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THE INFLUENCE OF CALCIUM AND STRONTIUM ON THE CARDIOTROPIC ACTIONS OF OUABAIN AND RHODOCHLORIN

by

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INTRODUCTION

In 1785, William Withering, a physician and botanist of Birmingham, England, published his famous book entitled, "An Account of the Foxglove and Some of Its Medical Uses: with Practical Remarks on Dropsy and Other Diseases" (Withering, reprinted in 1937). Withering gave an interesting account of how he learned from an old woman in Shropshire the use of digitalis in treating certain forms of dropsy. He recognized its potent cardiotonic action, for he wrote that, "It has a power over the motion of the heart to a degree yet unobserved in any other medicine, and this power may be converted to salutary ends". However, he apparently thought that the diuresis produced by digitalis was its primary pharmacological action. In 1799, John Ferriar was the first to recognize that the primary action of digitalis is on the heart and the diuretic effect is only of secondary importance. Today there is certainly no doubt of the importance of these two therapeutic effects of the cardiac glycosides in the treatment of congestive heart failure.

Although 180 years have lapsed since Withering first described digitalis, its mechanism of action on the myocardium is still far from clearly understood. Great strides have been made during the last 50 years to elucidate the chemistry and the pharmacological properties of the cardiac glycosides. Perhaps the works of Cushny (1925), Jacobs (1933), Elderfield (1935), Chen et al. (1938, 1943, 1954), and Stoll (1949) should be cited as among the major contributors

to this fascinating field. Literally hundreds of publications are presented in the literature on digitalis and its allied cardiac glycosides. The methods of investigation are both numerous and imaginative.

The importance of calcium for myocardial contractility has been known since the classical work of Ringer (1883a) and it has been long recognized that the positive inotropic action of the cardiac glycosides on myocardium is augmented by calcium (Weizsacker, 1910). Ringer was apparently also the first to report the cardiotonic properties of strontium (1883b). More recently, the electrical and contractile properties of strontium on the isolated cat papillary muscle (Garb, 1951) and on the isolated toad ventricle (Nayler and Emery, 1957) have been evaluated, and the positive inotropic effect of strontium is confirmed.

From a large number of chlorophyll derivatives extracted and purified by Hendrickson et al. (1957), rhodochlorin was demonstrated to be the most effective cardiac stimulant on the hypodynamic isolated frog heart (Berueffy, 1956). The basic chemical structure of the cardiac glycosides consists of a steroid (cyclopentaperhydrophenanthrene), common to heme and the chlorophylls. Because of the differences in chemical structure and the similar cardiotonic action of ouabain and rhodochlorin, their effects on the hypodynamic frog heart provide a fascinating comparative study.

This dissertation is a study of the influence of the

divalent cations, calcium and strontium, on the cardiotropic actions of ouabain and rhodochlorin. With the collaboration of the Department of Anatomy, an electron microscopic study of the frog myocardium was also undertaken, and special attention was given to the influence of calcium and ouabain on its ultrastructure.

Literature Review

A. Cardiotropic actions of calcium and strontium

1. Role of calcium in cardiac excitation-contraction coupling

In 1883, Ringer demonstrated that the frog heart ceases to contract and remains relaxed when calcium ions are absent from its perfusion fluid. Later, it was shown that in the absence of calcium when no contractions are recordable, the rhythmic spontaneous action potentials of frog heart are still present in an only slightly modified form (Locke and Rosenheim, 1907; and Mines, 1913). At the turn of the century, it was known from studies on skeletal muscle that depolarization of the fiber membrane is the electrical event responsible for initiation of the mechanical response (Biedermann, 1896). In retrospect, the above results obviously suggest that the action potential or depolarization of the muscle fiber surface permits or facilitates the entrance of calcium ions into the fiber and these calcium ions then initiate the mechanical response. However, Sandow (1952) was apparently the first to formulate a working hypothesis involving this mechanism, and he coined the term "excitation-contraction coupling" to describe this process. Considerable evidence to support this hypothesis has accumulated in the past decade. A number of outstanding investigations on this topic have been carried out on skeletal muscle, and it is necessary to point them out during this discussion.

Frank (1964) has posed the following questions which he feels must be answered in the affirmative before there could be a complete acceptance of Sandow's hypothesis. "(1) Does the introduction of calcium ions into the muscle fiber initiate a mechanical response? (2) Do calcium ions enter the fiber in an amount sufficient to initiate a mechanical response during an action potential or depolarization? (3) Do the calcium ions which enter the fiber have sufficient time to reach their intracellular site of action? (4) Can the link between the electrical and mechanical events in muscle contraction be severed by removing calcium from the surface of the muscle of fibers"? For a number of these questions there are now substantial answers.

(1) Does the introduction of calcium ions into the muscle fiber initiate a mechanical response? For skeletal muscles this question has been answered in the affirmative. In 1947, Heilbrunn and Wiercinski demonstrated that calcium was the only physiologically occurring cation which caused shortening when injected in concentrations of 1.25 mM into bits of frog adductor magnus muscle fibers. Niedergerke (1955) confirmed this finding under more physiologic conditions, where KCl, $MgCl_2$ and $CaCl_2$ were used in combination; when $CaCl_2$ was omitted no shortening of the fibers was observed with the interference microscope. It is well known that the myocardium is much more sensitive to extracellular calcium ions than skeletal muscles. Therefore, it is not unreasonable to expect that similar results would be

obtained by the introduction of calcium ions into a cardiac fiber.

(2) Do calcium ions enter the fiber in amount sufficient to initiate a mechanical response during an action potential or depolarization? It has been shown that there is an increased influx of calcium ions during mechanical responses in cardiac muscle (Winegrad, 1961; Winegrad and Shanes, 1962; Niedergerke, 1963a, 1963b; and Grossman and Furchgott, 1964a, 1964b) and in skeletal muscle (Bianchi and Shanes, 1959 and Shanes, 1961). Furthermore, this increased influx is roughly proportional to the strength of contraction. These are highly suggestive, but not direct proof, that the calcium influx as measured by the loss of Ca^{45} from the perfusion solution is sufficient to initiate a mechanical response, because it is not known how these ions initiate and maintain a contraction. A more direct proof, but by necessity less physiologic, is provided by Weber and Winicur (1961). They demonstrated in myosin and actin preparation that calcium (0.06 mg) is necessary for superprecipitation of 80% (2.0 mg/2.5 mg) of the actomyosin system in the presence of ATPase. They considered superprecipitation and high ATPase activity to be comparable to contraction. This represents a calcium to actomyosin ratio of 6/200, an estimation that would be compatible with Ca^{45} flux studies in intact cardiac tissues (see discussion below).

When one attempts to associate the increment of calcium influx with an increase in the force of contraction, there are a number of pertinent and related factors worthy of

consideration. These are (1) the source of this calcium; (2) the mechanism by which it enters the cell; (3) the time in the cardiac cycle during which the added calcium enters the cell; and (4) the possible mode of action inside the cell.

Niedergerke and Harris (1957) have demonstrated that the changes in extracellular sodium and potassium concentration associated with increased twitch tension were accompanied by increased uptake of Ca^{45} by the resting frog ventricular strip and by the tissue in potassium-contracture (Niedergerke, 1959). These studies dealt primarily with rapid changes in calcium transfer at the "cell surface" rather than in the "cell interior" and were based on a difference in time constant of calcium transfer and the rate of tension development. Similarly, a superficially bound calcium which is rapidly exchangeable has been postulated in guinea pig atrium (Grossman and Furchgott, 1964a). An additional pertinent observation is provided by Weidmann (1959) who demonstrated in turtle ventricle during a single heart beat that a sudden increase in the concentration of calcium in the extracellular fluid during the initial stages of a twitch produced a more rapid rate of tension development and a greater peak tension than occurred at a lower calcium concentration. A hypothesis that incorporates these data and correlates the rate of calcium influx during a contraction and the size of the contraction would be the following: the calcium that enters the cell with contraction comes from superficial sites and from the extracellular fluid;

the calcium that enters the cell from the extracellular fluid during depolarization passes through the same superficial sites to which calcium was bound in the resting state.

Weidmann's data also suggest that the increment in calcium influx occurs during the depolarization phase. In support of this hypothesis are the observations that the increased heart rate is associated with a shorter diastole, a shorter action potential but an increased calcium influx per beat, and an increased rate of rise of tension during the contraction (Trautwein, 1963). Furthermore, chelation of calcium by EDTA-Na_2 completely eliminates the overshoot of the action potential in frog ventricular strips (Bennett and Wong, unpublished data). Since the depolarization depends mainly upon sodium entrance into the cell, the latter finding is perhaps the strongest support that calcium enters the cell at the same time as sodium during depolarization and that calcium is important to the overshoot.

The possible mode of action of calcium inside the cell is a more perplexing question, and a quantitative consideration of molecular data here should prove interesting. If the molecular weight of myosin is about 500,000 (Davis et al., 1960), and its concentration in heart muscle is equal to that in skeletal muscle, taken to be 7.6 percent of wet weight (Huxley and Hanson, 1960), then each gram of heart contains 1.5×10^{-4} mM of this protein. The maximal

increment in calcium influx measured by Wingrad (1961) was 0.6×10^{-6} mM/Gm of guinea pig atrium. The ratio of the number of calcium ions entering the cell during contraction to the number of myosin molecules is about 1/250. Since each actomyosin unit contains one myosin molecule (Huxley, 1961), the ratio of calcium ions to actomyosin complexes would be 1/250. It is unlikely that only 0.4 per cent of the contractile protein is activated during the strongest contractions of the isolated atrium. Therefore, if calcium does initiate the contraction, each ion must ultimately have an effect on many actin-myosin units. One of the reactions in the contractile process, though not necessarily the one directly involving calcium, must involve either a chain reaction or the interaction of one molecule or ion with as many as 250 molecules or units. The latter could possibly occur by an enzymatic reaction or by a specific type of molecular alignment in which one molecule is in close association with many contractile units. Anyone of these systems could involve an electron-transport mechanism.

To sum up the answer to Frank's second question concerning excitation-contraction coupling: considerable evidence supports the hypothesis that the calcium entering the fiber during depolarization is sufficient to initiate a mechanical response and that the tension developed is proportional to the degree of calcium influx. Evidence has also been cited to show that membrane calcium is mobilized during each contraction (Neidergerke, 1963a, 1963b). Hence, the absolute concentration of calcium

reaching the contractile site during a twitch cannot be ascertained. Although autoradiographic studies with Ca^{45} have been carried out on skeletal muscle (Costantin and Podolsky, 1965) in an attempt to show the site of intracellular accumulation of Ca^{45} applied to the muscle fiber, their data could only be discussed in qualitative terms.

(3) Do the calcium ions entering the fiber have sufficient time to reach their intracellular site of action? Based upon consideration of the kinetics of diffusion and the time-distance relationship between excitation and activation in frog sartorius muscle, Hill (1929, 1948) found that no reasonable rate of diffusion could account for the known speed of contraction and therefore some other mechanism must be responsible. But as Hill himself pointed out, his calculation was based upon the diffusion coefficient of calcium ions moving through water. Although the rate of diffusion through tissue may be quite different, it can hardly be expected to be faster. Furthermore, the equation is based upon a perfect cylinder, which is usally not the case in living tissues. In slowly contracting myocardium with minute myofiber caliber such as in Rana pipiens, diffusion is quite adequate to account for the coupling time between excitation and contraction; but for fast contracting skeletal muscles, there is strong evidence indicating that the sarcoplasmic reticulum provides a special pathway for the coupling of excitation and contraction.

(4) Can the link between the electrial and mechanical

events in muscle contraction be severed by removing calcium from the surface of the muscle fiber? As mentioned above, this was the first evidence which indicated that calcium may act as the link between the action potential and contraction in cardiac muscle. More recently, Niedergerke (1956) has demonstrated that the potassium-induced contracture of cardiac muscle will be prevented by exposing the muscle to a calcium-free solution.

Thus based upon the criteria set forth by Frank, and the evidence provided above, there is little doubt that calcium is the physiological link between excitation and contraction.

In order to make the discussion complete, it should be pointed out that there are differences in excitation-contraction coupling between cardiac and skeletal muscles. Cardiac muscle is much more sensitive to extracellular calcium concentration than skeletal muscle. Luttgau and Niedergerke (1958) have postulated that the strength of contraction in cardiac muscle is quantitatively related to the ratio $[Ca]/[Na]^2$ in the extracellular fluid. This inhibitory action of sodium is easily demonstrated in cardiac muscle, but not in skeletal muscle. Caffeine can induce a contracture in skeletal muscle independent of extracellular calcium ions (Frank, 1962), and this effect has been explained by the release of intracellular calcium by caffeine. In cardiac muscle, low concentrations of caffeine produce a positive inotropic effect, but higher

concentrations completely inhibit the mechanical response (Suzuki, 1962). Caffeine does not induce contracture in cardiac muscle exposed to Ringer's solution, but can induce contracture of frog ventricular strips if the sodium in the bathing solution has been replaced by KCl, choline, chloride, or nonelectrolytes. Although the phenomenon of contracture is distinguished from that of normal contraction on the basis of the mode of excitation, both processes involve actual shortening of the contractile protein. Therefore, data obtained in the study of contracture have been considered to provide valuable evidence concerning contractile mechanisms in general.

2. Role of calcium in myocardial metabolism

It was postulated in the previous section that each calcium ion activated during a depolarization may be involved in an enzymatic reaction intracellularly to elicit a contraction from many actomyosin units; or each calcium ion may, by a specific type of molecular arrangement, activate many actomyosin units. It was also estimated that one calcium ion is capable of activating as many as 250 actomyosin units. A molecular arrangement of 250 actomyosin units around one calcium ion is indeed difficult to conceive. Electron microscopic studies have demonstrated that myocardium is not an anatomical syncytium (Moore and Ruska, 1957; Fawcett and Selby, 1958), but the myofibrils are arranged through intercalated discs and desmosomes. Therefore, it would be difficult to explain the contractile phenomenon solely on the basis of a mechanical chain reaction initiated

by the activation of a few actomyosin units. Most investigators favor an enzymatic role of calcium in myocardial contractility. Hence the discussion here on myocardial metabolism will necessitate the use of many biochemical data.

When one attempts to correlate *in vitro* biochemical data with *in vivo* experiments, the following factors should always be carefully considered: (1) *in vitro* studies are, at best, far from physiologic no matter how carefully one tries to simulate *in vivo* conditions; (2) the anatomical barriers are removed, especially in homogenate and subcellular particulate studies; (3) the removal of the anatomical barriers would naturally lose intracellular metabolic activators or inhibitors; (4) the substrate concentration would, therefore, not necessarily reflect the concentration required in an intact system. These are some of the inherent problems existing in all studies of biological systems in test tubes. Hence there are perhaps more conflicting data and controversies between laboratories in this field than other fields of investigation. Be that as it may, biochemical investigation has made valuable contributions to our knowledge of cellular composition, possible function of organelles and possible sites of drug metabolism. This mode of investigation has certainly become an important part of the overall experimental approach.

It is now generally agreed that the energy for muscle contraction comes either directly or indirectly from the splitting of high energy phosphate bonds. Although there may be other compounds that account for a small percentage

of the total high energy phosphate bonds of cardiac muscle, it is postulated that 90 percent or more of such bonds occur in adenosine triphosphate (ATP) and creatine phosphate (CP) (Wollenberger, 1958; and Wollenberger et al., 1960). It is no surprise then that much effort has been directed toward the study of the interaction between calcium and the ATP-ATPase system.

The splitting of high energy phosphate bonds from the ATP molecule requires the enzyme ATPase. Ebashi (1961) and Weber and Winicur (1961) have demonstrated that superprecipitation and ATPase activity of natural and reconstituted actomyosin do in fact depend upon calcium. Furthermore the synaeresis and maximal ATPase activity of myofibrils require calcium under conditions where the particulate relaxing factor (Marsh-Bendall factor) is irreversibly inactivated (Weber and Herz, 1962).

The demonstration of the relaxing factor resulted from the observation of Marsh in 1951 that the precipitation of a suspension of myofibrils was inhibited by a water soluble muscle extract. This led to the work of Bendall (1952, 1953) who showed that the muscle extract used by Marsh also produced a marked lengthening of glycerinated fibers previously shortened under the influence of ATP. Marsh (1952) and Bendall (1953) also demonstrated that the relaxing effect of this extract is strongly inhibited by calcium. This observation has been confirmed and amplified by many other investigators (eg. Ebashi, 1960). A cardiac relaxing

substance has been more recently demonstrated by Honig et al. (1962). Although 2.5×10^{-6}M soluble calcium inhibits skeletal relaxing substance, the latter workers found that up to 10^{-3}M of ionized calcium were without effect on cardiac relaxing substance. It was concluded that the mechanism by which cardiac relaxing substance influences ATP hydrolysis involves more than simple chelation of ionized calcium. Muscatello et al. (1961) have demonstrated that the relaxing substance resides in a subcellular tubular component, the sarcoplasmic reticulum. Scott (1932) showed by microincineration studies that calcium is also intimately associated with these sarcotubules. These findings have generated a great deal of interest in the interrelation of relaxing factor, calcium, and sarcoplasmic reticulum in muscle contraction. A working hypothesis at present is that the calcium that enters the cell during depolarization inhibits the relaxing system and thereby initiates contraction, or that it activates contraction, and relaxation occurs by the removal of the calcium by the relaxing system.

The activation of phosphorylase as a possible mechanism for positive inotropic action is still not widely accepted. Belford and Feinleib (1962) observed that the catecholamines, aminophylline and calcium ions all produced an increase in the glucose-6-phosphate level in the cat ventricle. The authors explained these effects in the same light as was proposed by Sutherland and Rall (1960), i.e.

these agents increase the production of cyclic 3',5'-AMP from ATP; cyclic AMP in turn increases the synthesis of active phosphorylase and this activation results in glycogenolysis and subsequent rise in the glucose-6-phosphate concentration. There are supporters for this "cyclic AMP-phosphorylase" hypothesis (Rall and West, 1963; and Hess et al., 1962). However, Mayer (1963) and Mayer et al. (1963) could find no correlation between active phosphorylase of a dog heart in situ and the increase in contractile force when small doses of epinephrine were employed. In addition, with small doses of either epinephrine or norepinephrine, no increase in the glucose-6-phosphate level was noted in the heart, although an increase in the contractile force was observed.

Although the enzymatic role of calcium in myocardial metabolism has not been clearly established, evidence at present suggests that the following two factors concerning calcium are important: (1) energy is provided by ATP and CP and the enzyme ATPase probably requires calcium as a co-factor for activation. (2) the physiologic relaxing factor may play an important role in modulating the force of contraction by regulating the concentration of calcium at the contractile site.

3. Role of calcium in excitability and automaticity of myocardium

In the previous sections evidence was cited to support the role of calcium in excitation-contraction coupling, and it was suggested that the underlying mechanism of this