ALTERED SARCOLEMMAL Na^+ - Ca^{2+} EXCHANGE IN HEARTS SUBJECTED TO HYPOXIA-REOXYGENATION

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In Partial Fulfillment of the Requirements
for the Degree of Masters of Science

by

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ABSTRACT

Although the occurrence of intracellular Ca^{2+} overload is known to be an important factor in hypoxia-reoxygenation injury, the exact mechanisms for this abnormality are not clear at present. Since Na^{+}-Ca^{2+} exchange in the sarcolemmal membrane is considered to be involved in Ca^{2+} - efflux, this study was undertaken to examine the effect of hypoxia-reoxygenation on this system. Isolated rat hearts were made hypoxic by perfusing with a substrate-free medium gassed with 95% N_2 and 5% CO_2 and then reperfused with oxygenated normal medium. Sarcolemmal vesicles were isolated from hearts of control, hypoxic and hypoxia-reoxygenated conditions and the Na^{+}-dependent Ca^{2+} uptake activity was measured at different times of incubation as well as at different concentrations of Ca^{2+}. Sarcolemmal ATP-dependent Ca^{2+} accumulation was also measured, as information on the status of this activity in conditions of hypoxia-reoxygenation is lacking. A significant decrease in Na^{+}-dependent Ca^{2+} uptake was observed in preparations from hearts made hypoxic for 10 min. Reoxygenation of 10 min hypoxic hearts resulted in a further depression of Na^{+}-Ca^{2+} exhange activity. ATP-dependent Ca^{2+} accumulation was also depressed in hypoxic as well as reoxygenated hearts. Hypoxia was found to markedly increase the resting tension and depress the ability of the heart to generate contractile force; reoxygenation resulted in partial recovery of these parameters. These results suggested a defect in the Na^{+}-Ca^{2+} exchange system and the ATP-dependent Ca^{2+} pump in the

heart sarcolemmal membrane and this may contribute to the occurrence of intracellular Ca^{2+} overload and functional abnormalities due to hypoxia-reoxygenation injury.

I. INTRODUCTION AND STATEMENT OF THE PROBLEM

Myocardial ischemia and subsequent reperfusion has been shown to have deleterious effects on cardiac function and ultrastructure (1, 2). Reduction in delivery of both oxygen and substrate due to lack of blood flow to the myocardium (3, 4) results in the development of ischemic injury. In fact, hypoxia-reoxygenation is believed to induce cell damage similar to that seen in the myocardium subjected to ischemia-reperfusion (5, 6). Although the occurrence of intracellular Ca^{2+} overload is thought to be an important factor in the genesis of irreversible hypoxic or ischemic cell damage (7), the exact mechanisms of this abnormality are not clear at present. Since Na^{+}-Ca^{2+} exchange and ATP-dependent Ca^{2+}-pump in the sarcolemmal membrane are thought to participate in the efflux of Ca^{2+} from the myocardial cell (7-10), any defect in their activities can contribute to abnormal intracellular Ca^{2+} concentrations. Myocardial ischemia induced by incubating rabbit ventricular slices in substrate-free medium in the absence of oxygen for 1 to 2 hr was found to depress Na^{+}-Ca^{2+} exchange activity in sarcolemma (11). Furthermore, global ischemia in isolated rat hearts for a period of 1 hr was also found to decrease the Na^{+}-dependent Ca^{2+} uptake activity in sarcolemmal vesicles and this depression was reversible upon reperfusion with oxygenated medium for 15 min (12). On the other hand, perfusing the isolated rat heart with hypoxic medium containing glucose for 60 min did not produce any depression in the Na^{+}-dependent Ca^{2+} uptake activity in sarcolemma and

it was suggested that ischemic damage may differ from that caused by hypoxia (12). In order to approximate ischemia, we chose to remove both oxygen and substrate (glucose) from the hypoxic perfusate. In this study we have examined the effects of hypoxia-reoxygenation on the sarcolemmal Na^{+}-Ca^{2+} exchange activity by employing isolated rat hearts perfused with substrate-free medium. Furthermore, changes in the ATP-dependent Ca^{2+} accumulation in heart sarcolemma due to hypoxia-reoxygenation were monitored. Although earlier studies have shown a depression in the sarcolemmal Ca^{2+} pump activity in dog hearts subjected to short term ischemia-reperfusion (13), no information regarding the effects of hypoxia-reoxygenation on ATP-dependent Ca^{2+} accumulation in the sarcolemmal vesicles is available in the literature. Alterations in contractile activity of the isolated hearts subjected to hypoxia and reperfusion were also measured in this study in order to gain some information regarding any relationship between changes in sarcolemmal Ca^{2+} transport and heart function.

II. REVIEW OF THE LITERATURE

Overview of myocardial membrane systems and the concept of hypoxia-reoxygenation damage. The low intracellular concentration of free Ca^{2+} (0.1 μM) in the resting myocardial cell is maintained primarily by three membrane systems; the sarcolemma, the sarcoplasmic reticulum and mitochondria (14, 15). The regulation of intracellular calcium concentration is crucial because Ca^{2+} ion signals modulate an array of reactions in heart cells, including the contraction and relaxation of the myofibrils (15) and several steps of myocardial metabolism (14). The sarcolemma and sarcoplasmic reticular membranes contain Ca^{2+}-transporting systems which utilize the metabolic fuel of the myocardium, ATP, solely for the purpose of maintenance of calcium homeostasis. Mitochondria may act as a Ca^{2+} "sink", buffering the myocardium from dramatic rises of intracellular Ca^{2+} concentration (15, 16). Although mitochondrial participation in the excitation-contraction cycle involving beat-to-beat delivery of calcium to the contractile proteins was previously speculated upon (17), this view has fallen from favour largely due to recently revised estimates of physiologic mitochondrial Ca^{2+} content (18, 19), wherein results indicate mitochondrial Ca^{2+} content to be 5-10 fold less than was previously described. The function of the sarcoplasmic reticulum has been evaluated using vesicular preparations (20, 21, 28). Calcium is thought to bind to high-affinity sites on the sarcoplasmic reticular transport enzyme, Ca^{2+}-ATPase,

followed by subsequent translocation into the lumen of the sarcoplasmic reticulum at the expense of phosphate bond energy (ATP hydrolysis) (22). Recent work suggests that GTP hydrolysis is associated with the incorporation of Ca^{2+} into an "intermeadiate" calcium pool in series with calcium translocation (23). The sarcoplasmic reticulum has been shown to respond to small quantities of exogenously-applied Ca^{2+} at its surface by releasing a comparatively large amount of Ca^{2+}, presumably to cytosolic targets (24, 25). These results have given rise to the concept of "trigger" or "activator" Ca^{2+}, which may originate from the extracellular space (24) and may initiate an amplified release of Ca^{2+} from sarcoplasmic reticular stores to raise intracellular concentration of Ca^{2+} to 10 μM. However, the mechanism of release of Ca^{2+} from the luminal stores of the sarcoplasmic reticulum to the cytosol remains poorly understood at present.

Influx of extracellular Ca^{2+} may initiate myocardial contraction directly, or via a combination of the above mechanisms (27). The sarcolemmal membrane mediates Ca^{2+} transport via several systems; the Na^{+}-Ca^{2+} exhange system (29, 30), the Ca^{2+}-stimulated ATPase otherwise referred to as the Ca^{2+}-pump (31) and the slow Ca^{2+} channels which are widely believed to be involved in the cardiac action potential and excitation-contraction coupling. Cardiac contractile tension has been shown to be dependent upon Ca^{2+} ion influx via the sarcolemmal Ca^{2+} channels (32) which

allow Ca^{2+} to flow into the intracellular space down the electrochemical gradient in response to membrane depolarization. The gating of these channels has been suggested to be a metabolically controlled process (33). Dhalla and associates (7, 35) suggest that a novel Ca^{2+}-dependent ATPase, which is activated by millimolar concentrations of Ca^{2+}, may be involved in the gating of the slow Ca^{2+} channel to permit Ca^{2+} entry into the myocardium. This enzyme has been isolated in pure form (34). Many of the aforementioned sarcolemmal, sarcoplasmic reticular or mitochondrial Ca^{2+}-transporting mechanisms are regulated by intracellular phosphorylation systems (35). Thus, the ability of the myocardium to regulate intracellular Ca^{2+} concentrations depends upon the proper functioning of each component of the three membrane systems.

The ischemic myocardium is subject to two principal metabolic alterations, specifically: (i) reduced delivery of oxygen and substrate, and (ii) buildup of metabolic by-products, including intracellular accumulation of protons and extracellular accumulation of K^{+} (3). Thus, ischemia produces a complex array of factors which influence the functional, metabolic and ultrastructural aspects of the myocardium. In view of the complex nature of ischemia-induced injury to the myocardium, many studies on hypoxia alone or hypoxia coupled with a lack of substrate have been done in order to examine the effects of features of ischemia in isolation. The goal of this thesis is to examine aspects of sarcolemmal Ca^{2+}-transport in hearts subjected to

conditions of hypoxia-reoxygenation.

Functional, metabolic and ultrastructural changes. The phenomenon of immediate rapid decline of contractility in hearts at the onset of ischemic or anoxic conditions is well documented (36, 40). Early reduction of myocardial mechanical capability induced by ischemia or hypoxia is reflected by a number of indices of contractile function. A reduction in developed tension and in the maximum rate of tension development, as well as shortened duration of contraction has been observed in isometric studies (37-39). Nayler and associates (36) described the effect of hypoxia on contractile characteristics, and defined a three-stage response to hypoxia, wherein an initial rapid decline in developed force was followed by a lagging increase in resting tension. The initial stage was characterized by the rapid decline in developed tension with no change in resting tension, and was thought to represent impaired excitation-contraction coupling in the myocardium. Possible explanations of the mechanism of this phenomenon include altered availability of calcium bound to the outer surface of the cell membrane (41); altered Ca^{2+} influx; impaired handling of Ca^{2+} in sarcoplasmic reticular stores (42); altered intracellular ionic composition (i.e. elevated phosphate levels) (43); or an altered supply of ATP (36). The early slow rise in resting tension (phase 2) accompanied by suppression of developed tension is postulated to be due to reduced availability of high energy phosphates (36).

Tissue calcium uptake is reputedly unchanged during the early stages of the phase 2 response (36). During the late period of the phase 2 response it is postulated that Na^+-K^+ ATPase may be progressively inhibited due to lack of ATP, and thus intracellular Na^+ would rise (36). An increased influx of Ca^{2+} to the intracellular space may occur via the sarcolemmal Na^+-Ca^{2+} exhange mechanism; and this cascade of cellular events which theoretically would result in increased intracellular Ca^{2+} concentration, may be similar to the proposed mechanism of digitalis-mediated positive inotropism (7). However, this hypothesis depends on the putative low affinity of the Na^+-K^+ ATPase enzyme for ATP and thus the inhibition of Na^+-K^+ ATPase activity by low cellular ATP concentration. This view is questionable because the affinity of the enzyme for ATP may be great enough to maintain activity even during decreased availability. Therefore, the possibility exists that factors other than ATP availability are responsible for the depression of Na^+-K^+ ATPase activity during hypoxia.

After prolonged periods of hypoxia (phase 3), resting tension rises rapidly. Increased contracture of the myocardium indicates that the concentration of intracellular Ca^{2+} is rising, due either to excessive entry of Ca^{2+} from the extracellular space or from release of internal stores. Reoxygenation of the myocardium at this phase of the hypoxia response is characterized by a further rapid entry of Ca^{2+} (5, 6, 44, 45). Recovery of myocardial function following a period of hypoxia varies with species (46), pacing of the

isolated heart duration of hypoxia and the inclusion or exclusion of substrate in association with the hypoxic challenge (6).

At the onset of ischemia or hypoxia, dramatic changes in cardiac metabolism are apparent. The process of mitochondrial oxidative metabolism is complex, and is dependent upon a structured sequence of events including cellular uptake of fatty acids, intracellular activation of these biomolecules and their subsequent transfer into the mitochondria followed by β-oxidative breakdown to intramitochondrial acetyl-CoA. Inclusion of this molecule into the tricarboxylic acid (TCA) cycle which drives oxidative phosphorylation results in ATP production, a process reliant on a continuous supply of molecular oxygen (47). Oxidative metabolism is considerably more complex than glycolysis because all of the substrates, metabolites and cofactors that move between the mitochondrial matrix and the cytosol must traverse a membrane barrier. Controlled changes in mitochondrial membrane permeability with regard to the above factors are thought to serve a regulatory function with regard to oxidative metabolism (48). Oxygen is the final electron acceptor of the electron transfer chain wherein ATP production occurs in parallel to the oxidation of NADH and $FADH_2$ (reduced coenzymes) which originate from carbohydrate and fatty acid oxidation via the TCA cycle and by β-oxidation. An ischemic or hypoxic challenge causes an immediate decline in oxidative

phosphorylation; fatty acids and fatty acyl carnitines are thought to accumulate in the intracellular space (49-52) and these lipophilic compounds have been shown to alter Na^+-Ca^{2+} exchange activity and Ca^{2+} permeability of cardiac sarcolemmal vesicles (53, 54). Specific examples of defects in pathways of cellular respiration when faced with an ischemic or hypoxic challenge exist. Schwartz et al. (55) demonstrated that a reduction of carnitine-mediated mitochondrial oxidation of palmitic acid in ischemic myocardium is due to an aberration in carnitine activation. Severe impairment of the electron transport chain, indicated from a reduction of the ratio of ADP phosphorylation:oxygen consumed was evident at the onset of ischemia and in severely ischemic myocardium (55). Furthermore, exogenous addition of cytochrome c increased respiration levels from that of ischemic myocardium. Thus, this cytochrome is sensitive to ischemia and may make a key contribution to the rapid decline in oxidative phosphorylation in these conditions. Transmembrane calcium ion movement is thought to contribute to the regulation of production of ATP in the mitochondria (56). Calcium uptake in the mitochondria has been associated with concomitant ATP hydrolysis (17). As well, Ca^{2+} moves into the intramitochondrial space across the membrane barrier through an electrophoretic Ca^{2+} uniporter, driven by the negative potential maintained inside the mitochondria by respiration (56). Therefore, mitochondria may serve to control cytosolic Ca^{2+} concentration by this uptake mechanism. Mitochondria

isolated from ischemic hearts exhibited decreased Ca^{2+} uptake (55). In addition, mitochondrial Ca^{2+} release from ischemic myocardium was similar to that wherein cellular respiration was blocked by KCN. Intracellular concentration of phosphocreatine, a high-energy compound associated with ATP production, decreases in ischemic myocardium (57).

Lactate, normally consumed by the myocardium, was found to be retained in elevated concentrations in ischemic hearts (57). Hypoxia is similarily associated with an intracellular accumulation of H^+ ions and lactate resultant from increased glycolytic activity (6, 58, 59). It can be assumed that with the onset of ischemia or hypoxia, a concomitant shift from oxidative metabolism to glycolytic metabolism might occur in the myocardium (3, 59, 60). Glycolysis in early ischemia occurs at a high rate and this rate is maintained for a short duration of time (61); a similar sequence of events occurs in conditions of hypoxia (62-64). The time course of the metabolic changes hitherto discussed is short, wherein high-energy phosphate levels are decreased 30 sec after coronary occlusion (57) in conjunction with rapid decrease in oxidative metabolism (55) and elevation of glycolytic activity as discussed above. In hearts made ischemic for a period of 10 min, ATP and CP levels were significantly reduced whereas the lactate:pyrurate ratio was increased (57). Altered substrate availability, pH, cofactor and coenzyme concentrations did not change the activities of glycolytic

or oxidative (TCA cycle) enzyme activities significantly even after 2 hrs of ischemia (57). Thus, injured myocardium retains the ability to undergo limited glycolysis, dependent upon substrate availability. ATP production via glycolysis is too low to support a constant level of available phosphate bond energy, and the metabolism of the myocardium takes a downhill course without reaching equilibrium (4).

The contractile proteins of the myocardium are not labile to short periods of ischemia (2). Myofibrillar ATPase activity in human myocardium sampled 4-6 hr postmortem was found to be similar to that of fresh tissue (65). Furthermore, Katz and Maxwell (66) noted that the structural conformation of actin is not altered after prolonged hypoxia or ischemia. Acidosis of the myocardium occurring in hypoxic or ischemic conditions was speculated to somehow damage the contractile proteins in a reversible fashion. However, negative inotropism initiated by acidosis may be the result of competition for the troponin C Ca^{2+} binding site by H^{+} and Ca^{2+} (67). Therefore, increased concentrations of Ca^{2+} are needed to activate the contractile proteins, and thus decreased Ca^{2+}-sensitivity of the contractile proteins in the wake of acidosis as a component of myocardial ischemia or hypoxia is a possibility (67).

Reperfusion of ischemic tissue may hasten the death of heart cells suffering irreversible ischemic injury or, on the other hand, may restore oxidative metabolism to near normal levels in reversibly injured heart cells (3).

Recovery of normal metabolism is dependent upon the duration of the ischemic or hypoxic challenge to the myocardium. Cytosolic phosphorylation potential is restored within 3 min in reperfused heart tissue after 15 min of ischemia (68). The adenine nucleotide pool remains unchanged from the previous ischemic depletion due to very slow resynthesis of precursors. Accumulated lactate, adenosine, inosine, hypoxanthine and H^+ ions are washed into the systemic circulation upon reperfusion (3). Reperfusion of irreversibly injured myocytes is associate with rapid cell swelling, postulated to be caused by increased osmotic load of the intracellular myocardium during ischemia; fluid is drawn into the cell upon reperfusion. Hypoxia-reoxygenation injury to the myocardium is associated with the occurrence of intracellular Ca^{2+} overload and will be dealt with in detail in a subsequent section, wherein emphasis will be placed on changes in sarcolemmal, sarcoplasmic reticular and mitochondrial membrane function.

Ultrastructural changes in the myocardium as a result of hypoxia-reoxygenation or ischemia-reperfusion injury have been studied extensively (1, 3, 69-72). Jennings (1) observed normal sarcolemmal, mitochondrial and nuclear membrane ultrastructure in hearts subjected to ischemia for a period of five minutes. Myofibrils from these hearts were moderately relaxed so that the "I" bands were present. Myocardium subjected to 10 min of ischemia was characteristically lacking in glycogen granules in

perinuclear, perimitochondrial and subsarcolemmal sites in the myocardium; this change was considered a marker of shifting ATP-producing metabolism within the cell (1). Futhermore, at the same duration of ischemia, some mitochondria showed a loss of cristae and matrix damage. Following longer durations of ischemia or hypoxia, a well-typified sequence of cellular changes are present, and include relaxation of myofibrils, intracellular edema, swollen sarcoplasmic reticulum and further damage to the mitochondria (73). Controversy exists as to the nature of sarcolemmal damage following reperfusion of ischemic myocardium (3). Jennings and associates (3) cite increased osmotic load due to the accumulation of glycolytic metabolites during ischemia as the cause of explosive cell swelling upon reperfusion, thus physically rupturing the sarcolemma and eventually resulting in cell nerosis. However, several studies of total ischemia in isolated perfused hearts have found the sarcolemma to remain intact in conditions of ischemia-reperfusion (70, 74). Furthermore, hearts subjected to hypoxia fail to show sarcolemmal disruption upon reoxygenation (5, 6, 45). Upon reperfusion or reoxygenation of ischemic or hypoxic myocardium, electron-dense bodies become apparent within the matrices of the mitochondia (1, 71). Sudden contraction of myofibrils may be related to the appearance of large quantities of Ca^{2+} in the intracellular space (73). The separate but related nature of ultrastructural damage to the myocardium due to ischemic injury and to reperfusion injury

is becoming evident; much research is currently pursuing aspects of myocardial ischemia-reperfusion injury with regard to this theme (75).

Changes of the functional integrity of sarcolemma, mitochondria and sarcoplasmic reticulum:Ca^{2+} overload and ischemia-reperfusion injury. Physiologic variation of the rate of Ca^{2+} ion influx to the cytosol of the myocardium has a direct influence on the magnitude of contractile force due to excitation/inactivation of Ca^{2+}-dependent myofibrillar ATPase transforming phosphate-bond energy into mechanical work (76). The concept of intracellular Ca^{2+}-overload was put forward by Fleckenstein and associates (76) in association with catecholamine-induced high-energy phosphate breakdown and cardiac necrotization; however, intracellular Ca^{2+}-overload is also recognized as a major factor in ischemia-reperfusion injury and hypoxia-reoxygenation injury (6, 7, 36, 42, 45). As stated above, ischemic or hypoxic injury to the myocardium appears to be aggravated during subsequent reperfusion or reoxygenation (75, 77). Several investigators have shown that no change in total tissue calcium content takes place during ischemia or hypoxia unless hearts were subjected to very long durations of these interventions (6, 36, 74), whereas tissue calcium is markedly increased upon reoxygenation of hypoxic hearts (78). Furthermore, tissue Ca^{2+} was found to be dramatically elevated as a result of reperfusion of dog myocardium made ischemic for a period of 40 min (79).

Sarcolemmal injury. The fact that cardiac sarcolemma is involved in beat-to-beat regulation of Ca^{2+} influx and efflux to and from the intracellular space and furthermore that dysfunction of this membrane is associated with various pathological conditions is well documented (7, 14, 42). The occurrence of intracellular Ca^{2+} overload in the myocardial cell during ischemia-reperfusion challenge may be due to either an increase in Ca^{2+} influx or a decrease in Ca^{2+} efflux across the sarcolemma, or both. Increased sarcolemmal Ca^{2+} permeability has been demonstrated in association with Ca^{2+}-depletion injury (80), an intervention which is in some ways similar to cell damage incurred by hypoxia-reoxygenation (81, 87). Treatment of isolated rabbit hearts with verapamil prior to induction of hypoxia failed to prevent a net gain in tissue Ca^{2+} (82). Verapamil is known to block the passage of Ca^{2+} through the slow Ca^{2+} channels (76, 83, 84), and therefore it may be deduced that these channels are not involved in the creation of intracellular Ca^{2+} overload, a view which has been confirmed in several studies (85, 86).

Ganote and Kaltenbach (88) hypothesized that the oxygen-induced myocardial enzyme release of isolated rat heart preparations previously made hypoxic was due to contracture of myocytes at the moment of reoxygenation thus stretching and rupturing the sarcolemmal membrane and potentiating the leakage of intracellular proteins to the perfusate. However, studies indicating a lack of entry of cellular marker molecules into the intracellular space

refute this view (70, 74). Furthermore, in studies where aequorin was used to determine intracellular Ca^{2+} concentration, Allan and Orchard (89) report that the onset of myocardial contracture in hypoxia occurs in the absence of significant increases in intracellular Ca^{2+} concentration. In retrospect, cellular disruption with attendant physical disruption of the sarcolemma is not a clear-cut phenomenon in ischemia-reperfusion injury of the myocardium, and almost certainly is not a determinant of hypoxia-reoxygenation injury.

In view of massive sarcolemmal disruption as a component of myocardial injury in conditions of short duration hypoxia-reoxygenation or ischemia-reperfusion, altered Ca^{2+}-transporting function of sarcolemmal ion channels, ATPase proteins or exchange proteins has been examined in many studies (7, 12, 13, 42, 90, 91). Cardiac sarcolemmal Na^{+}-K^{+} ATPase activity is depressed in samples of ischemic myocardium (90). As stated previously, depression of this activity is thought to indirectly increase the intracellular concentration of Ca^{2+}, via intracellular Na^{+} buildup and countertransport of Na^{+} for Ca^{2+} (76), but this view remains unproven. Furthermore, Laustiola et al. (92) failed to observe an increase of Ca^{2+} influx in hypoxic hearts; rather, a decrease in Ca^{2+} influx was observed. Short-term ischemia-reperfusion of dog hearts was found to depress Ca^{2+}-stimulated ATPase (Ca^{2+}-pump) activity (13). Sarcolemmal Na^{+}-Ca^{2+} exchange activity was

altered in rabbit ventricle when ventricular slices were incubated 1-2 hours in a substrate-free medium gassed with 95% N_2 and 5% CO_2 (11). In addition, Na^+-dependent Ca^{2+} uptake activity was decreased in sarcolemmal vesicles from isolated rat hearts subjected to global ischemia for a period of 1 hour, which was reversible upon reperfusion with oxygenated medium for a period of 15 min (12). Similarly perfused hearts in conditions of high-flow hypoxia yielded sarcolemmal vesicles with unaltered Na^+-dependent Ca^{2+} uptake activity; these authors suggested that damage suffered by the myocardium in conditions of ischemia was fundamentally different from that caused by hypoxia (12).

Since Ca^{2+} influx to the intracellular space is either reduced or remains normal in the early stages of myocardial ischemia or hypoxia (6, 36, 93, 94), it appears that increased concentrations of intracellular Ca^{2+} associated with ischemia-reperfusion injury are potentiated at the moment of reperfusion or reoxygenation (6, 94). Attendent to increased intracellular concentration of Ca^{2+} is the activation of Ca^{2+}-dependent proteases, lysosomal enzymes and phospholipases which may damage mitochondrial, sarcoplasmic reticular and sarcolemmal membranes (95-97). Prostaglandins are thought to be involved in the development of irreversible ischemic injury to the myocardium (98). Additionally, reperfusion is proposed to initiate Ca^{2+}-dependent proteolysis of xanthine dehydrogenase to xanthine oxidase which forms free radicals from xanthine or hypoxanthine; these are metabolic breakdown products of

adenosine and are known to accumulate in the ischemic myocardium (99). As well, polymorphonuclear leukocytes may invade the ischemic myocardium (100) and promote the formation of oxidation products of catecholamines (101). Therefore irreversible damage occurring in conditions of ischemia-reperfusion may be due to a host of mechanisms.

Changes of the mitochondrial membrane. The primary role of the mitochondrial lies in the production of ATP via oxidative phosphorylation; in addition, mitochondria may sequester large quantities of Ca^{2+} by ATP-dependent mechanisms in pathophysiologic conditions (15). Therefore, the function of mitochondrial Ca^{2+}-transporting ability, ATP-production and respiration levels have been monitored in ischemic and postischemic myocardium. Schwartz et al. (55) found that mitochondrial calcium uptake was diminished in severely ischemic myocardium. They also reported severe impairment of electron transport capability of the mitochondria, thusly indicative of alterations in cellular respiration. Furthermore, carnitine-stimulated oxidation of palmitic acid was decreased. Ultrastructural changes of the mitochondria in irreversibly damaged myocardium include disorganization of cristae and amorphous densities known to be calcium phosphate crystals (1, 68). Excessive fluxes of Ca^{2+} and PO_4^{2-} are contributory to the formation of these bodies; the rise in cytoplasmic concentration of inorganic phosphate is believed to result from uncontrolled liberation of phosphate bond energy by intracellular Ca^{2+}-activated

ATPases (76). Sharma et al. (73) found that cellular ATP:ADP ratio was decreased in association with ultrastructural damage to the mitochondria. Furthermore, the extent of mitochondrial damage was more severe in hearts subjected to ischemia-reperfusion as opposed to those subjected to ischemia only. Trump et al. (102) point out that an inverse relationship develops between intramitochondrial Ca^{2+} content and ATP content with the progression of duration of ischemic injury to the myocardium. However, reperfusion during early ischemia may result in rapid recovery of mitochondrial function, and that massive mitochondrial matrix disruption upon reperfusion only occurs after a comparatively longer duration of ischemia (55, 103). Therefore, the duration of myocardial hypoxia or ischemia may be viewed as a critical parameter in consideration of the extent of ischemia-reperfusion injury to the functional integrity of mitochondria.

Sarcoplasmic reticular membrane changes. Before the subcellular sarcoplasmic reticulum was positively identified by researchers, it was referred to as the "soluble relaxing factor" named thusly because of its in vitro effect on actomyosin proteins (104-106). The sarcoplasmic reticulum is now known to rapidly sequester Ca^{2+} on a beat-to-beat basis, and thus plays a central role in excitation-contraction cycle. The nature of release of sarcoplasmic reticular stores of Ca^{2+} to the contractile proteins is as yet undefined. A number of investigators have examined the possibility of altered sarcoplasmic reticular function in

conditions of hypoxia-reoxygenation or ischemia-reperfusion (20, 21, 93, 107-110). Early investigation by Schwartz et al. (55) led to the discovery that Ca^{2+}-uptake of the sarcoplasmic reticulum was depressed in hearts ischemic for 12 to 60 min. This finding was later confirmed in a recent study (110) wherein sarcoplasmic reticular vesicles isolated from ischemic canine endocardium (but not epicardium) was found to sustain a loss of <u>in vitro</u> Ca^{2+}-transport and Ca^{2+}-ATPase activities, which paralled the changes in the histology of the tissue. The changes in the above activities occurred at 15 min ischemia, and were maximally depressed at 30 min duration of ischemia. Lee and Dhalla (111) correlated depressed sarcoplasmic reticular Ca^{2+} binding and Ca^{2+}-uptake activities with the occurrence of intracellular Ca^{2+} overload, and therefore, it is possible that this mechanism may contribute to the elevated cytoplasmic Ca^{2+} concentrations observed in ischemia. Furthermore, other workers have reported depressed microsomal Ca^{2+} binding and uptake activities from rat hearts deprived of oxygen and substrate for a period of 10 min (112). Nayler and associates (3) found a depression of Ca^{2+} binding activity in hearts subjected to ischemia for a period of 60 and 120 min. It should be pointed out that cAMP-dependent phosphorylation of the sarcoplasmic reticular membrane by exogenous or endogenous protein kinase was not different in control or ischemic areas of the same heart (110).

Intracellular acidosis may be a potential mediator of sarcoplasmic reticular dysfunction in ischemic conditions (107). A decrease in tension development has been correlated to acidosis in hypoxia and ischemia (59, 60). Furthermore, Mandel et al. (114), have found that a decrease in the rate of formation and decomposition of high energy phosphate intermediates in cardiac sarcoplasmic reticulum occurs between pH 6.0 and 6.8. The ability of the sarcoplasmic reticulum to take up and thereafter release calcium was impaired at low pH (115), and myocardial acidosis was shown to cause a five-fold increase in the free Ca^{2+} concentration required for development of 50% maximum tension by the myofilaments (116). Krause and Hess (107) found a 50% decrease in oxalate-supported sarcoplasmic reticular Ca^{+} uptake activity at pH 7.1 from canine myocardium subjected to ischemia for a period of 7.5 min. A further significant depression of this activity was observed at pH 6.4 under otherwise identical conditions. Control Ca^{2+} uptake activities were significantly depressed at pH 6.4 as compared to Ca^{2+} uptake activities observed at pH 7.1. In addition, decreased Ca^{2+} uptake activity was correlated to decreased Ca^{2+}-stimulated, Mg^{2+}-dependent ATPase activity, and suggestion was made that the major effect of ischemia is to depress the sarcoplasmic reticular Ca^{2+}-ATPase activity (108). Therefore, depressed myocardial contractility observed in conditions of hypoxia-reoxygenation or ischemia-reperfusion may be partly due to a defect in the Ca^{2+}-transporting property of this membrane.

The present study was undertaken in order to examin changes in sarcolemmal Na^+-Ca^{2+} exchange and ATP-depend Ca^{2+} accumulation in hearts subjected to hyp reoxygenation. In addition, contractile activity o hearts was assessed in order to examine the possibi correlation between changes in sarcolemmal Ca^{2+} and heart function.

III. METHODS

Model of hypoxia-reoxygenation injury. Male Sprague-Dawley rats weighing 250-300 g were killed by decapitation and hearts were rapidly excised and placed in ice-cold buffer. Hearts were then arranged for coronary perfusion via nonrecirculating Langendorff technique at 37°C with oxygenated Krebs-Henseleit solution, pH 7.4, containing (in mM) NaCl 120.0, $NaHCO_3$ 250.0, KCl 4.6, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.25, and glucose 11.0. Hearts were electrically stimulated (Phipps and Bird, Inc. stimulator) at 240 pulses/min via a square wave of 1.5 ms duration at twice the threshold voltage. The coronary flow was maintained at 9 ml/min for different intervals by a Harvard peristaltic pump. Hypoxia was induced by perfusion with Krebs-Henseleit solution (without glucose) gassed with 95% N_2 and 5% CO_2, whereas reoxygenation in these hearts was carried out for 20 min with oxygenated Krebs-Henseleit solution containing 1.25 mM $CaCl_2$ and 11 mM glucose. In some hearts, 10 min of hypoxia was followed by reoxygenation of 1, 3, 5, and 10 min duration. Hearts perfused for appropriate time intervals with normal Krebs-Henseleit solution served as controls. Hearts were allowed to equilibrate for 10 min prior to any of the above experimental interventions.

Measurements of contractile characteristics. Contractile force development was monitored with a force displacement transducer (Grass FT. 03) on a Grass polygraph. Maximal rates of contractile force generation (+ dF/dt) and

maximal relaxation rates (- dF/dt) of isolated hearts were measured. A resting tension of 2 gm was applied to the heart at the beginning of each experiment and changes in this parameter due to hypoxia-reoxygenation were recorded.

Preparation of cardiac sarcolemma. Sarcolemma membrane was isolated from a pool of two hearts similarly treated according to the method of Pitts (117). Ventricles were washed, minced and homogenized in 0.6 M sucrose, 10 mM imidazole/HCl, pH 8.3 (3.5 ml/g tissue) with a polytron PT 20 (5X 20 sec, setting 5). The homogenate was centrifuged at 12,000 xg for 30 min and the pellet was discarded. After diluting (5 ml/g tissue) with 160 mM KCl, 20 mM 3- (N-morpholino) propanesulphonic acid (MOPS), pH 7.4 (KCl/MOPS), the supernatant was centrifuged at 96,000 xg for 60 min. The resulting pellet was resuspended in KCl/MOPS and layered over a 30% sucrose solution containing 0.3 M KCl, 50 mM $Na_4P_2O_7$ and 0.1 M Tris/HCl, pH 8.3. After centrifugation at 95,000 xg for 90 min (utilizing a Beckman swining bucket rotor), the band at the sucrose-buffer interface was taken and diluted with 3 volumes of KCl/MOPS solution. A final centrifugation at 96,000 xg for 30 min resulted in a pellet enriched in sarcolemma. The pellet was resuspended in 160 mM NaCl, 20 mM MOPS and the net sarcolemmal yield (mg protein/g tissue) was measured using the method of Lowry et al. (118). All isolation steps were carried out at 0-4^{o}C and assays were performed immediately after isolation of the membrane fraction.

Determination of Na^+-Ca^{2+} exchange and ATP-dependent Ca^{2+}-accumulation. Na^+-Ca^{2+} exchange measurements were carried out by the method of Reeves and Sutko (30) with a few minor modifications. Briefly, 10 µl Na^+-loaded sarcolemmal vesicles (10-15 µg protein) were diluted 1:50 in 160 mM KCl, 20 mM MOPS (KCl/MOPS), pH 7.4, and varying concentrations of $^{45}CaCl_2$ (39.4 mCi/mg) in a total volume of 500 µl and incubated at 37°C. After the appropriate span of time, the reaction was stopped by the addition of 100 µl of 5 mM La Cl_3 and KCl/MOPS, pH 7.4. Samples of 100 µl were filtered through a Millipore filter (pore size 0.45 µm) and washed with 1 ml of ice-cold 1 mM $LaCl_3$ and KCl/MOPS, pH 7.4. In parallel to these samples, non-specific calcium uptake was measured by placing Na^+-loaded vesicles in an equimolar medium containing 160 mM NaCl and 20 mM MOPS; these values were subtracted from the total Ca^{2+} uptake in Na^+-loaded vesicles for obtaining Na^+-dependent Ca^{2+} uptake activity. ATP-dependent calcium accumulation was determined by the method described by Caroni and Carafoli (10). The desired free Ca^{2+} concentration was maintained by the addition of ethylene glycol-bis-(β-amino ethyl ether)-N, N'-tetra-acetate (EGTA), and the free Ca^{2+} concentrations present were calculated as previously outlined (119). Filters were measured for radioactivity in 10 ml of scintillation fluid as described elsewhere (119). Non-specific Ca^{2+} accumulation was measured by determining vesicular calcium content in the absence of ATP and these values were subtracted from the total Ca^{2+} accumulation to

obtain the ATP-dependent Ca^{2+} uptake.

Gel electrophoresis. Electrophoresis of protein in sodium dodecyl sulphate-polyacrylamide gel (10%) was performed according to Laemmli (120). Before electrophoresis was performed, membrane proteins (1 mg) were solubilized at 37°C for 2 h in a mixture containing 1% sodium dodecyl sulphate (SDS), 1% mercaptoethanol, and 0.001% bromophenol blue in 10 mM sodium phosphate buffer (pH 7.0). Approximately 30 μg membrane protein was placed on the slab gel surface, and the gel was then run at room temperature for 3-4 h with a current of 30 mA. Gels were stained with coomassie brilliant blue and destained with 7% acetic acid. The resulting bands were scanned at 633 nm in an LKB 2202 laser densitometer. Molecular weights of the protein peaks were estimated by running known molecular weight standards (Sigma, Dalton Mark VI, SDS-6) under identical conditions.

Determination of membrane phospholipid, cholesterol content and membrane marker enzymes. Phospholipids and cholesterol in cardiac sarcolemmal fractions were determined by methods previously described (119). Na^+-K^+ ATPase activities were assayed as a marker of sarcolemmal purity according to procedures outlined elsewhere (121). K^+-EDTA-stimulated ATPase activity was measured by the procedure described by Martin et al. (122). Cytochrome c oxidase activity (123) and rotenone-insensitive NADPH cytochrome c reductase activities were measured as described (124) to

determine the extent of contamination of the sarcolemmal fraction with mitochondria and sarcoplasmic reticulum, respectively. To further exclude possible contamination by the sarcoplasmic reticulum, ATP-dependent Ca^{2+} uptake was also tested in the presence of 2 mM oxalate.

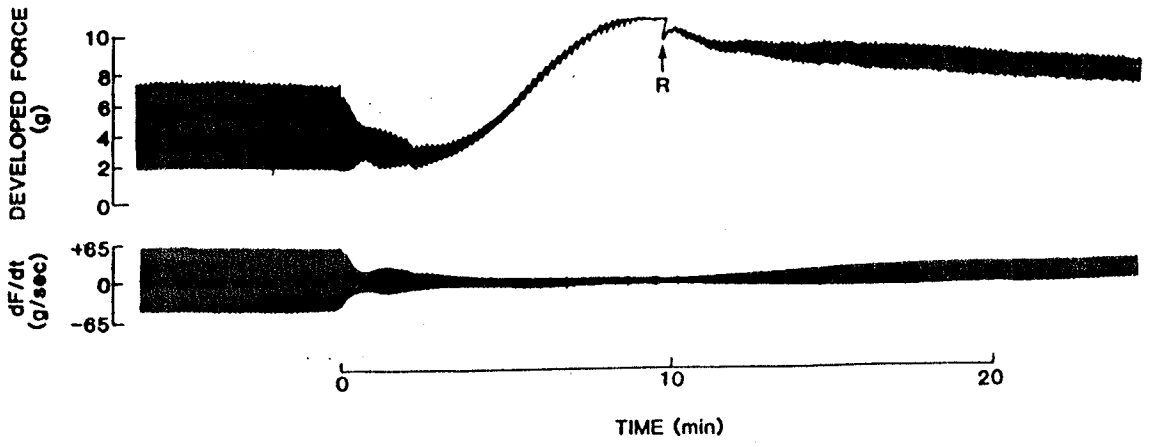
Determination of passive Ca^{2+} and Na^{+} accumulation. Sarcolemmal vesicles (50 μg) suspended in 160 mM KCl, 20 mM MOPS, were preincubated (in the absence of ATP) in the uptake medium (450 μl) containing 160 mM KCl, 20 mM MOPS, pH 7.4. Passive calcium accumulation was initiated by the addition of $^{45}CaCl_2$ bringing the total volume to 500 μl. Passive Na^{+} accumulation was carried out as follows; sarcolemmal vesicles (50 μg) suspended in 160 mM, 20 mM MOPS were added to medium containing 160 mM $^{22}NaCl$ (622.4 mCi/mg), 20 mmol/l MOPS (total volume was 500 μl). Vesicles were filtered and washed at the appropriate times and the radioactivity of filters was assayed.

Data analysis. Results are presented as a mean $\pm$ S.E. The statistical differences between mean values for two groups were evaluated by the student's t-test. For comparison of more than two groups, multiple analysis of variance was carried out and Duncan's new multiple-range test was used to determine differences between the means within the population.

IV. RESULTS

Changes in developed force and resting tension in hypoxic and reoxygenated rat hearts. To investigate a possible relationship between contractile characteristics and sarcolemmal function in hypoxia-reoxygenation, contractile force and resting tension alterations under varying duration of hypoxia were measured. As described by Nayler et al. (36), perfusion of the isolated rat hearts with hypoxic medium resulted in a marked depression in the contractile force development and dF/dt whereas reoxygenation resulted in a partial recovery (Fig. 1). Contracture, as reflected by increased resting tension of hearts exposed to hypoxia, was also apparent (Fig. 1). Five min perfusion of hearts with hypoxic medium depressed contractile force by 80% whereas resting tension was about 250% of the control value. (Table 1). Reoxygenation of the 5 min hypoxic hearts resulted in an 85% recovery of contractile force development and a return of resting tension to near control levels (118%) was observed. Perfusion of hearts with hypoxic medium over a duration of 10 min resulted in about 90% depression in contractile force (Table 1). Resting tension of the same hearts was increased 420% of the control value. Reoxygenation of these hearts for 20 min resulted in retention of 65% depression in contractile force while resting tension was 210% of the control value, which is relatively 50% of the state of contracture of the hypoxic hearts. Perfusion of hearts for

FIGURE 1.



Time effect of hypoxia on the rate of contractile force development (dF/dt) and the contractile force (C.F.) of electrically-stimulated perfused rat heart in a typical experiment. Perfusion of the heart with hypoxic medium was initiated at time 0 min; subsequent reoxygenation was at 10 min, as marked by "R".

TABLE 1. Contractile force development and resting tension of hypoxic and reoxygenated rat heart.

Time of hypoxia/ reoxygenation	Developed contractile force (%)	Increase in resting tension (%)
5 min hypoxia	19.5 ± 4.10	243 ± 8.51
5 min hypoxia + 20 min reoxygenation	84.5 ± 7.00	118 ± 9.51
10 min hypoxia	10.5 ± 0.790	420 ± 11.8
10 min hypoxia + 20 min reoxygenation	34.7 ± 3.09	210 ± 11.2
30 min hypoxia	0	453 ± 10.5
30 min hypoxia + 20 min reoxygenation	0	300 ± 17.2

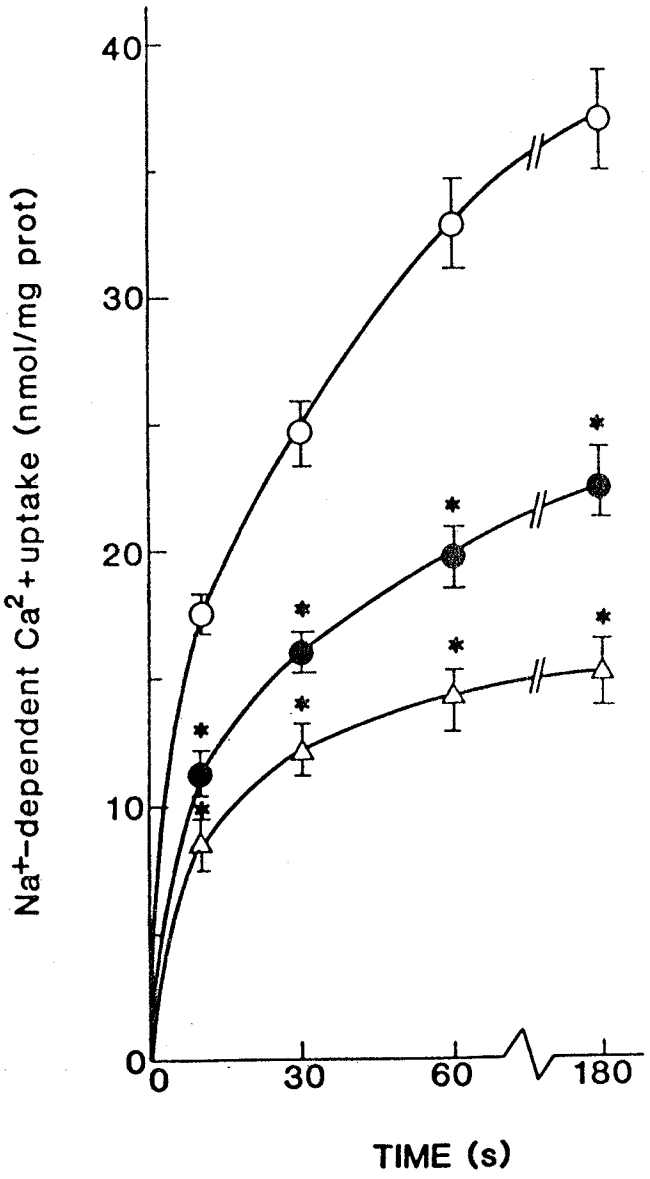
Each value is a mean ± S.E. of six experiments. The results are expressed as % of the control developed contractile force or resting tension before starting perfusion with hypoxic medium. Initial resting tension on hearts was 2 g; mean ± S.E. of contractile force development was 5.7 ± 0.32 g.

30 min with hypoxic medium completely abolished development of contractile force and resulted in a 450% increase in resting tension (Table 1). Reoxygenation of these hearts resulted in no improvement in developed contractile force whereas resting tension was reduced relative to hypoxic hearts (300% of the control value).

Alterations in sarcolemmal Na^+-Ca^{2+} exchange in hypoxia-reoxygenation. Perfusion of hearts with hypoxic medium for 5 min did not significantly alter Na^+-dependent Ca^{2+} uptake; reoxygenation of these hearts also resulted in no significant change (Table 2). A longer duration of hypoxic perfusion (10-30 min) resulted in a significant depression of Na^+-Ca^{2+} exchange activities in sarcolemmal vesicles of both hypoxic and reoxygenated hearts. However, no changes in the nonspecific Ca^{2+} uptake was detected under similar conditions (Table 2).

Figure 2 shows the effect of 10 min hypoxia and 20 min reoxygenation on the heart-sarcolemmal Na^+-dependent Ca^{2+} uptake in hypoxic as well as reoxygenated hearts. From Fig. 2 it can be seen that vesicles from hypoxic hearts showed a 35-40% depression in Na^+-Ca^{2+} exchange activity while a 50-60% depression was evident in vesicles from reoxygenated hearts as compared to control values at different times of incubation. In a separate set of experiments, the effects of hypoxia-reoxygenation were studied on the initial rates of Na^+-dependent Ca^{2+} uptake activities under carefully controlled conditions. Na^+

FIGURE 2.



Time course of Na^+-dependent Ca^{2+} uptake in sarcolemmal vesicles. The effect of perfusion of hearts for 10 min with hypoxic medium (●—●) and reoxygenation of hypoxic hearts for 20 min (△—△). Control hearts were perfused for 10 to 30 min with normal Krebs-Henseleit medium (○—○). Na^+-dependent Ca^{2+} uptake was determined in the presence of 40 μM Ca^{2+}. Each value is a mean ± S.E. of six experiments. *Significantly different from control values where $P < 0.05$.

TABLE 2. Effects of different times of hypoxia and reoxygenation on Na^+-dependent Ca^{2+} uptake.

Time of hypoxia/ reoxygenation	Na^+-dependent Ca^{2+} uptake (nmol/mg protein/10 sec)	Nonspecific Ca^{2+} uptake (nmol/mg protein/10 sec
Control	17.2 ± 1.39	2.92 ± 0.21
5 min hypoxia	20.4 ± 1.09	2.82 ± 0.19
5 min hypoxia + 20 min reoxygenation	18.0 ± 1.17	3.11 ± 0.24
10 min hypoxia	13.1 ± 0.96*	3.05 ± 0.23
10 min hypoxia + 20 min reoxygenation	10.3 ± 0.66*	3.02 ± 0.17
30 min hypoxia	12.7 ± 0.66*	2.88 ± 0.16
30 min hypoxia + 20 min reoxygenation	11.5 ± 0.86*	3.21 ± 0.29

Each value is a mean ± S.E. of four to six experiments. Control values are the result of perfusion of hearts for 5 to 30 min with normal Krebs-Henseleit solution. Nonspecific calcium uptake measured by placing Na^+-loaded vesicles in equimolar NaCl uptake medium. Calcium concentration was 40 μM in all experiments.
*Significantly different from control values ($P < 0.05$).

-dependent Ca^{2+} uptake values at 5 sec, 10 sec, and 15 sec incubation periods were 8.5 $\pm$ 0.4, 17.2 $\pm$ 0.7 and 23.1 $\pm$ 1.1 nmol Ca^{2+}/mg protein for the control hearts (n = 6), 6.2 $\pm$ 0.3, 13.0 $\pm$ 0.4, and 18.7 $\pm$ 0.7 nmol Ca^{2+}/mg protein for the 10 min hypoxic hearts (n = 5) and 5.1 $\pm$ 0.2, 9.8 $\pm$ 0.3 and 14.2 $\pm$ 0.5 nmol Ca^{2+}/mg protein for the reoxygenated hearts (n = 4), respectively. These results indicate that under the conditions employed in the study, the Na^{+}-dependent Ca^{2+} uptake activities in the control and experimental hearts were essentially linear during the 15 sec incubation period and that hypoxia-reoxygenation depressed the initial rates of Na^{+}-dependent Ca^{2+} uptake activities. It is pointed out that some workers have reported linearity of Na^{+}-dependent Ca^{2+} uptake in the heart sarcolemmal vesicles for 1 to 2 sec (11) and this may be due to differences in the experimental design employed in their laboratory.

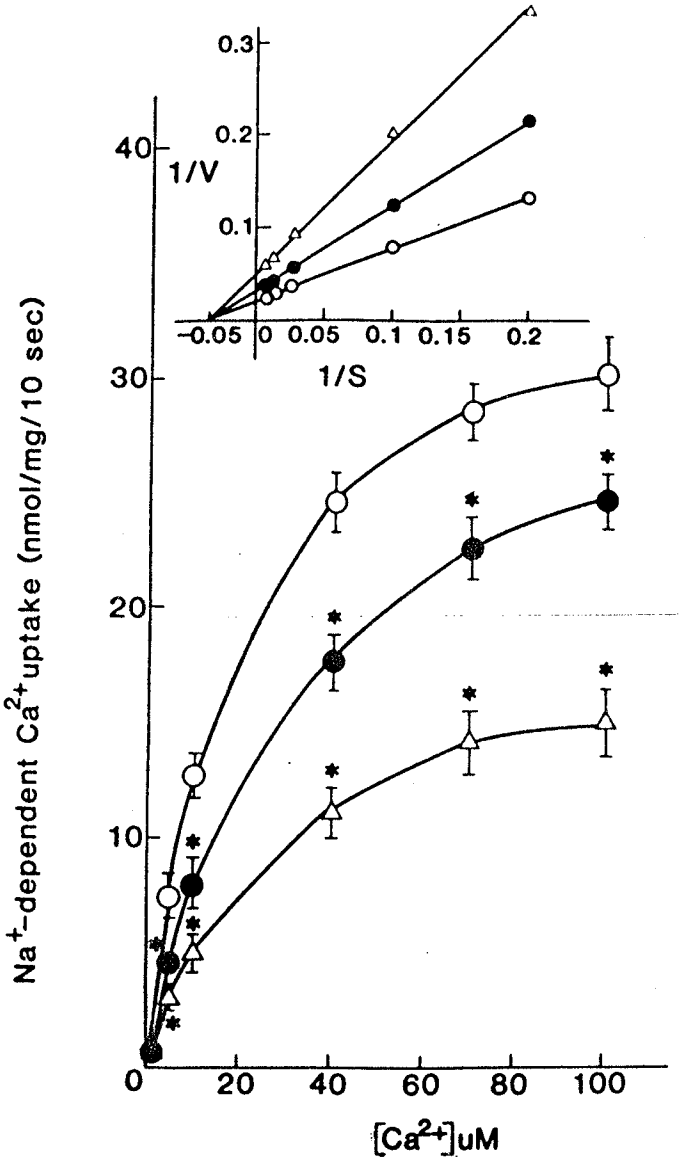
Sarcolemmal Na^{+}-dependent Ca^{2+} uptake activities were also examined in response to hypoxia (10 min) and reoxygenation (20 min) upon varying the concentrations of Ca^{2+} in the incubation medium (Fig. 3). A significant depression of Na^{+}-Ca^{2+} exchange activity was observed in vesicles of hypoxic hearts as compared to control values. Vesicles of reoxygenated hearts showed a further depression of Ca^{2+} uptake values. Representation of this data on a double-reciprocal plot illustrates the non-competitive nature of hypoxia-reoxygenation induced depression of Na^{+}-

Ca^{2+} exchange activities. The Ka (20-25μM Ca^{2+}) of the Na^{+}-Ca^{2+} exchange protein was apparently not affected by hypoxia or reoxygenation.

To examine the effects of duration of reoxygenation of hypoxic hearts on Na^{+}-Ca^{2+} exchange activities, reoxygenation of hearts made hypoxic for 10 min was carried out at 1, 3, 5 and 10 min duration (Table 3). Depression of Na^{+}-dependent Ca^{2+} uptake occurred after 1 min of reoxygenation and remained depressed for all subsequent periods reoxygenation. Non-specific Ca^{2+} uptake values were similar in control, hypoxic and reoxygenated samples. It should also be noted that 90-95% of the calcium accumulated in the Na^{+}-loaded vesicles from control, hypoxic and reoxygenated hearts was released within 3 min after exposure to 40 mM Na^{+}.

Alterations of ATP-dependent Ca^{2+} accumulation in hypoxia-reoxygenation. To examine the possibility of altered sarcolemmal Ca^{2+}-transport other than that attributed to Na^{+}-Ca^{2+} exchange, ATP-dependent Ca^{2+} accumulation was assayed in control, hypoxic and reoxygenated hearts (Table 4). Vesicles from hearts perfused for 5 min with hypoxic medium showed no change in the ATP-dependent Ca^{2+} accumulation from the control value. Reoxygenation of these (5 min) hypoxic hearts significantly depressed the vesicular ATP-dependent Ca^{2+} accumulation. Hearts perfused with hypoxic medium for 10 and 30 min yielded vesicles with significantly depressed ATP-dependent Ca^{2+} accumulation activity; this activity was depressed to a

FIGURE 3.



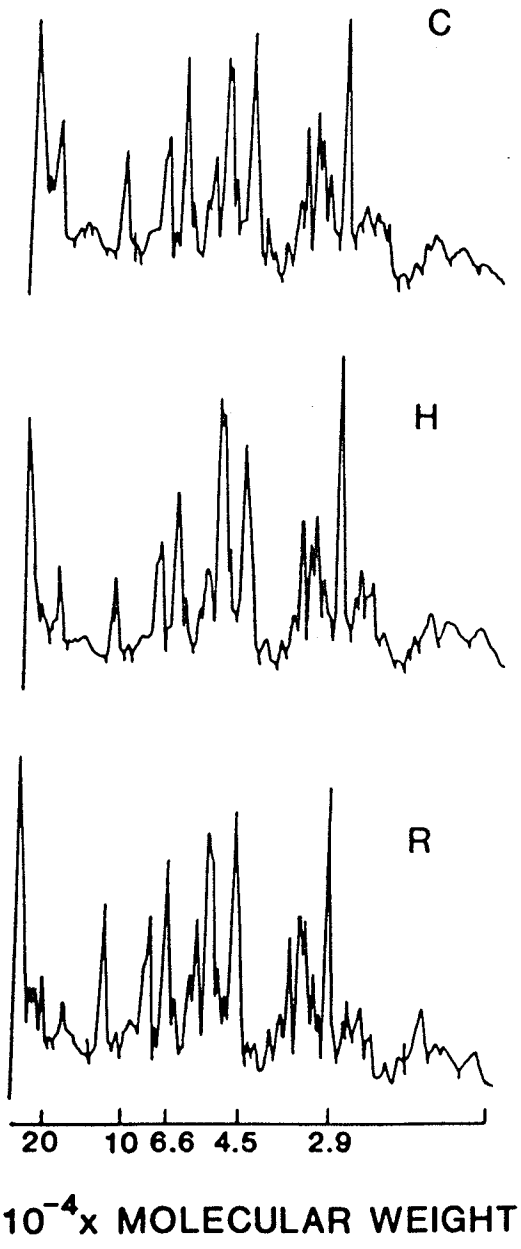
Na^+-dependent Ca^{2+} uptake as a function of calcium concentration in sarcolemmal vesicles. The effects of perfusion of hearts with hypoxic medium for 10 min (●—●), reoxygenation of hypoxic hearts for 20 min (△—△), and control hearts perfused for 10 to 30 min with normal Krebs-Henseleit medium (○—○). Na^+-dependent Ca^{2+} uptake was measured at 10 sec at all points. Each value is a mean ± S.E. of six experiments. *Significantly different from control values where $P < 0.05$.

TABLE 3. Effect of various times of reoxygenation of hypoxic (10 min) rat hearts on Na^+-dependent Ca^{2+} uptake.

Time of hypoxia/ reoxygenation	Na^+-dependent Ca^{2+} uptake (nmol/mg protein/10 sec)	Nonspecific Ca^{2+} uptake (nmol/mg protein/10 sec)
Control	18.8 ± 1.26	2.97 ± 0.65
10 min hypoxic	13.7 ± 1.38*	3.29 ± 0.92
1 min reoxygenation	10.8 ± 0.11**	2.50 ± 0.73
3 min reoxygenation	9.81 ± 0.34**	2.68 ± 0.60
5 min reoxygenation	9.87 ± 0.63**	3.70 ± 0.65
10 min reoxygenation	8.57 ± 0.65**	2.96 ± 0.54

Each value is a mean ± S.E. of four to six experiments. Control values are the result of perfusion of hearts for 10 to 30 min with normal Krebs-Henseleit solution. Nonspecific calcium uptake measured by placing Na^+-loaded vesicles in equimolar NaCl uptake medium. *Significantly different from control value ($P < 0.05$). **Significantly different from hypoxic value ($P < 0.05$). Calcium concentration was 40 μM in all experiments.

FIGURE 4.



Densitometric scans representative of sarcolemmal proteins separated by SDS gel electrophoresis. C: control; H: hypoxic (10 min); R: reoxygenated (20 min after 10 min hypoxia).

TABLE 4. Effects of hypoxia and reoxygenation on ATP-dependent Ca^{2+} accumulation.

Time of hypoxia/ reoxygenation	ATP-dependent Ca^{2+} accumulation (nmol/mg protein/5 min)	Nonspecific Ca^{2+} accumulation (nmol/mg protein/5 min)
Control	18.5 ± 1.14	5.22 ± 0.176
5 min hypoxia	19.4 ± 2.54	5.40 ± 0.108
5 min hypoxia + 20 min reoxygenation	14.7 ± 1.05*	5.52 ± 0.052
10 min hypoxia	13.4 ± 1.04*	4.72 ± 0.302
10 min hypoxia + 20 min reoxygenation	9.82 ± 1.10*	4.78 ± 0.264
30 min hypoxia	12.2 ± 0.046*	5.18 ± 0.146
30 min hypoxia + 20 min reoxygenation	9.8 ± 0.884*	4.90 ± 0.182

Each value is a mean ± S.E. of four to six experiments. Control values are the result of perfusion of hearts for 5 to 30 min with normal Krebs-Henseleit solution. Nonspecific Ca^{2+} accumulation was measured in the absence of ATP. Calcium concentration was 10 μM in all experiments. *Significantly different from control values ($P < 0.05$).

similar extent in vesicles of respective reoxygenated hearts. Non-specific Ca^{2+} accumulation was unchanged in control, hypoxic and reoxygenated vesicles.

Marker Enzyme Studies. In order to exclude the possibility that the observed alterations of Ca^{2+} transport may be due to differential purification of the sarcolemmal vesicles from control, hypoxic and reoxygenated hearts, activities of marker enzymes of different subcellular organelles were examined (Table 5). Sarcolemmal Na^+-K^+ ATPase activities were significantly depressed in hypoxic samples which is in agreement with previous work (42, 90). No recovery of this activity was observed in vesicles of reoxygenated hearts. The sarcolemmal Na^+-K^+ ATPase activities showed 18.5, 19.2 and 18.7 fold purification with respect to the homogenate activities in control, hypoxic and reoxygenated hearts, respectively. Approximately 10% of the sarcolemmal Na^+-K^+ ATPase activities in control, hypoxic and reoxygenated hearts were inhibited by 1 mM ouabain indicating that the sarcolemmal vesicles were predominantly of inside-out orientation. The Na^+-K^+ ATPase activities in all these preparations were inhibited completely by ouabain upon treating the vesicles with 0.2 mg/ml deoxycholate. K^+-EDTA-stimulated ATPase was undetectable in control, hypoxic and reoxygenated preparations ruling out the existence of myofibrillar contamination. Mitochondrial and microsomal contamination of sarcolemmal membrane fractions from control, hypoxic and reoxygenated samples were negligible as indicated by

TABLE 5. Sarcolemmal yield and marker enzyme activities from sarcolemmal vesicles of control, hypoxic and reoxygenated rat heart.

	Control	10 min hypoxia	10 min hypoxic + 20 min reoxygenated
Sarcolemmal yield	1.2 ± 0.46	1.3 ± 0.31	1.3 ± 0.39
Na^+-K^+ ATPase	23.2 ± 1.31	17.4 ± 1.55*	16.3 ± 1.92*
Ouabain sensitive Na^+-K^+ ATPase	2.4 ± 0.71	1.8 ± 0.79	1.5 ± 0.86
Cytochrome c oxidase	49.1 ± 4.60	51.2 ± 5.32	45.6 ± 3.71
K^+-EDTA ATPase	ND	ND	ND
NADPH cytochrome c reductase	4.2 ± 0.41	4.1 ± 0.39	4.0 ± 0.48

ATPase activities are expressed in μmol Pi/mg protein/hr whereas cytochrome c oxidase and rotenone-insensitive NADPH cytochrome c reductase, which are presented in nmol cytochrome c/mg protein/min. ND, not detectable. Sarcolemmal yield is expressed in mg/g wet heart. Perfusion of hearts for 10 to 30 min with normal Krebs-Henseleit medium were taken as control.

*$P < 0.05$ vs. control.

relatively low activities of cytochrome c oxidase and rotenone-insensitive NADPH cytochrome c reductase, respectively. Furthermore, the activities of cytochrome c oxidase and rotenone-insensitive NADPH cytochrome c reductase were 0.2 and 0.3 fold of those in heart homogenate; no difference in purification factor of these enzymes existed in control, hypoxic and reoxygenated samples. ATP-dependent Ca^{2+} accumulation in membrane vesicles from the control and experimental hearts was not augmented by the presence of 2 mM oxalate, which has been shown to increase ATP-dependent Ca^{2+} accumulation in sarcoplasmic reticulum. These data suggest that the relative contamination of control, hypoxic, and reoxygenated samples was minimal but similar.

Passive flux of Na^+ and Ca^{2+} sarcolemmal vesicles of control, hypoxic and reoxygenated hearts. Passive Ca^{2+} accumulation and passive Na^+ accumulation were assayed in separate experiments to detect variations of membrane permeability in sarcolemmal vesicles from control, hypoxic and reoxygenated hearts. As shown in Table 6, passive Na^+ accumulation in sarcolemmal vesicles over a duration of 30 min was not different in control and experimental samples. A similar passive uptake of Ca^{2+} in vesicles from control, hypoxic and reoxygenated hearts was also apparent (Table 7). These results are consistent with the view that the permeability characteristics of control, hypoxic and reoxygenated heart sarcolemmal vesicles are similar to each other.

Sarcolemmal composition during hypoxia-reoxygenation. Table 8 illustrates phospholipid and cholesterol concentration in vesicles from control, hypoxic and reoxygenated hearts. Phosphatidic acid was decreased whereas diphosphatidyl glycerol was increased in reoxygenated samples. It should be noted that intamembronol level of major phospholipids as well as of phosphatidylserine and phosphatidylinositol were unchanged in hypoxic and reoxygenated samples (Table 8). No major differences were observed in the protein composition of control, hypoxic and reoxygenated samples (Fig. 4) as densitometric scans demonstrate the absence of new peaks when representative gel patterns were compared; differential contamination of the sarcolemmal fractions under study therefore, seems negligible.

TABLE 6. Passive Na^+ accumulation of sarcolemmal vesicles of control, hypoxic and reoxygenated rat heart.

Perfusion	Passive Na^+ accumulation (nmol/mg protein) Incubation Time (min)				
	1	3	5	10	30
Control	63.0 ± 4.7	285 ± 17.4	548 ± 43.1	722 ± 57.1	759 ± 48.4
10 min hypoxia	67.0 ± 6.2	271 ± 23.8	529 ± 46.5	709 ± 57.1	749 ± 59.2
10 min hypoxia + 20 min reoxygenation	70.5 ± 5.2	262 ± 20.9	523 ± 49.1	699 ± 48.3	744 ± 45.8

Values are mean ± S.E. of 4 experiments. Sarcolemmal vesicles were suspended in 160 mM $^{22}NaCl$, 20 mM 3-(N-morpholino) - propanesulphonic acid (MOPS) buffer, pH 7.4 and were incubated at 37°C for different times. Hearts perfused with normal Krebs-Henseleit for 10 to 30 min were taken as controls.

TABLE 7. Passive Ca^{2+} accumulation of sarcolemmal vesicles of control, hypoxic, and reoxygenated rat heart.

Perfusion	Passive Ca^{2+} accumulation (nmol/mg protein) Incubation Time (min)					
	0.25	1	3	5	10	30
Control	2.2 ± 0.2	4.2 ± 0.51	7.1 ± 0.69	9.0 ± 0.86	11.9 ± 1.1	12.7 ± 1.3
10 min hypoxia	2.3 ± 0.16	3.7 ± 0.32	7.2 ± 0.78	9.2 ± 0.94	10.2 ± 0.77	11.8 ± 0.93
10 min hypoxia + 20 min reoxygenation	2.1 ± 0.22	4.0 ± 0.60	7.9 ± 0.81	9.3 ± 0.80	11.2 ± 0.91	12.3 ± 1.01

Values are mean ± S.E. of 4 experiments. Sarcolemmal vesicles were suspended in uptake medium containing 40 μM $^{45}CaCl_2$, 160 mM KCl, 20 mM 3-(N-morpholino) - propanesulphonic acid (MOPS) buffer, pH 7.4 and were incubated at 37°C. Hearts perfused for 30 min with Krebs-Henseleit solution for 10 to 30 min were taken as controls.

TABLE 8. Phospholipid composition and cholesterol content of sarcolemma from control, hypoxic and reoxygenated rat hearts.

	Control	10 min hypoxic	10 min hypoxic + 20 min reoxygenated
Phospholipids, (nmol Pi/mg)			
Phosphatidylcholine	132 ± 10.3	164 ± 10.3	155 ± 10.2
Lysophosphatidylcholine	4.6 ± 1.9	5.6 ± 0.7	3.5 ± 0.3
Phosphatidylethanolamine	109 ± 8.1	134 ± 13.1	123 ± 7.7
Sphingomyelin	25.4 ± 2.4	30.8 ± 4.1	24.3 ± 1.7
Phosphatidylserine	23.5 ± 2.8	26.4 ± 2.5	21.9 ± 1.8
Phosphatidylinositol	13.1 ± 1.6	16.2 ± 1.2	12.4 ± 0.8
Diphosphatidylglycerol	20.2 ± 0.9	22.5 ± 3.2	27.7 ± 1.3*
Phosphatidic Acid	2.7 ± 0.06	2.9 ± 0.04	2.0 ± 0.03*
Total Phospholipid	435.6 ± 33.8	493.5 ± 24.6	488.3 ± 21.9
Cholesterol, nmol/mg	98.2 ± 4.1	113.1 ± 5.9	98.1 ± 2.9
Cholesterol:phospholipid molar ratio	0.231 ± 0.01	0.233 ± 0.01	0.203 ± 0.01

Values are ± S.E. of 6 experiments using separate membrane preparations. Hearts perfused 10 to 30 min with normal Krebs-Henseleit solution were taken as controls.

*Significantly different from the control values ($P < 0.05$).

V. DISCUSSION

Ischemic-reperfusion injury is generally regarded to result in the loss of ability of myocardial cells to maintain normal calcium homeostasis and that this may lead to intracellular accumulation of calcium and subsequent cell death (3, 79). Likewise, hypoxia-reoxygenation injury of the myocardium has been documented to produce an intracellular Ca^{2+} overload (36, 42) and the mechanisms for such an event are considered to reside in membrane-bound Ca^{2+} transporting systems. In this study we have demonstrated a depression in the initial rate as well as capacity of Na^{+}-dependent Ca^{2+} uptake in sarcolemmal vesicles obtained from hearts perfused with hypoxic medium (substrate-free) for a period of 10 min or more. This finding is in contrast to other investigators (12) who failed to detect a change in the sarcolemmal Na^{+}-Ca^{2+} exchange activity upon perfusing hearts for 90 min with hypoxic medium (in the presence of glucose). Although these workers were able to show a depression in the initial rate of Na^{+}-dependent Ca^{2+} uptake in sarcolemmal vesicles using globally ischemic rat hearts (12) the capacity of Na^{+}-Ca^{2+} exchange was not altered in their experiments. Since their control values for the Na^{+}-dependent Ca^{2+} uptake in the sarcolemmal vesicles were rather low (1.248 nmol Ca^{2+}/mg protein/10 sec at 75 μM Ca^{2+}) in comparison to our study (28 nmol Ca^{2+}/mg protein/10 sec at 70 μM Ca^{2+}), it is possible that the differences in these studies may be due to different methods employed for the determination of Na^{+}-Ca^{2+}

exchange activities. It should be noted that Bersohn and associates (11) have also reported a 50% depression of initial rate, but not of the capacity of the Na^+-dependent Ca^{2+} uptake in sarcolemmal vesicles obtained from rabbit ventricular slices which were incubated in glucose-free Tyrode's solution in the absence of oxygen for 1 hr. Since the control values for the sarcolemmal Na^+-dependent Ca^{2+} uptake reported by these workers (20 nmol Ca^{2+}/mg protein/10 sec at 40 μM Ca^{2+}) were comparable to those reported in this study (17 nmol Ca^{2+}/mg protein/15 sec at 40 μM Ca^{2+}), the observed discrepancy in results with respect to changes in the capacity of Na^+-Ca^{2+} exchange in hypoxic heart may be due to the experimental models employed in these studies. The depression in the sarcolemmal Na^+-dependent Ca^{2+} uptake in hypoxic heart was associated with a decrease in the V_{max} value without any changes in the affinity of the Na^+-Ca^{2+} exchange system for Ca^{2+}. Similar results have also been reported by Bersohn et al. (11) by employing the above mentioned in vitro model of ischemia.

Rapid exacerbation of cell damage occurring immediately after reoxygenation of hypoxic hearts is a characteristic of hypoxia-reoxygenation damage in the myocardium. Our results indicate that a rapid further depression of Na^+-Ca^{2+} exchange activity occurs in syncrony with reoxygenation of hypoxic hearts. Depression of Na^+-Ca^{2+} exchange activities may be a contributing factor to eventual necrosis of the myocardial cell via a buildup of intracellular Ca^{2+} in the

myocardium as this system may be involved in the efflux of Ca^{2+} from the cardiac cell (9). Furthermore, the observed depression of sarcolemmal ATP-dependent Ca^{2+} uptake in hearts perfused with hypoxic (substrate-free) medium for 10 min or more as well as upon reoxygenation is in agreement with results by Chemnitius et al. (13) who studied repeated ischemia-reperfusion injury in canine hearts. These workers have reported a decrease in both the initial rate and capacity of ATP-dependent Ca^{2+} uptake in sarcolemmal vesicles following ischemia and reperfusion. Because ATP-dependent Ca^{2+} uptake is thought to be involved in the extrusion of Ca^{2+} from the intracellular space, a depression of its activity can also be seen to contribute to the occurrence of intracellular Ca^{2+} overload. It should be pointed out that the observed depression in the sarcolemmal Ca^{2+} transport activities in experimental hearts are not due to differences in the orientation of the membrane vesicles or cross contamination with other organelles. The vesicles of control, hypoxic and reoxygenated hearts were to a predominant and similar extent of inside-out orientation; this was evident from the activities of ouabain-sensitive Na^+-K^+ ATPase. Although Na^+-K^+ ATPase activities were depressed in hypoxic samples and did not recover in reoxygenated samples, this did not represent a change in sarcolemmal purity because Na^+-K^+ ATPase activities in the sarcolemmal preparations from the control and experimental hearts were purified to an equal extent with respect to the heart homogenates. Furthermore, marker enzyme activities

revealed minimal but equal extent of cross contamination with fragments of mitochondria and sarcoplasmic reticulum. Gel electrophoretic patterns revealed no new peaks in the hypoxic or reoxygenated preparations. In addition, no difference was observed between the control and experimental preparations with respect to nonspecific Ca^{2+} binding as well as passive accumulation of Ca^{2+} and Na^{+} ions.

The exacerbated cell damage that occurs during reoxygenation of the hypoxic myocardium is far from being understood. Although hypoxia for 5 min was found to markedly depress the contractile force development and increase the resting tension, no changes in the sarcolemmal Na^{+}-dependent Ca^{2+} uptake or ATP-dependent Ca^{2+} accumulation were evident at this time. A slight depression in contractile force and a slight increase in resting tension were apparent upon reperfusing the 5 min hypoxic hearts and these effects were associated with a significant depression in ATP-dependent Ca^{2+} accumulation in sarcolemmal vesicles. Perfusion of hearts with hypoxic medium for 10 min or more resulted in a further increase in the resting tension, which may reflect the occurrence of intracellular Ca^{2+} overload (87). Sarcolemmal vesicles obtained from hearts perfused with hypoxic medium for 10 min or more showed significant depressions in the sarcolemmal Ca^{2+}-transporting activities. Furthermore, inability of these hypoxic hearts to recover their contractile activities was associated with further decreases in the sarcolemmal Ca^{2+} transporting activities.

These alterations may have incurred via the "turning-on" of various Ca^{2+}-dependent mechanisms resulting in contractile failure and cell damage (7). Activation of phospholipases may have contributed to the small changes in phospholipid concentration extracted from vesicles of hypoxic and reoxygenated hearts. However, no major changes in sarcolemmal phospholipid composition were evident except that diphosphatidylglycerol concentration was significantly elevated and phosphatidic acid was significantly depressed in the reoxygenated samples only. The significance of diphosphatidylglycerol change in altering the sarcolemmal Ca^{2+} transport activities is unknown, but accumulation of phosphatidic acid within the sarcolemma has been correlated with increased Na^{+}-Ca^{2+} exchange activity (125). Therefore, it is possible that decreased concentrations of phosphatidic acid which are apparent in reoxygenated samples may contribute to depressed Na^{+}-Ca^{2+} exchange activities; however, in hypoxic samples, Na^{+}-dependent Ca^{2+} uptake was not associated with any changes in phosphatidic acid. Thus, this mechanism may only provide the basis for further depressing Na^{+}-Ca^{2+} exchange activities upon reoxygenation after hypoxia. Another mechanism which may explain differences in Ca^{2+} transporting activities of vesicles from hypoxic and reoxygenated hearts involves the generation of free radicals in the myocardium (6, 126). Buildup of hypoxanthine as a result of ATP breakdown and the conversion of xanthine dehydrogenase to xanthine oxidase via a Ca^{2+}-activated protease is hypothesized to occur in ischemic

myocardium (99). With subsequent reintroduction of molecular O_2 during reoxygenation, the additional formation of free radicals may alter structural conformation of the sarcolemmal membrane. Therefore, it can be hypothesized that free radical binding to the protein mediating Na^+-Ca^{2+} exchange is a mechanism of further depression of these activities in reoxygenated samples.

In conclusion, the results described in this study provide evidence for a depression of Na^+-dependent Ca^{2+} uptake activities of cardiac sarcolemmal vesicles in hearts subjected to hypoxia-reoxygenation injury. There was a further depression of these activities in vesicles from reoxygenated hearts as opposed to hypoxic samples. This defect was characterized by a depression in initial rate of Ca^{2+} uptake, as well as a reduction in capacity of the Na^+-Ca^{2+} exchange system. ATP-dependent Ca^{2+} accumulation was similarly depressed in hypoxia-reoxygenation injury. These changes in the ability of sarcolemma to mediate Ca^{2+} efflux from the cardiac cell may represent one of the mechanisms involved in the development of intracellular Ca^{2+} overload known to be associated with hypoxia-reoxygenation injury in the myocardium.

VI. REFERENCES

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