one should consider the fact that the possibility of demonstrating contraction-related binding of cardiac glycosides by heart muscle is very small when conventional techniques of measuring uptake of radioactively labeled drugs are used. Because the number of “active” sites to which a glycoside could bind is probably very small relative to the number of “inactive” or “nonspecific” sites which also bind the drug, large differences in binding to the active or receptor sites would probably be undetectable against the background of unaltered binding to the nonspecific sites. The more rapid uptake Kuschinsky et al. found in contracting atria might be related to the availability of more receptors because of the contractile activity, but also might be simply a result of augmented diffusion to all binding sites due to the mechanical activity. Far more refined techniques and more ingenious experimental designs are needed to identify binding of a drug to specific receptor sites in most tissues.

The concept of a contraction-dependent reaction does not require specification of the site or mechanism of action of the glycoside. In fact, it is compatible with several possible modes of action. However, two major factors must be considered: (1) the mode of reaction of the glycoside with the receptor and (2) the accessibility of the glycoside to the receptor site. The reaction between a drug and receptors is usually expressed as a form of a mass action; the major determinants of the reaction are the concentration of the drug and the association and dissociation constants. It is implied that the receptor is always "receptive." It should be considered, however, that sometimes the receptiveness of the receptor varies with the functional state of the cell, perhaps because of changes in the
conformation of the receptor. Thus it is possible that the receptor for glycosides in the myocardium undergoes cyclic changes that augment the interaction between drug and receptor. Such cyclic changes might be associated with changes in certain parts of the cell, such as depolarization of the membrane, molecular interaction of the contractile proteins, or functional alteration of the excitation-contraction coupling system (sarcotubular system), any one of which may be the location of the receptors. Also to be considered is the second factor, namely accessibility to the receptor. Thus, the contraction-dependent action we have observed may be due, not to phasic drug-receptor interactions based on active site receptiveness, but to phasic alterations in accessibility of the drug to an active receptor site. For example, if the cardiac glycosides act upon an internal structure but the cell membrane is permeable to them only during brief periods (e.g., during depolarization of the membrane), the drugs would be delivered to the receptor in surges with each heart beat, and the effect would be "beat" dependent. Our present data do not permit differentiation between the possibilities of phasic changes in receptor accessibility and receptiveness.

Interrelationships of cardiac glycosides and calcium have been noted for many years and are discussed critically by several authors (15-17). In recent years a number of investigators have found that cardiac glycosides increase the exchange of calcium in heart muscle (18-21). Also, several workers have reported a direct relationship between the frequency of heart contractions and the exchange of calcium (22-24). Govier and Holland (25) showed that the calcium exchange in isolated rabbit atria is related to the number of beats during the period of exposure to the radioactive calcium and that the augmented exchange produced by ouabain is related in magnitude to the number of atrial contractions. Grossman and Furchgott (20) and Govier and Holland (25) found that calcium exchange was not affected by ouabain in quiescent heart muscle. On this basis, they have postulated the existence of a pool of tissue calcium that becomes exchangeable during the contractile response and have suggested that the positive inotropic action of the cardiac glycosides correlates with increased influx of calcium. Although Grossman and Furchgott are cautious in the interpretation of their data, Govier and Holland suggest that ouabain "has made more calcium available for contraction, perhaps by increasing the magnitude of the contraction-dependent pool" instead of inducing an increase in contractile force that secondarily increases exchange of calcium. Langer (24) has described the kinetics of a pool or system of calcium that is readily exchangeable, is related to heart frequency, and is closely related to maintenance of contractile tension. Although Langer did not study the effect of a cardiac glycoside, the system he described would appear to be similar to the pools described by Govier and Holland and Grossman and Furchgott.

Current concepts concerning excitation-contraction coupling and the role of calcium in this system (26, 27) are compatible with the hypothesis of contraction-dependent action of ouabain but do not help in specifying a site or mechanism of action. There is no doubt that calcium-binding vesicles can be obtained from heart muscle, presumably as fragments of the sarcotubular system (28-31). Several investigators have reported effects of ouabain on the binding of calcium by cardiac sarcotubular vesicles. Lee et al. (32), Lee and Choi (33), and Carsten (34) found inhibition of uptake of calcium by the vesicles. In contrast, Klaus and Lee (35) found a ouabain-induced release of calcium but no inhibition of uptake. They also found that the physiologically less potent dihydro-ouabain was less potent than ouabain in releasing calcium from the vesicles. Carsten (34), however, demonstrated inhibition only in aged vesicles in which the uptake of calcium was less than that of fresh vesicles. Carsten also found that ouabain antagonized the vesicle-produced inhibition of myofibrillar ATPase of skeletal muscle, but again only after the inhibitory
activity of the granules declined after aging for several days. Whether these effects can be definitely related to the positive inotropic effect is still doubtful. While an increased intracellular concentration of free calcium is compatible with increased contractile activity, achievement of the elevated calcium by inhibition of calcium uptake would tend to prolong relaxation, an effect not observed with cardiac glycosides. However, ouabain-induced release with no impairment of uptake of calcium would be more compatible with the known physiological action of ouabain in therapeutic concentration, that is, augmented contractile tension, increased speed of contraction, and increased rate of relaxation. If these demonstrations of ouabain action on the sarcotubular system are valid for the intact myocardium, it is not clear how the action relates to the contraction-dependent aspect of the positive inotropic action of ouabain. This action on sarcotubular vesicles from heart muscle should be tested for specificity to see if equivalent action is obtained on vesicles from skeletal muscle, a muscle which does not respond to ouabain with a sustained increased contractile force. However, more definitive evidence is needed before assigning a major site of action of cardiac glycosides on the calcium exchange system of sarcoplasmic reticulum.

The small effect that occurs when quiescent atria are exposed to low concentrations of ouabain and then washed (Fig. 8) and the greater effect of toxic concentrations (Fig. 9) can be explained in two ways: (1) There may be a minor part of the ouabain effect which is not dependent on contractile activity (as Vincenzi contends). (2) There is some permeation of glycoside molecules during the exposure to quiescent muscle into sites from which the glycoside is not rapidly removed during washout. That is, molecules of drug may become sequestered (although remaining inactive) during the exposure and, on resumption of contractile activity, exert their effect. Sequestration of glycoside molecules and removal into the bath fluid with dilution would explain the reversal of the effect of ouabain seen in Figure 9. One possible site of sequestration is the transverse tubular system (invaginations of the sarcolemma).

Conclusion

The positive inotropic action of cardiac glycosides on heart muscle depends primarily on contractile activity of the tissue. The glycosides have little effect (except in toxic doses) when they are added to quiescent heart muscle and then removed before the tissue is induced to contract. The effect of the glycoside on the tissue in vitro can be arrested, but not immediately reversed, by washing with glycoside-free medium. Recovery from the effect is slow and appears not to be contraction dependent. These results suggest that the binding of cardiac glycosides to myocardial receptors and the positive inotropic action are determined by the number of contractions and not by the time of exposure. These experiments do not specify a site of action.

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