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The Current Concept on Mechanism of Positive Inotropic Action of Cardiac Glycosides on the Heart

Kwang Soo Lee

Chung-Ang University, College of Medicine and Columbia University, College of Physicians and Surgeons

INTRODUCTION

Digitalis Glycosides have been in use for over 200 years and still are considered to be the most effective agents for the treatment of cardiac failure. This longevity is due to the fact that very low concentrations of these agents produce a highly specific positive cardiotonic action which attracted attention from both biologists and clinicians. Particularly the illucidation of the mechanism of positive inotropic action appealed to many investigators since it may reveal the cause of cardiac failure as well as the intricate workings of cardiac contraction. Thus, almost all major areas of cellular function in the myocardium have been explored and this has resulted in accumulation of voluminous information on this subjects.

Inspite of all endeavors of investigations in the past, the considerable controversy with regard to the true mechanism of inotropic action has persisted for some time. However, recent advancement in this subject has made it possible to formulate the fairly definitive answer to this problem on a reasonably firm experimental basis. Although there are still some unsolved problems, a broad line of limitation appears to clearly define the area in which the true mechanism of the drug action resides. The broad concensus being formed with respect to the subcellular mechanism of inotropic action of cardiac glycosides emerges mainly through the recent developments in the three fields of cardiac physiology. The first is the clearer understanding of excitation-contraction coupling processes in heart muscle and the localization of sites of action of cardiac glycosides in this process. The second is the identification of Na^+-K^+ activated ATPase as the pharmacological receptor of digitalis. The third is the current advance of our knowledge on the sarcolemmal structure and transmembrane ion movements based on the newly available methodology.

Excitation-contraction coupling processes as the site of action

Broadly speaking, the cardiac contraction may be increased through alterations in three major components of muscular function, namely the contractile machinery, the energy metabolism and the excitation-contraction coupling processes. Thus, effects of cardiac glycosides (CG) on these major areas have been investigated rather extensively. The brief review on these studies is in order.

In early sixties, effects of CG on the contractile machinery, cardiac actomyosin has been eagerly pursued. It was found that the rate of polymerization of cardiac actin was accelerated by ouabain or digitoxin whereas skeletal muscle action was compleltely uninfluenced by even higher concentrations of CG (Harvey & Daniel, 1952) which was confirmed by others (Wollenberger, 1954; Zabor, 1956). However, this effect of CG was unrelated to the cardiotonic effect of CG (Wollenberger, 1954). Also when the cardiac actin is more purified, the effects of CG on actin disappear (Jenny, 1967). With regard to myosin, changes in the physicochemical properties of myosin prepared from animals treated with digitoxin have been observed (Olson *et al.*, 1961). However, several investigations employing myosins of different degrees of purity and actomyosin isolated from normal as well as failing hearts, indicate that various CG in a wide concentration ranges do not influence ATPase activities of these preparations, whether calcium in different amount is present or not (Jenny, 1965; Luci & Kritcher, 1967; Storing *et al.*, 1966). Thus, no convincing evidence of a direct action of CG on myosin has been established. Similarly effect of CG on cardiac actomyosin was investigated by number of workers. Superprecipita-

tion of natural cardiac actomyosin was found to be accelerated by CG (Storing *et al.*, 1966), which was not confirmed in experiments employing more purified or reconstituted actomyosin (Jenny *et al.*, 1967; Katz, 1966). Also most investigators found no significant effect of CG on cardiac actomyosin of different purities (Katz & Repke, 1969; Kay & Green, 1964; Read & Kelsey, 1954; Storing *et al.*, 1966). In addition, the possibility of indirect effects such as CG influence on the Ca sensitivity of the contractile machinery appears to be unlikely (Fabiato & Fabiato, 1973; Nayler, 1973). It is safe to conclude then that CG do not appear to exert a direct influence on the contractile machinery of cardiac muscle.

Since the excellent review of Wollenbeger (1949) on the metabolic action of the cardiac glycosides, the voluminous literature has accumulted on CG effects on the metabolism of normal and failing hearts. Many investigations on the effect on cardiac oxygen consumption show that CG stimulte both the myyocardial oxygen consumption and the contractile force simultaneously at the inotropic stage and the increase in oxygen consumption is the result of the increase in cardiac work induced by the inotropic effect of CG (Clancy *et al.*, 1967; Coleman, 1967; Gousios, 1967; Lee, 1960). Investigations on various cellular components such as mitochondria and sarcoplasmic reticulum (relaxing factor) indicate that CG do not have direct effects on the isolated cellular components and any metabolic effect of CG requires the integrity of cellular structure (Lee, 1965; Lee *et al.*, 1969; Lee & Klaus, 1971). Many isolated enzyme systems participating in anaerobic glycolysis in electron transport through the respiratory chain have been investigated and none of these enzymes tested was found to be definitely influenced by CG (Helmreich, 1950; Reiter & Barron, 1952; Saunders *et al.*, 1950). The above results suggest strongly that the metabolic effects of CG are mediated through some other primary effects of CG which require the presence of intact cellular membrane for manifestation.

Thus the current view points the excitation-contraction coupling processes to be the site where digitalis exerts its influence causing the positive inotropic action. The excition-contraction coupling processes will be summerized briefly. The propagated membrane excitation is followed by the transmembrane action potential and internaly propagated conduction of the membrane excitation through transversely and longitudinally oriented membrane-limited channels in the sarcoplasmic reticulum, reaching the contractile element, actomyosin throughout the muscle cell (Ebashi & Endo, 1968; Huxley & Taylor 19; Sandow, 1965; Simpson & Oerteils, 1962), Although the detailed mechanism for the spread of excitation from the surface membrane into muscle fibers is not clarified, it is generally accepted that the depolarization of the surface membrane releases an activator in muscle, triggering the contraction of actomyosin. The data provided by numerous innvestigators show this activator to be calcium (Allen & Blinks, 1978; Baker *et al.*, 1969; Barry *et al.*, 1981; Fabiato & Fabiato, 1979; Hawath, 1949; Langer, 1968; Weber & Winnicur 1900; Winegrad, 1968; Wood *et al.*, 1969). In cardiac muscle, the activator cacium ion also enters into myocardial cell during the plateau phase of the action potential through slow channels (Beeler & Reuter, 1970a; 1970b). The rise of intracellular calcium concentration through the above processes upon excitation enables the activator to bind troponin, the key protein through which the regulatory action of calcium is mediated to the contractile system. Troponin is bound to actin through tropomyosin and this troponin-tropomyosin system prevents the interaction of myosin and actin in the absence of cacium. Binding of calcium to the calcium receptor protein, troponin releases the preventive effect of these modulatory proteins on the interaction of actin and myosin, thus leading to activation of the contractile system (Ebashi *et al.*, Ebashi & Endo, 1968; Ebashi & Kodama, 1966). Thus calcium is assigned the central role in the excitation-contraction (E-C) coupling processes and the level of intracellular calcium concentration attained during the excitation determines the contractile tension. The transient intracellular Ca^{++} rise during systolic contraction of cardiac muscle has been demonstrated (Allen & Blinks, 1978; Morgan & Blinks, 1982). It is generally believed that activation of mammalian cardiac muscle releases Ca^{++} *from junctional sarcoplasmic reticulum (terminal cisternae)* (Fabiato, 1983; Sommer & Johnson, 1980). However detailed mechanism of Ca^{++} release upon stimultion and return of Ca^{++} during relaxation have many complexities which have not been completely clarified at present. It is generally agreed that the mechanism of inotropic action of CG is associated with this Ca^{++} movement during the E-C coupling processes which is mainly brought by

the inhibition of Na^+-K^+ activated ATPase by CG (Akera & Brody, 1978; Langer, 1981; Lee *et al.*, 1969; Schwartz *et al.*, 1975). Thus, this review is mainly concerned with the currently developed concept of the interelationship between the inhibition of Na^+-K^+ activated ATPase by CG and the altered calcium movement during E-C coupling processes.

Na^+-K^+ activated ATPase as pharmacological receptor for CG

Since the discovery of Na^+-K^+ activated ATPase by Skou (Skou, 1957; 1960; 1961) it is firmly established that this ATPase is the sodium pump located on the cell membrane. It is now well known that the Na^+-K^+ ATPase activity is specifically inhibited by CG (Albers, 1967; Albers *et al.*, 1968; Glynn, 1964; Schwartz *et al.*, 1975). In fact, the inhibition of Na^+-K^+ ATPase by CG is so specific that the intimate correlation between the inhibition of the active transport of Na^+ and that of the Na^+-K^+ ATPase activity is one of the major reasons to believe the identity of these two cellular functions. Thus, CG have become the most important tool of those who engage in the exploration of membrane transport mechanism. In general, it appears that the concentration of CG necessary for 50% inhibition of Na^+-K^+ ATPase is of the order of 10^{-6} to 10^{-7} M which is on the borderline between therapeutic and toxic concentrations. In intact membrane preparations, cardiac glycosides decrease the sodium pump activity only when the drugs are present in the extracellular space. This suggests that the receptor for the drug resides on the external surface of the membrane (Caldwell, 1960; Hoffman, 1966; Whittam, 1962; Whittam & Ager, 1964). The close reltionship of Na^+-K^+ ATPase to a receptor for cardiac glycosides is indicated by the fact that the most highly purified enzyme preparations, possessing only two major polypeptides retain their sensitivity to the drug (Hokin *et al.*, 1966; Jorgensen, 1974; Kyte 1972; Lane *et al.*, 1973). Since digitalis receptor resides on the external surface of the cell membrane and the active site of ATPase activity lies on the internal surface of the membrane, it is presumed that the drug inhibition is an allosteric chain of events in that conformational changes of the sodium pump must be invoked to connect drug interaction on the extenal surface to inhibition of ATP hydrolysis at the internal side. CG are found to bind only a certain form of enzyme and it forms the complex (E_2-P) which is considered to be acylphosphate (Schwartz *et al.*, 1975).

Sodium-potassium activated ATPase inhibition and inotropism

Repke (1963) was the first to propose that the effect of cardiac glycosides on Na^+-K^+ ATPase is responsible for the inotropic effect of the drug. Originaly he postulated that the stimulatory effect of CG on the Na^+-K^+ ATPase by very low concentrations might be related to the cardiotonic action but later changed to the view that Na^+-K^+ ATPase inhibition not the stimultion is the cause of inotropism. Since then, attempts to correlate digitalis receptor for inotropism to the inhibition of Na^+-K^+ ATPase have been made by numerous investigators. There are imposing alley of data indicating the parallel relationship between the Na^+-K^+ ATPase effect and inotropic action of cardiac glycosides (Akera & Brody, 1978; Glynn, 1964; Lee & Klaus, 1971; Lee *et al.*, 1969; Porterfield *et al.*, 1986; Repke, 1964, Repke & Portius, 1963). A significant relationship was found between the sensitivity of Na^+-K^+ ATPase enzymes of different species to the cardiotonic sensitivity of these species. For example, the ouabain sensitivity for various Na^+-K^+ ATPase preparations varied directly with sensitivity *in situ* of those animals from which the enzyme was prepared (Erdmann *et al.*, 1973). The relationship between species sensitivity and cardiac Na^+-K^+ ATPase-ouabain interaction was extended further by Akera *et al.* (1969) showing the direct relationship between dissociation *in vitro* of ouabain from Na^+-K^+ ATPase and the washout of the pharmacological effect of the drug on isolated hearts. In addition to the parallelism between the inhibitory effect on the ATPase and the inotropic effect of CG, both of these effects are antagonized by potassium and are decreased by the decreased extracellular sodium concentration or the temperature of incubation medium (Lee *et al.*, 1969; Repke, 1964; Repke & Portius, 1963). Above and other many parallelisms suggest a causal relationship between the inhibition of Na^+-K^+ ATPase activi-

ty and the inotropic effect of CG and a majority of studies support the concept that inhibition of the enzyme is basic to the inotropic action of CG (Akera *et al.*, 1970; Allen *et al.*, 1975; Besch *et al.*, 1970; Hougen, 1978). However, there are some data which conflict with the idea that Na^+-K^+ ATPase inhibition is necessary requirement for the inotropic effect of CG. Okita among others presented data suggesting dissociation of Na^+-K^+ ATPase inhibition from digitalis inotropy (Okita, 1973; 1977; Okita *et al.*, 1973). He finds the kinetic differences between digitalis inotropy and Na^+-K^+ ATPase inhibition as well as different affinities of CG to inotropic receptor and Na^+-K^+ ATPase in cardiac muscle. Some studies have shown a positive inotropic effect at low concentrations of CG without measurable inhibition of Na^+-K^+ activated ATPase (Murthy *et al.*, 1974; Rhee *et al.*, 1981). In fact, some investigations show that there is an actual increase in potassium gradient indicationg that the Na^+ pump activity is stimulted instead of inhibition with low-dose CG in Purkinje fibers (Noble, 1980). Also Godfraind's laboratory has shown evidence tht the Na^+-K^+ pump is stimulted by low doses of glycoside accompanied by positive inotropy (Ghysel-Burton & Godfraind, 1979; Godfraind & Ghysel-Burton, 1977). These studies are consistent with no change or even stimulation of Na^+-K^+ pump activity coincident with augmentation of force. In view of these data, it has been proposed that CG may induce an increase in the affinity for Ca^{++} by Na^+-K^+ ATPase (Gervais *et al.*, 1977) or by an associated membrane macromolecule (Lullmann & Peters, 1979) resulting in an increase in superficial membrane-bound Ca (Bailey & Fawzi, 1982; Besch & Schwartz, 1970; Nayler 1973; Schwartz *et al.*, 1975). According to these concepts, the binding of CG to Na^+-K^+ ATPase or even to other sarcolemma components alters the affinity of cardiac cell membrane to Ca^{++} in such a way to result in an increased Ca^{++} release during the excitation. However, the stimulatory effect of CG on Na^+-K^+ pump at low-doses may be explained on the basis of catecholamine release by CG as will be discussed in the later section.

Another suggestion presented is that CG interacts with the sodium pump in such a manner to allow a calcium-potassium efflux exchange mechanism without involving the alteration of $[Na^+]_i$ (Lindenmeyer & Schwartz, 1975). Thus a proposal is made that digitalis influences the operation of a carrier-mediated calcium-potassium exchange mechanism during the plateau phase of the action potential (Morad & Greenspann, 1973).

However, there are also numerous studies indication that these alternative possibilities do not likely to explain the inotropic mechanism of CG. As discussed in review by Langer (1977; 1981), there is no evidence of alteration of Ca^{++} binding sites of cardiac sarcolemma directly induced by CG and no correlationship exists between an increase in potassium efflux and the inotropic action of CG. Above alternate proposals have been advanced mainly due to the lack of clear data demonstrating that there is a definite rise in intracellular sodium concentration due to the inhibition of sodium pump at the time of inotropic effect of CG.

Thus, the role of Na^+-K^+ activated ATPase inhibition by CG occupies the central theme in the currently accepted "sodium pump lag" theory which claimes the causal relationship between the pump inhibition and inotropism. In late sixties, numerous studies indicated that both the maximal extent and the onset of the inotropic effect of CG are depressed in low Na^+ medium (Caprio & Farah, 1967; Farah & Witt, 1963; Reiter, 1963). The sodium dependence of the positive inotropic action on heart muscle resembles the Na dependence of the inhibitory action on the active sodium transport system at the cell membrane and on isolated Na^+-K^+ ATPase. It has been repeatedly asserted that the inhibitory action of CG on the membrane ATPase is facilitated by sodium and reduced by lowering the sodium concentration of the medium (Repke, 1964; Repke & Portius, 1963). In view of the highly specific inhibition of Na^+-K^+ ATPase by CG and the long known intimate involvement of alteration of sodium and potassium movement across the cell membrane in the inotropic effect of CG (Glynn, 1964), it is believed by the most investigators that the ionic shift resulting in net gain of Na^+ and net loss of K^+ by the cell would be observed at the time of inotropism. A large number of investigators report loss of potassium and gain of sodium in various cardiac preparations after the application of rather high dose of CG (Areskog, 1962; Bliss & Adolf, 1961; Brown *et al.*, 1962; Carslake & Weather all, 1962; Lee, 1963; Lee *et al.*, 1961; Regan *et al.*, 1956; Tuttle *et al.*, 1961). Employing the chemical method of anaysis, it is

shown that there is an increase in $[Na^+]$ of cardiac cell associated with the inotropic effect of CG (Akera & Brody, 1978; Biedert *et al.*, 1979; Langer, 1977; Langer *et al.*, 1975; Langer & Serena, 1970). However some studies report that a positive inotropic effect of CG in low dose levels is not accompanied by change in intracellular Na^+ concentration (Bentfld *et al.*, 1977; Busse *et al.*, 1979; Lee & Klaus, 1971; Schwartz *et al.*, 1975). Thus, in contrast to the well established toxic effect of CG on the myocardial potassium and sodium content, the effect of low, therapeutic concentrations of CG on cellular sodium and potassium remained controversial. In 1978, Akera and Brody attempted to correlate changes in the Na^+-K^+ ATPase activity, sodium pump activity, transmembrane sodium movement and cardiac contractility by computer simultion following the CG administration. Their simulation studies indicate that moderate inotropic doses of CG which inhibit 20-40% of Na^+-K^+ ATPase cause an enhancement of the intracellular sodium transient but fail to effect an accumulation of myocardial sodium. This suggested that the $[Na^+]_i$ change caused by CG inhibition of sodium pump would be too small to be measured by the then available methods. Thus, they suggested that a prolonged Na^+ transient due to pump inhibition affects Na^+ concentration in a subsarcolemmal pool that may regulate Na^+-Ca^{++} exchange and therby modify contractile state without changing bulk Na content in the cell at the end of contractile cycle.

The important advancement in this crucial problem of causal reltionship between $[Na]_i$ and CG inotropism has been made recently by the use of ion-sensitive microelectrodes for the measurement of $[Na^+]_i$. Lee *et al.* (Lee & Dagostino, 1982; Lee *et al.*, 1980) were the first to employ the sodium sensitive electrode in contraction heart muscle to demonstrate the $[Na^+]_i$ alteration during the inotropic action caused by non-toxic, low concentrations of CG in cardiac muscle. Since the ion sensitive microelectrodes measure the Na^+ activity (a_{n_a}) instead of $[Na^+]_i$, the measured value is much more meaningful than the values obtaind by the chemical anaysis since measurement of total cellular ion content or concentration may not reflect the relevant ion activity (Lev & Armstrong, 1975; Walker & Brown, 1977). They showed a close correltion between the increment of $a^i_{n_a}$ and that of contractile tension of sheep cardiac Purkinje fibers following the exposure to dhydroouabain (Lee *et al.*, 1980, Lee & Dagostino, 1982). Further stuudy on the effect of strophanthidin was conducted on canine cardiac Purkinje fibbes employing both Na^+ sensitive and Ca^{++} sensitive intracellular electrodes. In this study, strophanthidin was used instead of dihydroouabain since previous works indicated that dihydroouabain did not show a stimulatory effect on the sodium pump whereas ouabain and strophanthidin did produce a stimulatory effect at low doses (Noble 1980; Schwartz *et al.*, 1975). Lee and Dagostino (1982) found that $a^i_{n_a}$ in contracting fibers was, on average, 1.5 mM greater than quiescent fibers which had an average $a^i_{n_a}$ value of 7.4 mM. These values are consistent with those of Cohen *et al.*, (1982). Upon stimultion, intracellular sodium ions increase due to the entrance of Na^+ into the cell during each action potential, reaching a steady state level in about 2 minutes. The cessation of stimultion is followed by the temporary hyperpolarization as shown by the previous worker (Vassalle, 1970) which decays to a stable resting level. These findings suggest that the Na^+-K^+ pump rate in cardiac muscle cells depends on the level of intracellular Na^+ concentration as shown previously (Deitmer & Ellis, 1978; Eisner & Lederer, 1980; Eisner *et al.*, 1981; Godsby & Cranefield, 1979; Glitsch & Pusch, 1980) and the rate of Na^+-K^+ pump in the beating fibers is enhanced during the activity which is manifested as a transient hyperpolarization upon cessation of stimulation. Under such experimental conditions, the simultaneous and continuous measurement of $[Na^+]_i$ and twitch tension shows that an increase in $a^i_{n_a}$ and that of contractility have a parallel relationship during both onset and recovery periods of inotropic effect of strophanthin. There is no apparent dissociation between $a^i_{n_a}$ and contractility during onset and recovery periods. Also there is no sign of initial stimulatory phase of sodium pump induced by the non-toxic concentration of strophanthin.

Im and Lee in 1984, analysed the quantative relationship between developed tension (T) and intracellular Na^+ activity ($a^i_{n_a}$) of cardiac Purkinje fibers using a simple model equation $T = \beta (a^i_{n_a})^r$. The data obtained under various expeimental conditions fitted well on a single line described by the equation

and this indicated that the developed tension is a linear function of $(a^{i}_{n_a})^r$ where r is the power of intracellular sodium activity. Similarly, the linear relationship was found between the alteration of $a^{i}_{n_a}$ and twitch tension induced by strophanthidin in $a^{i}_{n_a}$ ranges near the normal value of around 8 mM.

In 1984 Fozzard and his associates conducted the similar studies in ventricular muscle employing the ion selective microelectrodes (Lado *et al.*, 1984; Sheu & Fozzard, 1982). They found proportional elevations in intracellular Na^+ activity after exposure of myocardium to glycoside concentrations producing graded increases in inotropic state.

Further anaysis of the relationship among intracellular sodium activity and strophanthin inotropy was conducted in canine cardiac Purkinje fibers by Vassale & Lee (1984). They measured simultaneously the alteations of electrical, mechanical and intracellular sodium ion activities in electrically driven cardiac Purkinje fibers following the application of strophanthidin in bone-toxic concentrations under various experimental conditions. The positive correlationship existed between the increased $a^{i}_{n_a}$ and the inotropic action of CG. However the availability of Ca^{++} to the cardiac muscle influenced the relationship of $a^{i}_{n_a}$ and contractility. These findings were further confirmed by subsequent studies (Grupp *et al.*, 1985; Lee *et al.*, 1985). Several studies by Eisner and Fozzard groups were conducted in sheep cardiac Purkinje fibers employing the ion selective microelectrodes exploring the relationship between the intracellular sodium activity and contractility as altered following exposure to CG (Eisner *et al.*, 1983; 1984; Wasserstrom *et al.*, 1983). These studies established the essential role of an increase in intracellular sodium activity in the action of digitalis at low and high concentrations.

Another approach to this causal relationship has been made by Smith and his associates employing monolayer cultures of chick embryo ventricular cells (Barry *et al.*, 1981; 1982; 1985; 1985; Biedert *et al.*, 1979). In their studies, they investigated the effects of glycoside-induced sodium pump inhibition on contractility, Na^+ content and altered Na^+-Ca^{++} exchange mechanism in cultures of ventricular cells. It was found that following the application of ouabain and dihydroouabain, an increase in Na^+ content of heart cell paralleled that of contractility and both returned to the control levels on the washout of drugs. The similar correlation of the Na^+ content and contractility was observed when the same degree of sodium pump inhibition was obtained by altering K^+ concentration of the culture medium. From these studies, they arrived at the same conclusion as that with ion selective electrodes, nanely the positive causal correlation between the inotropic action and an increased intracellular sodium concentration induced by CG. Thus, the latest studies in the past few years conducted by several different groups employing the ion sensitive intracellular electrodes or cultured cell preparation have firmly established the causal relationship between the increase in intracellular sodium activity due to the inhibition of Na^+ pump and the inotropic action of CG.

The Ca movement and CG action

The sodium pump is believed to reside on the sarcolemma and possibly on invaginated tubular system into the cell. If the increased $[Na^+]_i$ induced by the inhibition of sodium pump is ultimately responsible for the inotropic effect of CG, some link must exist between $[Na^+]_i$ and the intracellular component directly involved in contraction, the sarcomere. This link is presumed to be calcium. It is well known that Ca plays the central role in excitation-contraction coupling process and the degree of increase in the intracellular Ca^{++} transient during systole determines the contractile strength in cardiac contraction (Allen & Blinks, 1978; Ebashi & Endo, 1968; Fabiato & Fobiato, 1979; Morgan & Blinks 1982; Wier, 1980). It has been known for some time that there is a close relationship between the Ca^{++} movement and CG effect. The effect of calcium on myocardial contractility has been known since the early work of Ringer (1883). The effect of CG on the myocardial contractility is very similar to that of Ca^{++} and these agents share many common effects such as the abolishment of staircase phenomenon (Moulin & Wilbrandt, 1955). The CG and Ca ffects are synergistic or additive and interdependent (Caviel & Wilbrandt, 1958; Lee *et al.*, 1961; Salter *et al.*, 1949). Furthermore, the protective effect of a low $[Ca^{++}]_o$ level against the toxic CG action is well known (Gulner & Kallman, 1957). All these observations indicate the close relationship between the CG action and the calcium movement. The effect of

CG on myocardial Ca turnover was first measured by Harvey and Daniel (1952) who determined the influence of digitoxin on the rate of Ca^{++} efflux from guinea pig hearts employing Ca^{45}. No significant change in Ca^{45} efflux could be found and this result was confirmed by others in different heart muscles (Barry *et al.*, 1985; Wakabayashi & Goshima, 1981; Holland & Sekul, 1959; Klaus & Kuschinsky 1962; Langer & Serena, 1970, Sekul & Holland, 1960). However some experiments report a significant reduction of calcium efflux from frog hearts following ouaban application (Wilbrandt & Caviezel, 1954). In view of the complexity of myocardial calcium turnover, a comprehensive analysis of the calcium metabolism in heart muscle requires that the total calcium content and the degree of attainment of the steady equilibrium of calcium exchange must be known in addition to information on unidirectional fluxes. Some experiments included measurements of all these parameters. In experiments on isolated, electrically stimulted atria of quinea pigs, it was observed that the steady state equilibrium of the cellular calcium with the radioactive extracellular calcium had been attained between 60 and 120 min. The simultaneous determination of the total cellular calcium content revealed that the specific activity of the myocardial calcium after equilibrium was lower than that of the incubation medium (Klaus & Kuschinsky, 1962). This result indicated that only a part of the cellular calcium was exchangeable and this has been confirmed in many other experiments (Grossman & Furchgott, 1964; Klaus 1963). The amount of radioactive calcium present in the tissue after attainment of equilibrium was significantly greater in the presence of CG than in control, whereas the rate of calcium efflux was apparently not influenced by CG. From these results, an increase in myocardial calcium content in the presence of CG would be expected. However no significant change in the total cellular calcium content has been detected after the application of CG to heart muscles (Govier & Holland., 1964; Grossman & Furchgott, 1964; Klaus, 1963). It appears that these negative data on the total calcium contant of heart muscle in the presence of CG is mainly due to methodological difficulties to detect the minute amount of change occuring after the administration of CG. Recent studies employing more advanced methods for the measurement of intracellular Ca^{++} movement have largely overcome most difficulties in the past to give much clearer view on the role of $[Ca^{++}]_i$ in the digitalis action. Advent of the Ca^{++} sensitive intracellular microelectrode and Ca^{++} sensitive photoprotein in recent years has helped largely to solve the relationship between the alteration of $[Na^+]_i$ and that of $[Ca^{++}]_i$ following exposure of heart muscle to CG.

Blinks and his associates, employing Ca^{++} activated photoproten, aequorin showed that intracellular calcium transients are augmented during the positive inotropic effect of acetylstrophanthidin in aequorin-injected heart muscle (Allen & Blinks, 1978; Blinks *et al.*, 1982; Morgan & Blinks, 1982). Their works are confirmed by Wier and Hess (1984) who investigated $[Ca^{++}]_i$ alteration during the positive inotropic effects of ouabain, strophanthin and acetylstrophanthin in canine cardiac Purkinje fibers employing the same photoprotein. They found that CG increased $[Ca^{++}]_i$ transient during contractile activity but did not alter the $[Ca^{++}]_i$ during diastole. Sheu and Fozzard (1982) and Lee and Dagostino (1982) reported proportional elevations in intracellular Na^+ and Ca^{++} activities measured with ion-selective microelectrodes after exposure of myocardium to glycoside in concentrations producing graded increases in inotropic state. Their studies employing the Ca^{++} sensitive intracellular microelectrodes show definitely that there is an elevation in intracellular Ca^{++} activity after exposure of myocardium to nontoxic glycoside concentrations prducing graded increases in inotropic state. The reltionships among the tension, intracellular sodium and calcium activities in heart muscle were further investigated by Lado *et al.*, (1984). Their results confirm many of the previous findings that the increase in $[Na^+]_i$ is intimately associated with the increases in $[Ca^{++}]_i$ and contractility. Thus investigations employing the ion sensitive intracellular electrode in contracting heart muscle firmly establish the positive correlationship between an increase in $[Na^+]_i$ on one hand and increases in $[Ca^{++}]_i$ and contractility on the other. The above positive correlationship is also supported by studies employing the cultured myocytes.

In experiments employing cultured heart cells, Smith and his corraborators (Barry *et al.*, 1981; 1985; Biedert *et al.*, 1979) showed the close quantitative relationships among the degree of glycoside-induced

sodium pump inhibition, increases in exchangeable cellular Na^+ and Ca^{++} contents and the positive inotropic response. Also Morris *et al.* in 1986 showed in cultured neonatal rat cardiac myocytes hat the inhibition of Na^+ pump by ouabain increases the intracellular calcium concentration by 30% as indicated by fluorescent Ca^{++} indicator, Fura-2. Thus it is clear now that the intracellular calcium concentration is increased at the time of inotropic action of digitalis. This increased intracellular calcium is brought by increase in $[Na^+]_i$ which is the result of the Na^+-K^+ activated ATPase inhibition by CG. The critical question then, is illucidation of the mechanism through which the CG induced increase in intracellular sodium concentration eventually leads to an increase in the intracellular Ca^{++} concentration. The cellular Ca^{++} concentration would involve transmembrane and intracellular movements of Ca^{++}. However the Ca^{++} movement across cell membrane has been considered to be the major factor and most investigations are concerned with this aspect.

The brief comment on some important relevant development in our knowledge of the myocardial sarcolemma will be made before the discussion of the transmembrane Ca^{++} movement. The main components of the sarcolemma are lipids and proteins which constitutes unit membrane of bilayer. This bilayer contains phospholipid with hydrophobic fatty acid chains directed inward from the outer and inner surfaces. Sugars, amino acids and hydrophilic polar heads of the phospholipids exetnd into the water phase at both inner and outer surfaces of membrane. The integral proteins are embeded in the lipid bilayer and these are important features of the fluid mosaic membrane model of Singer (Singer & Nicholson, 1972). These proteins have both hydrophilic and hydrophobic segments and some of these proteins extend through the entire bilayer membrane from outer to inner surface. As these proteins float in the lipid bilayer, many of these proteins about on one another forming triads or tetrads of higher order combinations. As the protein come into apposition, it is proposed that they from a central, open core or hydrophilic channel of a few angstroms in diamenter. It is these channels which extend through the lipid bilayer through which the various ions such as Na^+, K^+ and Ca^{++} are selectively conducted across membrane. Passage of these charged ions through the channels is modulated by a gating process which is presumed to open or close the channels. It appears that the phosphorylation and dephosphorylation of the integral protein which make up the channels control the movement of these ions across membrane (Greengard, 1978). The movements of ions across membrane are influenced by membrane potentials and ionic concentration gradients, namely electrochemical gradients. Cardiac glycosides may alter the passage of Na^+ and Ca^{++} through these channels of heart cell membrane.

Another mechanism involved with the moving of ions across membrane are ion pumps or ion carriers. In contrast to the channels in which charged ions flow dependent upon the electrochemical gradients, these pumps and carriers move ions against electrochemical potentials, namely they involve the active transport and/or facilitated transport mechanism. Evidence will be presented that CG induced $[Na^+]_i$ modifies the $[Ca^{++}]_i$ concentration through above two membrane transport mechanisms, namely, through channel and carrier mechanisms. In addition the possibility that CG may modify the intracellular (not across cell membrane) Ca^{++} movement through the CG-induced increase in $[Na^+]_i$ will be discussed along with the possible participation of factors involving neurotransmiter release.

The Na^+-Ca^{++} carrier mediated exchange mechanism and inotropism

The discussion of quantitative aspect on $[Ca^{++}]_i$ in heart muscle would contribute for the understanding of Ca^{++} movement during contraction. Solard *et al.* (1974) provides the information about the quantity of Ca^{++} required to activate and increase force development in the mammalian heart. Approximately 3.5 μ moles Ca^{++}/kg wet wt are required to develop about 90% of maximum force development. To increase force from 40% to 80% maximum doubling of contractile tension, requires an additional 9 μ moles/kg wet wt. Recently various laboratories reported the intracellular calcium ion activity ranging between 90 nM-100 mM indifferent cardiac tissues as measured by Ca^{++} sensitive microelectrodes (Corey *et al.*, 1980; Dahl & Isenbery, 1980; Lado *et al.*, 1984; Lee & Dagostino, 1982; Lee *et al.*, 1980; Sheu & Fozzard, 1982). These values corresponds to $[Ca^{++}]$ of approximately 300 nM. It should be noted that all above values obtained by Ca^{++} sensitive electrodes are

steady state [Ca^{++}] since the time resolution of the electrodes does not allow recording of cyclic change occuring during the contractile activity. Thus, it is difficult to estimate the quantity of Ca^{++} required to activate full tension from the steady state data obtained by Ca^{++} sensitive electrodes. Lee and Dagostino (1982) showed that temporary interruption of stimulation produuced 10-30% decrease in steady state a^i_{Ca} in heart muscle. These latest figures on intracellular calcium concentraion obtained by *in vivo* application of the ion-sensitive electrodes give us an idea about the probable magnitude of Ca^{++} required for tension development in heart muscle. The steady state $[Ca^{++}]_i$ is definitely increased by CG as shown by recent works employing the improved ion selective electrodes. And yet many previous studies employing the voltage clamp technique (Greenspan & Morad, 1975; Mc Donald *et al.*, 1975; Thysum, 1974; Tritthart, 1974) failed to identify the alteration of Ca^{++} movement across cell membrane following the exposure to CG and this suggested that CG induced Ca^{++} movement across membrane is not electrogenic, meaning that Ca movement is coupled with another cation movement. Thus, Langer originially suggested the possibility that CG may augment Ca^{++} influx through a carrier system that couples Na^+ movements to Ca^{++} movements. Subsequently the existence of Na^+-Ca^{++} carrier exchange mechanism is experimentally verified in the perfused squid axon by Baker *et al.*, (1969) and further experimental evidence for such coupling system in heart muscle was presented by Reuter and his associates (Glitsch *et al.*, 1970; Reuter, 1973) and Tillisch and Langer (1974). It is generaly believed that the Na^+ activity gradients ($a^o_{n_a}/a^i_{n_a}$) across the cell membrane influences Na^+-Ca^{++} exchange across the cell membrane and the decrease in Na^+ activity gradients leads to the increased intracellular calcium concentration (Ellis & Deitmer, 1978; Li & Vassalle, 1983; Marban *et al.*, 1980). This is confirmed by recent studies employing ion selective electrodes or isotope flux methods in contracting isolated or cultured heart muscle (Lev & Armstiong, 1975; Lee *et al.*, 1985) Although the CG-induced increase in the intracellular Na^+ concentration which decrease the sodium activity gradients is clearly shown to lead to a net gain in cellular Ca^{++} through Na^+-Ca^{++} exchange mechanism, it remains to be settled whether an increase in the $[Na^+]_i$ (lowering the Na^+ activity gradient) functions primarily to increase Ca^{++} influx or decrease calcium efflux. Either would produce a gain in myocardial calcium. Based on rasults obtained in a study on guinea pig atrial and sheep ventricular tissue where it was found that perfusion with solutions in which the ratio of $[Na^+]_o/[Na^+]_i$ is greatly decreased by reducing sodium from the medium, reduced Ca^{++} efflux, Reuter initially postulated that an increase in intracellular sodium concentation would primarily decrease efflux from the cell (Reuter & Seitz, 1968). In later work, however, Reuter and colleagues showed that the positive inotropic effect of a reduction in extracellular K^+ concentration in guinea pig papillary muscle was dependent on the presence of extracellular Na^+ and proposed that the mechanism of low $[K^+]_o$-induced inotropy is an increase in Ca^{++} influx via enhanced Na^+-Ca^{++} exchange (Reuter, 1974). The early work of Niedegerke (1963) and Langer (1964) indicated an increase in influx of Ca^{++} during the activity in heart. Glitsch *et al.* (1970) demonstrated that $[Na^+]_i$ affected Ca^{++} influx in guinea pig atria and Langer and Serena (1970) working with an arterially perfused rabbit septal preparation, demonstrated that strophanthidin caused an increase in Ca^{++} content without any decrease in Ca^{++} efflux. Also studies on the squid axon (Baker *et al.*, 1969) emphasize the inward movement of Ca^{++} as coupled to the outward movement of Na^+ rather than vice versa. Several studies were conducted on cultured myocardial cells on the effect of reduced $[Na^{++}]_o$ on Ca^{++} fluxes across the cell membrane. Langer *et al.*, (1976) showed that the moderately reduced sodium ion gradient ($[Na]_o/[Na^+]_i$) augmented influx of Ca^{++} but did not alter Ca^{++} efflux in cultured myocardial cells. Recent studies by Smith and his associates on this aspect employing cultured myocytes also support an increase in Ca^{++} influx and not a decrease in Ca^{++} efflux when $[Na^+]_o/[Na^+]_i$ is decreased (Barry *et al.*, 1982; 1984; 1985). Barry and Smith (1984) report that Ca^{++} efflux via Na^+-Ca^{++} exchange in cultured ventricular cells is very sensitive to the transsarcolemmal $[Na^+]$ gradient and is greatly magnified by increases in $[Na^+]_i$ or decreases in $[Na^+]_o$. Their findings suggest that an increase in $[Na^+]_i$ induced by Na^+ pump inhibition results in an increase in Ca^{++} content by increasing Ca^{++} influx rate rather than by decreasing the rate constant for Ca^{++} efflux. No decrease in the Ca^{++} efflux rate constant is produced by a decrease in the transmembrane sodium gra-

dient resulting either from an increase in $[Na^+]_i$ or by a decrease in $[Na^+]_o$. The direction of Ca^{++} efflux via Na^+-Ca^{++} exchange depends on the stoichiometry of the exchange process, membrane concentration gradients for Na^+ and Ca^{++} and membrane potential (Mullins 1981). Estimates of the stoichiometry of the exchange process in various heart preparations indicate a 3 Na^+ to 1 Ca^{++} ratio (Bridge & Bassingthwaighte, 1983; Pitts, 1979; Wakabayashi & Goshima, 1981). Thus, the exchange process would be expected to be electrogenic and the reversal potential, $E_{rev} = 3E_{na} - 2E_{ca}$. according to Coraboeuf *et al.* (1981), En_a is 60 mV and Ec_a is 120 mV. Then E_{rev} would be -60 mV. Thus Na^+ entry with Ca^{++} extrusion would occur only at membrane potentials more negative than-60 mV and Ca^{++} influx via this mechanism should increase with membrane depolarization above-60 mV. This assumption is supported by the results of Coraboeuf *et al.* (1981) and Morris *et al.* (1986). It appears likely, then, that Ca^{++} enters the cell during systole in greater amounts via Na^+-Ca^{++} exchange after Na^+ pump inhibition (Eisner *et al.*, 1983).

In considering above results obtained *in vivo* heart muscle or in cultured heart cell, it appears that an increase in Ca^{++} infflux play more important role than a decrease in Ca^{++} efflux in incroeasing $[Ca^{++}]_i$ following the inhibition of sodium pump by CG resulting in a increase in $[Na^+]_i$.

CG effects on calcium channel: The slow inward current and inotropism

In view of the importance of the slow inward calcium current (Is_i) in cardiac excitation-contraction coupling processes, Katz (1972) and Fozzard (1978) proposed without actual experimental basis that digitalis might increase the cardiac contractility by increasing Is_i. The degree of contribution by Is_i for the activation of contractile activity in cardiac muscle is unclear at pesent. New and Trautwein in 1972 and Tritthard *et al.*, in 1973 estimated, using voltage clamping technique, the amount of calcium that enters the cell by slow inward currennt during cardiac muscle activity and arrived at the opinion that the amount entering the cell by slow inward current is far too low to account for full activation of contraction. Several electrophysiological studies were made on the effect of CG on the slow current but almost all the published evidence argued against increased Is_i by CG (Diacono & Dietrich, 1977; Greenspan & Morad, 1975; Josephson & Sperlakis, 1977; MeDonald *et al.* 1982 Thysum, 1974). However recent publications by mainly two groups of workers Eisner and Tsien present firm evidnce that cardiac glycosides do increase Is_i and this drug action contributes to the inotropic action of CG (Eisner & Lederer, 1980a; 1980; Eisner *et al.;* 1981; 1983; Marban & Tsien, 1979; 1981; 1982 a;).

Weingart *et al.* (1982) showed that strophanthidin can increase Is_i and alter its repriming kinetics in Purkinje fibers and the changes in Is_i were appropriate to help explain simultaneous variations in contractility. These findings are in agreement with previous work in frog atrial trabeculae (Einwachter & Brommundt, 1978). The works by Marban and Tsien also contribute for the illucidation of the role of Is_i in inotropic action of CG(Marban, 1981; Marban *et al.*, 1980; Maran & Tsien, 1979a; 1979 b; 1981; 1982 a; 1982 b). They find in ferret ventricular muscle and calf Purkinje fibers that the enhancement of Is_i follows a time course similar to the development of the positive inotropic effect induced by the therapeutic, nontoxic amount of CG. Then, question arises whether the increased Is_i is due to CG effect on calcium channel directly or indirectly through some other mechanism. Marban and Tsien found that the response of myocardial Is_i and twitch force to ouabain depended strongly on a previous history of driven action potential. The ouabain-induced increase in Is_i could be reversed by withdrawal of action potential stimulation over a period of several minutes. Also enhancement of Is_i in Purkinje fibers could be blocked by replacement of Ca^{++} by Sr which is known to travel calcium channel as same as Ca. These manaeuvers are not expected to interfere with access of CG molecules to Ca channels. These results indicate that CG increase Is_i not by the direct effect on calcium channels but secondarily through some other effect on cardiac muscle. The reasoning that this "some other" CG effect is the Na^+ pump inhibition by CG is supported by the following data. Lederer and Eisner (1982) confirmed the work of Weingart *et al.* (1978), namely the CG induced increase in Is_i and investigated the inotropic effects of low $[K^+]_o$ on Is_i. Since low $[K^+]_o$ induced only the inhibition of Na^+ pump (Eisner & Lederer, 1979; 1980 a) removal of K^+ from the medium should mimic any effect of cardiotonic steroids on Is_i, if CG

increasd Is_i through the inhibition of Na^+ pump. It is found that K removel does indeed increase Is_i suggesting that Na^+ pump inhibition mediates the effect. It is also found that the increases of Is_i and tension could be reversed by adding TI, Rb, C_s or Na_4 ions whose actions are attributed to the known ability of these cations to activate the external site of the Na^+ pump (Eisner & Lederer, 1980 a; 1980 b) in the K-free superfusage. By using "activater" cation one can distinguish between channel effect and an effect mediated via the Na^+ pump. The results show that the increased Is_i comes from the Na^+ pump inhibition and not from the direct effect on the slow current channel. In view of these data and the well known inhibition of Na^+ pump by CG, it is fairly certain that the increased Is_i comes from the CG inhibition of Na^+ pump.

With regard to the question of Na^+ pump inhibition by CG *pe se* or the resulting $[Na^+]_i$ inrease being the immediate cause of the increased Is_i the following data point to the latter to be the likely candidate. The previous history of frequency of stimulation influence (accelerate) the CG effect on Is_i (Marban & Tsien, 1982 b). High frequency of stimulation would alter $[Na^+]_i$ through Na^+ influx during action potential through rapid and slow components (Reuter & Scholz, 1977; Rougier *et al.*, 1979; Wiggins & Bentolila 1980) and influence subsequent CG effect if $[Na^+]_i$ increase is the cause of the increased Is_i brought by CG. Lederer and Eisner also used the electronic Na pump current (Eisner & Lederer, 1979; Gadsby & Cranefield, 1979) as a measure of Na^+ pump rate and showed that the effects of Na^+ pump inhibition, namely increased Isi by low $[K^+]_o$ are mediated not by the instantaneous pump rate but by a time-dependent change in a variable, such as intracellular Na^+ or Ca^{++} activity that depends on Na^+ pump activity. The similar effects of K-free solutions on Is_i magnitude is shown in frog atrial muscle (Goto *et al.*, 1977; Tsuda, 1979), although there are quantitative differences in the relative magnitude and phasic differences of the increase of Is_i in frog and mammaian heart muscles. Various intervention-such as K^+ removal from the medium, veratridine and high frequency of stimulation which are known to increase $[Na^+]_i$ all increase Is_i as well as contractile tension in a similar manner as CG (Deitmer & Ellis 1978; Eisner *et al.*, 1981; Ellis, 1977, Marban & Tsien, 1982 b). Thus, it is reasonable to suppose that the rise in $[Na^+]_i$ brought by CG not only augments contraction but also enhances Is_i.

The next question is the mechanism through which intracellular sodium concentration influences the slow inward current channel. Number of studies suggest the possibility that the increase in $[Ca]_i$ brought by an increase in $[Na]_i$ due to CG inhbition of Na pump mediates the enhancement of Is_i. Isenberg in 1977 reported that the injection of calcium ions into a Purkinje fiber produced an increase in Is_i. The CG induced enhancement of Is_i in Purkinje fibers is blocked by injection of EGTA into ventricular muscle but catecholamine inuced increase in Is_i is not blocked (Marban & Taien, 1982 b). Since EGTA injection into ventricular muscle prevents CG induced increase in Is_i but does not block the catecholamine induced increase in Is_i, it is presumed first; that intracellullar Ca^{++} is required for CG action on Is_i, second; that CG effect on Is_i is not mediated by catecholamine. Another experimental basis to suggest that Ca^{++} may be the mediater through which CG influence Is_i comes from results obtained with replacement of Ca by Sr. Both Ca and Sr traverse slow inward current channels (Vereeke & Carneliet, 1971) and activate tension and both are transported by the Na^+-Ca^{++} exchange mechanism (Reuter, 1974). However in systems where Ca^{++} is believed to modulate ionic conductances, Sr is a weak substitute for Ca(Meech, 1980 Gorman & Hermann, 1979). In the presence of Sr instead of Ca^{++}, CG induced Is_i change is abolished but the CG induced increase in tension persists despite the absence of Is_i enhancement (Marban & Tsien, 1982 b).

It appears that the tension is increased due to the increased $[Sr^{++}]_i$ induced by the increase $[Na^+]_i$ resulting from CG inhibition of sodium pump. But Sr appears ineffective in substituting or Ca in the process which increases Is_i conductance. These results strongly suggest that the increased $[Na^+]_i$ subsequent to sodium pump inhibition brings about an increase in $[Ca^{++}]_i$ which in turn increases Is_i.

The biochemical, enzymatic basis for the influence of $[Ca^{++}]_i$ on Is_i comes from recent development in membrane biochemistry. Dog heart membranes enriched for sarcolemma show Ca^{++}-calmodulin dependent protein kinase activity and phosphate incorporation into proteins of molecular weight of 8000-21000 (Jones *et al.* 1981. Lamers *et al.* 1981). This system shows high divalent cation spcificity,

strongly prefering Ca over Sr. (Notice the same preferance to Ca^{++} over Sr^{++} in Is_i increase induced by CG as shown by Marban & Tsien 1982 b). These protein brands phosphorylated by Ca^{++} also incorporate phosphate in response to cyclic AMP. The regulation of ionic channels in heart is thought to be controlled by phosphorylation of ionic channels by cyclic AMP-dependent protein kinase (Rinaldi *et al.*, 1981; Sperelakis & Schneider 1976; Tsien 1973). It should be noted that in sarcoplasmic reiculaum membranes, phosphorylation of the same regultory protein, phospholamban can be brought about by either cyclic AMP-dependent or Ca^{++}-calmodulin-dependent protein kinases (Katz & Remtulla, 1978; Kirchberger *et al.*, 1974; Le Peuch, 1979) If the above proteins which are phosphorylated by Ca^{++} calmodulin-dependent protein kinase in sarcolemma are same as ones phosphorylated by the cyclic-AMP dependent protein kinase as shown in sarcoplasmic reticulum membranes, then cylic AMP and Ca^{++} may regulate cacium channels through a final common pathway. In such a case, it is reasonable to presume that increase in $[Ca^{++}]_i$ brought by the increased $[Na^+]_i$ in the presence of CG may influence the final common pathway through Ca^{++}-calmodulin-dependent protein kinase. The alteration of the final common pathway by CG, in turn, regulates Ca^{++} channel to facilitate Is_i resulting in an increse in Is_i and contractility. In summary then, the enhancement of Is_i induced by CG has a close temporal correlation with the increase in contractile tension. The increase in Is_i corresponds to an enhanced Ca^{++} influx that may be an contributing but not a major factor in the inotropic action of CG. The enhancement of Is_i by CG may be mediated by the phosphorylation of regultory proteins which control ionic channels of heart muscle by increasing $[Ca^{++}]_i$ which is brought by an increase in $[Na^+]_i$ due to Na^+ pump inhibition.

Neurotransmitter release and inotropy

One series of studies which raises doubts concerning the concept that inhibtion of the Na^+-K^+ ATPase is a necessary condition for the production of increased myocardial force development is evidence showing that the Na^+-K^+ pump is stimulated instead of inhibited by low doses of CG (Ghysel-Burton & Godfraind, 1979; Godfrairid & Glysel-Burton, 1977; Langer, 1981). Tanz (1964) found that pretreatment of cats with reserpine resulted in an aleration of response of isolated papillary muscles to ouabain and this was confirmed by others in subsequent works (Foster, 1967; Spann & Sonnenblick). In the isolated auricle preparation from rabbits pretreated with reserpine or guanethidine, a positive correlation was found between the effect of ouabain and the catecholamine content of heart (Denis *et al.*, 1963; Levy & Richards, 1965). From these data a suggestion was made that at least part of the positive inotropic effect of CG is mediated through activation of beta-adrenergic receptors or the release of catecholamines (Dahlla & Mclain, 1964; Tanz & Marcus, 1966). Recently, Hougen *et al.* (1981) find ouabain evoked release of catecholamines from sympathetic nerve endings. Noble (1980) reports that increased force by contraction occurs in Purkinje fibers with low-dose glycoside when there is no reduced K^+ gradient and even an increased K^+ gradient as derived from reversal potential measurements. This indicates no change or stimulation of Na^+-K^+ pump activity coincident with augmentation of contractile force.

The demonstration of increased Na^+-K^+ pump activity directly conflicts with the well accepted hypothesis that Na^+ pump inhibition is basic to the production of positive inotropy. However the careful analysis of early reports (Spann & Sonnenblick, 1965; Tanz, 1964; Tanz & Marcus, 1966) in light of recent investigation (Lechat *et al.*, 1983) appears to provide a reasonable explanation. Recent studies by Smith and his associates investigated the relationship of low dose and high dose effect of CG (Houghen *et al.*, 1981; Lechat *et al.*, 1983). They found in guinea pig left atria that 10^{-8} M isoproterenol stimulated Na^+-K^+ pump activity as monitored by $^{86}Rb^+$ uptake. Ouabain in low concentrations (3×10^{-9} M) caused a significant increase in Na^+ pump activity but at concentrations greater than 10^{-8} M the pump was consistently inhibited. The ß-blockade by propranolol abolished both the stimultory effects of low-dose ouabain and isoproterenol. If the atria were depleted of endogenous catecholamines by administration of reserpine, then 3×10^{-9} M ouabain produced only a decrease in Na^+ pump activity. These results clearly indicate that low dose CG causes release of

catecholamine which augments Na^+-K^+ pump activity which abscures a smaller, direct inhibitory effect of CG. The increased level of catecholamine would be expected to produce a positive inotropic response. It should be noted however that catecholamine release induced by the low dose CG appears to be not the sole cause of increased Is_i induced by CG (Marban & Tsien 1982 b). It appears, then, there is no doubt that the primary action of the glycoside is to inhibit the Na^+-K^+ pump. In addition, However, low-dose glycoside is capable of releasing catecholamines in the myocardium. The neurotransmitter thus released stimulates Na^+-K^+ pump while producing the catecholamine-induced inotropy and at the same time masks the smaller inhibitory effect of CG on Na^+ pump.

The above explanation probably accounts for the seemingly contradictory results of low dose of CG (stimualtion) and that of higher dose of CG (inhibition) on Na pump activity inspite of the common inotropic effect on the contractility observed by both doses.

Intracellular Ca^{++} release and inotropy

Numerous investigations reveal the presence of Na^+-Ca^{++} exchange mechanism through which Ca^{++} may be released from mitochondria in exchange of Na^+ in the cytoplasm (Carafoli *et al.*, 1974; Crompton *et al.*, 1976; 1977; Kim, 1978; Shin *et al.*, 1982). Kim (1978) showed that there was a siginificant release of Ca^{++} from mitochondria which has taken up calcium previously on the presence of ATP by an increase of sodium concentration in media as low as 1 mM. It has been shown that CG increases the intracellular sodium concentration by more than 2 mM at the time of inotropy (Lee *et al.*, 1985; Lee & Dagostino, 1982). Thus, it is clear that an increase in $[Na]_i$ brought by the therapeutic dose of digitalis would be expected to release Ca^{++} intracellularly in a significant amount. The role of this increase in intracellular Ca^{++} concentration brought by an enhancement of $[Na^+]_i$ has been largely overlooked by most investigators mainly due to the unclarified role of mitochondria in the excitation coupling processes. The speed of movement of Ca^{++} through mitochondria is too slow to be accounted for the significant role in the contraction-relaxation cycle. However, the role of mitochondria in the regulation of intracellular Ca^{++} concentration at the steady state can be easily visualized.

It is this steady state intracellular calcium concentration which would be expected to be raised in the presence of greater amount of Ca^{++} released from mitochondria by an increase in $[Na]_i$ brought by CG. Lado *et al.* (1984) investigated the alteration of intracellular Na^+ and Ca^{++} activities employing ion sensitive electrodes under various experimental conditions. They found that a small change in Na^+ electrochemical gradient was accompanied by much larger change in Ca^{++} electrochemical gradient and attributed this finding to the non-linear properties of Na^+-Ca^{++} exchange in heart muscle. However, this unproportionally larger increase in $[Ca^{++}]_i$ in the face of small $[Na^+]_i$ increase may be due to Ca^{++} release by sodium from mitochondria. It is probable that CG-induced increase in $[Na^+]_i$ may release mitochondrial Ca^{++} which in turn contribute to the inotropic action of CG. This aspect must wait for further investigation to be clarified.

CONCLUSION

It is now well documented that sarcolemma [Na^+-K^+] activated ATPase is the digitalis receptor at the heart muscle. The receptor for the drug resides on the external surface of the membrane. The interaction of CG-receptor initiates an allosteric chain of events connecting drug interaction in the external surface to inhibition of ATP hydrolysis at the internal site. The sodium pump inhibition by CG results in rise in the intracellular sodium concentration. The subsequent reduction in the sodium activity gradient brought by the increased $[Na^+]_i$ causes the rise in intracellular calcium concentration through both Na^+-Ca^{++} carrier exchange and ionic channel mechanisms. Although the Na^+-Ca^{++} carrier system is involved with both Ca^{++} efflux and influx across sarcolemma in exchange with Na^+, evidence indicate that an increase in $[Na^+]_i$ caused by CG stimulates mainly the carrier mechanism to increase movement of Na^+ outward and Ca^{++} inward in the carrier system. The cardiac glycosides also influence the calcium channel mechanism and increase the slow calcium inward current. It appears that

the increased $[Na^+]_i$ not the sodium pump inhibition perse facilitates the calcium channel mechanism. This influence of the increased $[Na^+]_i$ on calcium channel may be mediated by calcium ion which is mobilized by CG. There is evidence that low dose of CG may cause a release of catecholamine and/or reduction in its reuptake. The subsequent increase in the neurotransmitter may augment sodium pump activity, slow inward calcium current and contractility. This catecholamine effect may contribute to the primary action of CG which is based on the inhibition of Na^+-K^+ activated ATPase. Also the possibility exists that the improved mobilization of intracellular calcium by CG is contributed by some other mechanisms such as release of mitochondrial calcium or alteration of calcium binding sites induced by CG. Thus, CG brings about the increased intracellular Ca^{++} level through the Na^+-Ca^{++} exchange carrier system, the channel mechanism, catecholamine release and other possible means of intracellular calcium mobilization. There is no doubt that the primary cause of the increase in intracellular calcium level during activity is the increased intracellular sodium level which is induced by CG-inhibition of sodium pump. Thus, through these multiple mechanisms cardiac glycosides increase the concentration of calcium at the force-producing myofilaments during the course of active tension development and enhance the force of cardiac contractility.

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