SUBCELLULAR BASIS OF THE CARDIOTOXIC EFFECTS OF COBALT, NICKEL AND MANGANESE

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ABSTRACT

The effects of Co^{++}, Ni^{++} and Mn^{++} on the contractile force of the isolated rabbit heart and the activities of the rabbit heart myofibrils, mitochondria, fragments of sarcoplasmic reticulum (microsomes) and sarcolemma were examined in order to establish the sites of action of these divalent cations. Co^{++}, Ni^{++} and Mn^{++} in concentrations from 0.1 to 1 mM were found to depress the magnitude and the rate of change of contractile force developement to varying degrees; however, Ni^{++} was found to be more effective than Co^{++} or Mn^{++}. Both Co^{++} and Ni^{++}, but not Mn^{++}, increased the resting tension of these hearts. Electron microscopic examination of the Co^{++}, Ni^{++} and Mn^{++} treated hearts revealed no ultrastructural changes due to perfusion with these divalent cations.

Although Co^{++}, Ni^{++} and Mn^{++} were found to increase ATP hydrolysis by myofibrils, these cations were less active than Ca^{++} or Mg^{++}. Myofibrillar ATP hydrolyzing activities due to Mg^{++} ATPase and Ca^{++} - stimulated ATPase were 0.032 and 0.186 μ moles Pi/mg protein/min respectively. Both myofibrillar Mg^{++} ATPase and Ca^{++} - stimulated ATPase activities were inhibited to varying degrees by 0.05 to 1 mM Co^{++}, Ni^{++} and Mn^{++}, except Mg^{++} ATPase activity was not affected by 0.05 mM Ni^{++} significantly. The order of potency for inhibiting Mg^{++} ATPase was $Mn^{++} > Co^{++} > Ni^{++}$ whereas that for Ca^{++} - stimulated ATPase was $Ni^{++} > Co^{++} > Mn^{++}$. In contrast to the depressant effect of Co^{++} and Ni^{++}, the inhibitory actions of Mn^{++} were not apparent at 0.25 to 1 mM concentrations of Mg ATP.

ATP hydrolysis by mitochondria was stimulated by Ca^{++}, Mg^{++}, Co^{++}, Ni^{++} and Mn^{++} in 0.05 to 4 mM concentrations. The mitochondrial ATPase activity (1 μ mole Pi/mg protein/min) in the presence of Mg^{++} was significantly depressed by 0.25 - 4 mM Ni^{++} and 1 - 4 mM Co^{++} or Mn^{++}. Mitochondrial calcium binding (52 n moles/mg protein/5 min) and uptake (131 - 186 n moles/mg protein/5 min, under different experimental conditions) activities were depressed to varying degrees

by 0.01 - 1 mM Co^{++}, Ni^{++} and Mn^{++}. The order of potency for inhibiting mitochondrial calcium accumulation was $Ni^{++} > Co^{++}$ and Mn^{++}. Mitochondrial ADP : O ratio and RCI were decreased by 0.05 - 0.10 mM Co^{++}, Ni^{++} and Mn^{++}.

Co^{++}, Ni^{++} and Mn^{++}, like Ca^{++} and Mg^{++}, were found to stimulate microsomal ATP hydrolysis. Both Mg^{++} ATPase (1.41 μ moles Pi/mg protein/min) and Ca^{++} - stimulated ATPase (0.44 μ moles Pi/mg protein/min) activities were decreased in the presence of 0.25 - 4 mM Co^{++}, Ni^{++}, Mn^{++} and the order of potency was $Ni^{++} > Co^{++} > Mn^{++}$. Microsomal calcium binding (43 n moles/mg protein/5 min) was not affected by 0.1 - 2 mM Co^{++}, Ni^{++} or Mn^{++}. On the other hand, microsomal calcium uptake (1064 n moles/mg protein/5 min) activity was decreased to a varying degree by 0.25 - 2 mM Co^{++}, Ni^{++} and Mn^{++} and the order of potency was $Ni^{++} > Mn^{++} > Co^{++}$. The inhibitory effects of these divalent cations on calcium uptake activity were observed at 10 - 100 μ M concentrations of calcium.

The ability of sarcolemma to hydrolyze ATP was stimulated by 0.1 to 4 mM concentrations of Ca^{++}, Mg^{++}, Co^{++}, Ni^{++} and Mn^{++}. The sarcolemmal Ca^{++} ATPase (22.8 μ moles Pi/mg protein/hr) and Mg^{++} ATPase (21.6 μ moles Pi/mg protein/hr) activities were depressed by 0.25 to 4 mM Co^{++}, Ni^{++} and Mn^{++} and the order of their potency was $Ni^{++} > Co^{++}$ and Mn^{++}. The sarcolemmal Na^{+} - K^{+} ATPase activity (9.4 μ moles Pi/mg protein/hr) was also decreased by 0.10 to 4 mM concentrations of Co^{++}, Ni^{++} and Mn^{++}. The sarcolemmal calcium binding in the presence of 0.1 mM Ca^{++} (98 n moles/mg protein/5 min) was depressed by 0.25 mM or higher concentrations of Co^{++}, Ni^{++} and Mn^{++} whereas that in the presence of 1.25 mM Ca^{++} (772 n moles/mg protein/5 min) was decreased by 2 - 4 mM Co^{++}, Ni^{++} and Mn^{++}. The sarcolemmal adenylate cyclase activities in the absence (124 p moles cyclic AMP/mg protein/min) and presence of 2 mM NaF (517 p moles cyclic AMP/mg protein/min) were decreased by 0.1 to 4 mM Co^{++} or Ni^{++} and stimulated by 0.1 to 4 mM, Mn^{++}.

These results clearly indicate that Co^{++}, Ni^{++} and Mn^{++} are capable of altering the functional activities of cardiac myofibrils, mitochondria, sarcoplasmic reticulum and sarcolemma. Changes in the functions of subcellular components in

addition to sarcolemmal alterations may participate in eliciting cardiodepressant actions depending upon the concentrations of these divalent cations. Displacement of calcium from some sarcolemmal sites by these divalent cations may make less calcium available for contraction. In addition these cations may enter the cell and act as a "false - coupler" of the events leading from excitation to contraction. On the basis of sensitivities of different subcellular components to divalent cations, ii is concluded that changes in mitochondrial calcium transport activities may play a predominant role in modifying the contractile activity due to Co^{++} and Ni^{++} whereas changes in mitochondrial oxidative phosphorylation and myofibrillar ATPase activities may be of primary importance for the cardiodepressant effect of Mn^{++}.

I. INTRODUCTION AND STATEMENT OF THE PROBLEM

Various cellular components such as sarcolemma, mitochondria, sarcoplasmic reticulum and myofibrils are generally considered to be intimately involved in heart function and metabolism. The participation of these organelles in contractile events is based on their ability to regulate the intracellular concentration of calcium which has been recognized to play a central role in the cardiac excitation - contraction coupling and relaxation processes. For example, various enzyme systems such as adenylate cyclase, Na^{+} - K^{+} ATPase and Ca^{++}/Mg^{++} ATPase in sarcolemma are believed to be involved in the regulation of calcium movements across the cell membrane. Furthermore, sarcoplasmic reticulum and possibly mitochondria are known to accumulate calcium by energy dependent mechanism and in addition mitochondria generates the major portion of ATP, through the process of oxidative phosphorylation. On the other hand, myofibrils by virtue of their calcium binding and ATP hydrolyzing abilities provide energy for contractile work. Thus any alteration in the functions of these cellular components can be conceived to modify the ability of myocardium to generate contractile force.

Over the past two decades, the actions of various interventions on myocardial contractility have been explained on the basis of their effects on one or more of the cellular organelles. Because of the lack of techniques available for monitoring the effects of different interventions on these cellular components in vivo, it is essential to study their interaction under in vitro conditions after separating these organelles into various fractions. In spite of the limitations of this approach, useful information on the mechanisms of action of different agents can be obtained by examining their dose - response relationships under a wide variety of experimental conditions.

Although various divalent cations such as Co^{++}, Ni^{++} and Mn^{++} are known to depress myocardial contractility, their mode of action is far from clear. Electrophysiological, mechanical and calcium flux studies have indicated an impairment of the excitation - contraction coupling process by these heavy metals;

however, the exact site of their action is poorly understood. We believe that these cations act on myocardium through their interaction with different organelles, depending upon their concentration, and affect various sites involved in the regulation of calcium. Since no reports concerning the interaction of these divalent cations with myocardial organelles as a basis for their cardiopressant action has yet appeared in the literature, it was the purpose of this study to provide some information in this regard. It was designed to establish the subcellular basis of the cardiodepressant effect of Co^{++}, Ni^{++} and Mn^{++}.

In one series of experiments, the effects of different concentrations of Co^{++}, Ni^{++} and Mn^{++} on the contractile force developement by the isolated perfused rabbit heart were investigated. An electron microscopic examination of these hearts was carried out to determine any changes in the myocardial ultrastructure due to these divalent cations. In another set of experiments, myofibrillar, mitochondrial, sarcoplasmic reticular and sarcolemmal fractions were isolated from unperfused rabbit hearts and the effects of different concentrations of Co^{++}, Ni^{++} and Mn^{++} on various biochemical parameters of these fractions were studied. The ATP hydrolyzing abilities of these cellular fractions in the presence of Ca^{++} and Mg^{++} were measured. Furthermore, sarcolemmal calcium binding, adenylate cyclase and $Na^{+} - K^{+}$ ATPase activities were examined. The effects of these cations were also tested on the calcium accumulating abilities of mitochondrial and sarcoplasmic reticular fractions. In addition, mitochondrial oxidative phosphorylation activities were measured in the absence and presence of Co^{++}, Ni^{++} and Mn^{++}.

II. REVIEW OF THE LITERATURE

A. Regulation of Calcium Movements in Heart:

The importance of calcium in heart function has been recognized since Ringer (1) made the observation that heart was unable to contract when perfused with a medium lacking in calcium. Subsequently, it was shown that cessation of the mechanical activity of the heart on perfusion with a calcium - free medium was not associated with any change in the surface electrical activity (2, 3). By demonstrating that an intracellular injection of calcium is capable of initiating contraction, Heilbrunn and Wiercinski (4) further suggested that calcium is an essential link between excitation and contraction. Niedergerke (5, 6) showed that the calcium causing contraction exists in the ionized form and the force of contraction is related directly to the amount of ionized calcium in the cell. Since increasing the extracellular concentration of calcium during depolarization enhanced the mechanical activity of heart, it was claimed that calcium is the only ion present in body fluids which is capable of coupling excitation of the cell membrane to the contractile response of the cardiac muscle (7 - 9). These early electro-mechanical observations in addition to morphological and biochemical evidence (10 - 15) have established the fact that calcium is the final mediator in the excitation - contraction coupling process of the heart muscle. According to the current concept of cardiac excitation - contraction and relaxation processes (16 - 19) four cellular organelles namely sarcolemma, sarcotubular system, mitochondria and myofibrils are mainly involved in regulating calcium movements. Electrical depolarization initiated by the pacemaker is propagated along the sarcolemma and is believed to enter the cell through a transverse tubular system. This process is associated with an influx of calcium from the extracellular space and a release of calcium from superficial sites in the sarcolemma. In addition, there is a release of calcium from intracellular stores such as sarcoplasmic reticulum and possibly mitochondria through direct and indirect mechanisms. All these sources of calcium contribute in raising the concentration of free intracellular calcium from approximately 10^{-7} M to 10^{-6} - 10^{-5} M. This calcium binds to troponin and

relieves the inhibition exerted by troponin - tropomyosin system upon actin and myosin whereas hydrolysis of ATP due to stimulation of actomyosin ATPase provides energy for contraction of the myofibrils. The cytoplasmic concentration of free calcium is then lowered by a variety of mechanisms involving the sarcoplasmic reticulum, sarcolemma and possibly mitochondria and this process is believed to be associated with relaxation of myofibrils. Thus the cardiac contraction and relaxation cycle is generally viewed as the reflection of raising and lowering the intracellular concentration of free calcium. Furthermore, the molecular mechanisms of contraction and relaxation can be readily understood in terms of the functions of various membrane systems and contractile apparatus which intimately participate in regulating the movements of calcium in heart muscle. Although it is recognized that other cellular components such as the nucleus may also be involved in the regulation of intracellular calcium (20), no definitive information on this aspect is available in the literature at the present time.

Recently, extracellular calcium has been implicated in coupling excitation with contraction in cardiac muscle. Electrophysiological studies have provided evidence for a slow inward current of the cardiac action potential to be due to calcium (21 - 27). The magnitude of this current during the plateau phase of the action potential was relatively unaffected by external sodium ion concentration or tetrodotoxin, a specific antagonist of sodium influx. However, it was dependent upon external calcium concentration and was sensitive to calcium antagonists such as verapamil and its methoxy-derivative, D600 (28). Various cat ions such as Ni^{++}, Co^{++}, Mn^{++} and La^{+++} were found to depress contractile force by inhibiting the slow inward calcium current (29, 30). The threshold of the calcium current (about - 40 mV) must be reached before activation of the contractile apparatus takes place. Other voltage - clamp studies have failed to demonstrate a clear relationship between changes in the slow inward calcium current and contractile activity during the "staircase phenonemon" (23, 31, 32) or Na^{+} - free perfusion (23, 31).

On the basis of total charge due to the calcium current it has been calculated that only 5 to 10 μ moles calcium influx/kg of heart weight occurs

during depolarization (24, 25). However, biochemical studies concerning measurements of myofibrillar calcium binding, ATPase activity and isometric tension at different concentrations of calcium revealed that about 85 μ moles of calcium/kg heart weight were necessary for full tension developement by the myocardium (33). From such experiments, it is clear that the calcium influx as measured by voltage - clamp techniques is not sufficient to fully activate the contractile apparatus upon depolarization. This point has been emphasized by Bassingthwaighte and Reuter (34) who presented evidence to support the view that additional calcium must be released from intracellular sites for full cardiac contraction. This, however, does not undermine the essential role played by extracellular calcium because La^{+++}, which does not penetrate the myocardial cell membrane, was found to uncouple excitation from contraction (35, 36).

The possibility that a small quantity of extracellular calcium entering during depolarization, as measured by voltage - clamp studies, could trigger the release of calcium from intracellular sites was suggested by some investigators (37, 38). This concept of "trigger calcium" was substantiated by Fabiato and Fabiato (39 - 41) when they showed that concentrations of calcium lower than that required for activating contraction of myofilaments directly were able to produce transient contractions in the cardiac cells with disrupted sarcolemma. The data from homogenized rat hearts (42) and chemically treated frog ventricles (43) have also been interpreted to support the hypothesis of regenerative calcium release from intracellular sites. However, it should be noted that calcium - induced calcium release can only be demonstrated clearly in preparations which are heavily loaded with calcium or treated with caffeine. Therefore, interpretation of results obtained from these unphysiological preparations should be taken with some caution.

Although release of calcium from the lateral cisternae of the sarcoplasmic reticulum directly by the wave of depolarization, travelling along the sarcolemma into the transverse tubular system, (44) can be conceived to occur in cardiac muscle, it should be noted that the sarcoplasmic reticulum in heart is not well developed (45, 46). Although Lee et. al. (47) demonstrated a release of calcium from the sarcoplasmic reticulum under the in vitro conditions, it is not clear

whether this effect was due to electrolysis or heat produced by the electric current. On the basis of the observation that changes in pH are associated with changes in calcium release from the sarcoplasmic reticulum (48) and the oscillation of intramuscular pH during contraction - relaxation cycle of cardiac muscle (49) it can be conceived that depolarization leads to changes in pH of the myoplasm and thereby causes a release of calcium from intracellular stores. Some investigators (50, 51) have suggested that release of calcium from intracellular stores is mediated by a rise in the intracellular sodium concentration whereas others (52) have attributed this event to an increase in the level of cyclic AMP. Irrespective of the mechanism of the release of calcium from the intracellular sites upon depolarization, the central role played by extracellular calcium in cardiac contraction can be readily appreciated by a rapid decline in contractile force upon perfusing hearts with calcium - free medium without any changes in the electrical activity (53).

Since myocardial cells such as the atrial and Purkinje cells (54), as well as the ventricular cells of embryonic or neonatal chickens have no transverse tubules (55), it appears that this membranous system is not essential for the rapid propagation of excitation throughout the cell or for the coupling of excitation to contraction. It should be noted that the time between excitation and the onset of contraction of cardiac muscle is about 20 m sec. Thus it is not essential that the wave of excitation release calcium from the sarcoplasmic reticulum almost simultaneously at the level of each sarcomere. On the other hand, current evidence points to the sarcolemmal basement membrane as the source of coupling calcium in the cardiac fiber (56). Accordingly, the calcium which is presented to the area of the contractile filament is released from the superficial cell membrane and travels inward to the area of the sarcomeres by a simple process of diffusion. Calcium ion diffuses in the myoplasm at a rate of 1 μ/msec and would take 5 msec to reach the contractile unit of the cardiac cell with an average diameter of 10 μ.

Although it is recognized that the superficial sites in heart sarcolemma are in equilibrium with calcium in the interstitial space, it is difficult to determine the amounts of calcium released from the superficial sites and entering from the extracellular space during depolarization. In this regard it should be noted that

the voltage - clamp studies do not detect the non - electrogenic movement of calcium into the myocardium as well as that released from the superficial sites in sarcolemma upon depolarization. Niedergerke (57) has already claimed that external calcium enters the myocardial cell through a carrier system. The existence of such a non - electrogenic carrier system, which moves calcium inward and sodium outward, has been shown by some investigators (58 - 60). A calcium - potassium exchange carrier has also been proposed for the myocardium (61). Thus, it appears that calcium enters the myocardial cell from the extracellular spaces and superficial sites in sarcolemma upon depolarization via electrogenic and non - electrogenic (carrier) mechanisms.

From the foregoing discussion, it is clear that different sources such as extracellular, sarcolemmal and intracellular sites participate in raising the myoplasmic level of free calcium upon depolarization of the cardiac cell. Data from calcium flux studies (13, 15, 62 - 68) have provided evidence regarding the existence of calcium in several compartments in cardiac muscle. It is generally believed that one compartment, which probably represents extracellular and sarcolemmal sources of calcium, is essential to the coupling process in cardiac muscle whereas the other compartment, which probably represents intracellular sources (sarcoplasmic reticulum and mitochondria), is intimately involved in the maintenance of contractile force (68, 69). Although these compartments have not been morphologically defined, some reports (70 - 72) indicate that certain interventions may modify the myocardial contractile force by influencing one or more of these calcium compartments. Because available techniques do not permit the exact localization of calcium compartments affected by various agents in the intact cardiac muscle, it is necessary to separate different cellular components into fractions and examine the effects of different interventions on their functions under in vitro conditions.

B. Interaction of Calcium with Cell Components:

Although considerable information is available on the electrical behaviour of the heart cell membrane, very little is known about its molecular composition and structure because of the difficulties involved in obtaining

sufficient material uncontaminated by intracellular organelles for biochemical analysis. The sarcolemma has two components, a thin electron dense plasma membrane and a much thicker, amorphous layer, basement membrane which coats the plasma membrane (73). The sarcolemma with its basement membrane invaginates at regular intervals into the myocardial cells forming the transverse tubular system which comes into close apposition with the lateral sacs of the sarcoplasmic reticulum, terminal cisternae. The basement membrane has been studied histochemically and has shown staining characteristics indicative of mucopolysaccharide or mucopolysaccharide - protein complex (74) with a high density of negatively charged sites, which are believed to selectively bind cations, notably calcium (75). There is a growing awareness that many cellular functions are directly controlled by macromolecules outside the cell, either as components of the plasma membrane or as cell - surface associated material (76). It is therefore possible that the basement membrane plays a crucial role in regulating myocardial function and metabolism. The importance of this layer in cardiac calcium movements has been recognized recently (15, 77) and it may well be that this site may be the source of "trigger" calcium. Whether or not this site is the same as the superficial calcium binding sites in sarcolemma is an open question at present. It also remains to be determined if the basement membrane plays any role as a calcium accumulating system outside the myocardial cell as proposed by Hajdu and Leonard (78) for maintaining a 3 - 4 fold higher calcium concentration in the microdomain of the cell in comparison to the extracellular space. Although it is conceivable that the basement membrane may be intimately involved in limiting calcium permeability across the plasma membrane, extensive research is required to gain insight into the functional aspect of this layer.

In contrast to the basement membrane, plasma membrane is considered to contain various enzyme systems which may be involved in the regulation of ionic permeability and modulation of myocardial contractility. Cytochemical evidence suggests the localization of both $Na^+ - K^+$ ATPase and adenylate cyclase in heart cell membrane (79, 80). The cardiac membrane fractions enriched in sarcolemma, obtained by employing different procedures, also showed high specific activities of

both $Na^+ - K^+$ ATPase and adenylate cyclase (81 - 86). The adenylate cyclase catalyzes the transformation of ATP into cyclic AMP which has been recognized as an important regulator of heart function and metabolism (87). On the other hand, $Na^+ - K^+$ ATPase has been shown to control myocardial function through the movements of sodium and potassium across the cell membrane (88). Although both enzymes are lipoproteins and require Mg ATP as a substrate, they differ in their responses towards cardioactive agents such as ouabain and epinephrine (89). For example, ouabain inhibited the sarcolemmal $Na^+ - K^+$ ATPase activity while epinephrine stimulated the sarcolemmal adenylate cyclase activity. The inhibition of $Na^+ - K^+$ ATPase by cardiac glycosides through a wide variety of mechanisms has been considered to augment calcium influx (18, 26, 88, 90). On the other hand, activation of adenylate cyclase is believed to be associated with an increase in calcium influx through the cyclic - AMP - protein kinase system (24, 91, 92). The role of $Na^+ - K^+$ ATPase in regulating calcium movements in heart under different experimental conditions has been emphasized (93), and dibutyryl cyclic AMP has been shown to enhance the uptake of calcium into the myocardium (91, 92). At any rate, both $Na^+ - K^+$ ATPase and adenylate cyclase in heart sarcolemma have been shown to be inhibited by calcium (89, 90, 94) but the exact significance of this effect is not clear at present.

The existance of an ATP - dependent "calcium pump," involved in lowering the intracellular concentration of free calcium in the myocardial cell during the relaxation phase, has been postulated in the heart sarcolemma (95). Although some investigators were able to show Ca^{++} - stimulated Mg^{++} dependent ATPase in heart sarcolemma (86, 96), the low enzyme activity reported by these investigators can be easily taken to be due to some technical artifact or contamination by the sarcoplasmic reticulum. The heart sarcolemmal preparation obtained by hypotonic shock - Li Br treatment was found not to exhibit Ca^{++} - stimulated Mg^{++} dependent ATPase (97). Furthermore, heart sarcolemma did not show ATP - dependent calcium binding (97, 98). It should be mentioned here that the inability to demonstrate a "calcium pump" in heart sarcolemma may be due to predominantly right - sided out orientation of the sarcolemmal membrane vesicles. Thus further