PHOSPHOLIPID N-METHYLATION AND THE CALCIUM PARADOX OF THE HEART

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by

Sujata Majumder
Department of Anatomy
Faculty of Medicine
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SUJATA MAJUMDER

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Table of Contents

List of Tables

Table of Figures

ABSTRACT

Synthesis of N-methylated phospholipids by phosphatidyl-ethanolamine(PE) N-methylation, sites I, II and III activities were examined in sarcolemmal(SL) and sarcoplasmic reticular(SR) membranes from rat hearts subjected to Ca^{2+}-paradox. Perfusion(5min) with Ca^{2+}-free medium followed by reperfusion(5min) with medium containing 1.25mM Ca^{2+}, produced a marked rise in resting tension without any recovery of contractile force. Five minutes of perfusion with Ca^{2+}-free medium did not affect either the SL or SR N-methyltransferase system. Ca^{2+} readmisson for 2.5-5 min in the case of SL and 1-5 min in the case of SR induced a time dependent depression which was limited only to SL site II and SR site I activities. These alterations were not influenced by different (1-20min) Ca^{2+}-free perfusion times. No alteration at any time of Ca^{2+}-free perfusion or reperfusion was observed in the mitochondrial N-methyltransferase system. These results indicate that intracellular Ca^{2+} overload during Ca^{2+} paradox is associated with a defective SL and SR PE-methyltransferase system.

In an attempt to decipher the possible causes of the observed paradox induced alterations, the kinetics of the inhibition as well as the effect of the sulfhydryl-group modifying reagents (dithio-threitol, DTT; reduced glutathione, GSH; oxidized glutathione, GSSG; and fatty acids (oleic and arachidonic acids), were studied in separate experiments. These studies indicated that: (1) the enzyme protein itself, at least at the level of the sulfhydryl groups, is not affected by the paradox injury; (2) fatty acids may

play a role in the inhibition but only at the level of the SL N-methyltransferase system; (3) the two N-methylation systems, i.e. SL and SR, seem to be modulated by different factors and mechanisms as is obvious from their differential behavior to fatty acid challenge and their dissimilar kinetic profiles during paradox injury.

INTRODUCTION AND STATEMENT OF PROBLEM

Previous studies have shown that PE N-methylation affects certain well defined influences on the Ca^{2+} regulatory systems of the myocardial cell, at various subcellular levels (35,44,45,46). These influences are attributed to changes in the lipid microenvironment around these Ca^{2+} transport-related systems, effected by the methyltransferase-dependent formation of N-methylated phospholipids, which thus alters lipid-protein interactions leading to a modification of the protein function (2). These changes afforded to the Ca^{2+} regulatory systems tend to lead to a lowering of the cytosolic Ca^{2+} concentration and, therefore, a prevention of cellular Ca^{2+} overload (35,44,45,46). The activity of the PE N-methyltransferase system has also been observed to be altered in certain pathophysiological conditions, where myocardial Ca^{2+} overload is significant (47,58,62,63,64). Direct correlation was also found in certain of these pathophysiological conditions, between changes in PE N-methylation and Ca^{2+} regulatory activities, although in other cases such correlation was not evident.

Since PE N-methylation appears to function towards lowering the cytosolic level of Ca^{2+} and preventing intracellular Ca^{2+} overload, we hypothesized that the enzyme itself should be affected during an actual overload situation. Thus, the present study focusses on examining the status of the PE N-methyltransferase system in a typical model of myocardial Ca^{2+}-overload, the calcium paradox.

REVIEW OF LITERATURE

Phospholipid N-methylation in myocardial membrane systems

All biological membranes are made of a lipid bilayer in which are embedded biochemically active proteins. These membrane proteins may be structural or functional and, in the latter case, they may be receptors, enzymes, G-proteins etc. In any event the structural and/or functional integrity of the proteins are maintained to a large extent by the lipid environment in which they exist(1). The lipid bilayer of biological membranes is mainly composed of phospholipids, which provides an excellent fluid matrix for protein organization and movement. In most eukaryotic cells the two most abundant phospholipids are phosphatidylethanolamine (PE) and phosphatidylcholine (PC) (2). The phospholipids are asymmetrically distributed in all plasma membranes. More particularly, in the heart sarcolemma the negatively charged phospholipids, phosphatidylserine and phosphatidylinositol, are located exclusively in the inner or cytoplasmic leaflet; 75% of PE is in the inner leaflet; 93% of sphingomyelin and 43% of PC are in the outer leaflet (2,3). The conversion of one phospholipid to the other and therefore, the providing of certain unique composition of lipids, is of major importance in the modulation of membrane functions. The major direct route for the conversion of PE to PC in biological membrane is via the transmethylation pathway (4). It should be clarified at this point that PC formation occurs mainly through the CDP-choline pathway (1) and that

transmethylation caters for > 30 to 40% of the total PC synthesis in liver and for 5 - 10% only in other tissues including the heart (4). Evidence is however present which supports the concept that transmethylation of PE to PC, if it occurs in sufficient amount or is localized in a limited area of the lipid bilayer, could affect both membrane structure and function (2). The transmethylation process is catalyzed by the membrane-bound PE N-methyltransferase system. The schematic representation in Fig. 1 shows that AdoMet approaches the N-methyltransferase system from the cytoplasmic side of the plasma membrane, where the PE molecules undergoing methylation are located. The sequential addition of three methyl groups from the physiological donor S-adenosyl-L-methionine (AdoMet), to the amino head group of a PE molecule synthesizes PC on the outer surface of the membrane via the intermediate formation of the monomethyl (phosphatidyl-N-monomethylethanolamine, PMME), and dimethyl (phosphatidyl-N, N-dimethylethanolamine, PDME) derivatives (5,6). The first methyltransfer reaction is the rate-limiting step of the process (4). Essentially, phospholipid methylation can be seen as an enzyme-mediated movement of methylated phospholipids across the bilayer, from the inner to the outer surface, causing reorganization of the bilayer and changes in physical properties of the membrane.

Several reports have shown the presence of the N-methylating system in a variety of tissues and cell types including brain (7), liver (8), basophilic leukemia cells (9), erythrocytes (10) and other tissues (5). The existence at sarcolemmal (SL) mitochondrial

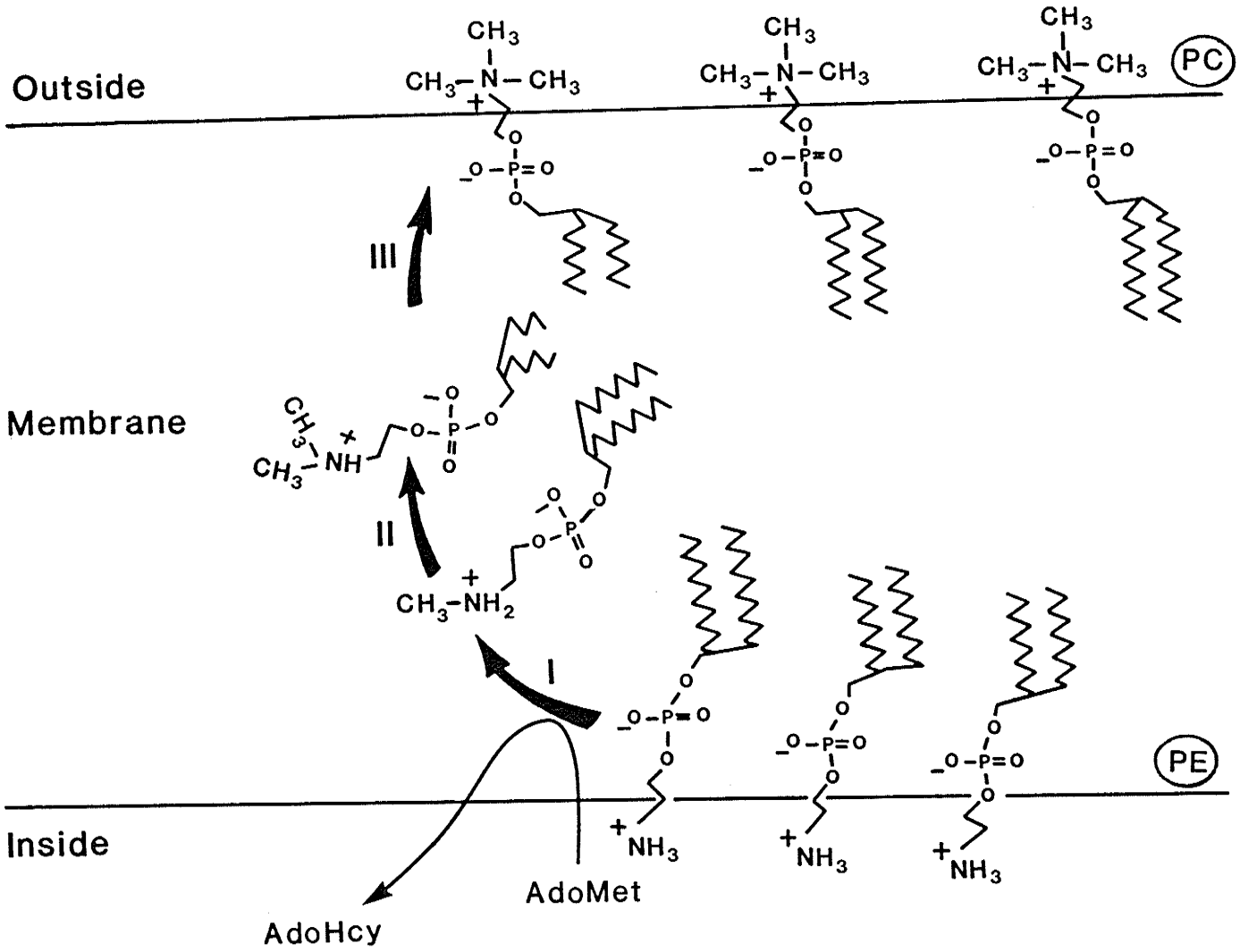

Fig. 1 Schematic representation of the enzymatic N-methylation of phosphatidylethanolamine (PE) to phosphatidylcholine (PC).

and sarcoplasmic reticular (SR) level of a N-methyltransferase system where three different catalytic sites effect PE N-methylation in cardiac tissue has been shown on the basis of kinetic parameters, pH profile, sensitivity to divalent cations and pathobiological behaviour (6). Site I shows very low apparent K_m (0.1 μM) for AdoMet, partial requirement for Mg^{2+}, optimum pH of 8.0, and a major formation of PMME (11,6). Site II and III do not require Mg^{2+}, have a higher apparent K_m for AdoMet (3.6 and 119 μM respectively), and optimum pH of 7.0 and 10.5 respectively (11). These latter two sites are involved in the major formation of PDME (site II) and PC (site III) (11). In other words, each assay condition selectively highlights only one of the three sites and favors the synthesis of only one methylated phospholipid, while the formation of the other two is minimized but not completely abolished. As far as the subcellular distribution of the enzyme system is concerned (12), the 1000 *g* fraction (sarcolemma, nuclei, celldebris, myofibrils) showed good specific activities for all three catalytic sites and due to the very high protein yield, this fraction carried the highest total activity (70% to 80%) (6). The 10,000 *g* fraction, which is enriched in mitochondria, also showed a good specific activity but, due to the lower protein yield the total enzyme activity is comparatively lower (10% to 13%) (6). A certain amount of activity, lower than that of the above two fractions, is associated with the sarcoplasmic reticular fraction (13,14). In cardiac tissue the methyltransferase enzyme system has been proven to be particulate in nature by virtue of the fact that

the enzyme activity is absent in the soluble fraction (12). Myofibrils do not exibit phospholipid methyltransferase activity (12). The kinetic parameters for each of the three catalytic sites are similar in SL, SR and mitochondria. Thus the characteristics of the methyltransferase molecule might be similar in its different subcellular localizations. Cardiac membranes exhibit also an enzymatic methylation of intramembrane components of a heterogeneous group of non-polar lipids (15).

Effect of transmethylation on membrane properties

Hirata and Axelrod reported that enzymatic methylation of phospholipids plays an important role in the transduction of receptor-mediated signals through the membranes of a variety of cells (5). The methylation which results in a rapid vectorial rearrangement of phospholipids increases membrane fluidity (5). Administration of the ß-agonist, L-isoproterenol, on reticulocytes which had ß-adrenoceptors coupled to adenylyl cyclase, caused a dose-dependent increase in the incorporation of ^{3}H-methyl groups into phospholipids (5). On the basis of this and of related experiments, a mechanism was suggested which could explain the link between phospholipid N-methylation and signal tansduction. The proposed mechanism was that binding of agonist (hormone) to receptor, would enhance the methylation of phospholipids, which in turn would increase the fluidity of the membrane. The increased fluidity would allow a better coupling of the post-receptor structures to adenylyl cyclase thus favoring the signal

transduction (5). The transmethylation reaction also seems to regulate signal transduction at the receptor level. In fact, it has been found that receptors which are often hidden in membranes become available to specific ligands upon increased conversion of PE to PC(5). Of further note, it has been recently shown that the intramembranal accumulation of N-methylated phospholipids in the heart modifies the localized lipid environment of some membrane-bound Ca^{2+} transport systems, thereby influencing their functions.

The calcium regulatory system

It is clear that calcium movements are closely related to cardiac electrophysiological events, contractile function, membrane integrity and energy metabolism. It is also quite evident, however, that the maintenance of appropriate concentration of this cation is mandatory, since abnormally high or low concentrations are associated with cardiac dysfunction and death. The cytosolic concentration of Ca^{2+} is normally maintained between 10^{-7}M during relaxation and 10^{-5} during contraction, in contrast to the very high concentration (1.25 mM) of the cation in the extracellular space (16). This large concentration gradient between the inside and outside of the cell is very effectively maintained by the Ca^{2+}-regulatory systems located both at the level of the sarcolemmal membrane and the membranes of intracellular organelles such as the sarcoplasmic reticulum and mitochondria (17,18,19). Starting with an intracellular concentration of 10^{-7}M (resting level), the

pathways involved in increasing the cytosolic Ca^{2+} level to 10^{-5} M or activating level are:

1. The slow Ca^{2+} channels

The opening of the slow Ca^{2+}-channels is initiated by a wave of depolarization originating at the sino-atrial node (11). The inward movement of calcium through the SL channels is energy dependent and may invlove a plasma membrane protein, the Ca^{2+}-dependent ATPase. This has been proposed to be primarily a gating mechanism whereby the channels are opened at the expense of ATP to allow passive movement of Ca^{2+} down its concentration gradient (20,21,22).

2. Membrane-bound calcium

The function of the slow Ca^{2+}-channels and of the Ca^{2+}-dependent ATPase is linearly related to the Ca^{2+}-bound to the external surface of the sarcolemma (23,24,25,18,26,27). This Ca^{2+} pool seems to be bound to low affinity-high capacity sites of negatively charged membrane components (sialic acid residues, phospholipids, proteins) (18,28,29,30,31) and seems to represent a superficial, rapidly exchangeable pool that enters the cell upon depolarization and is responsible for maintaining the cardiac contractile function (18,28,32).

3. The Na^{+}-Ca^{2+} exchanger

The second pathway for the influx of Ca^{2+} into the cytosol is the energy-independent Na^{+}-Ca^{2+} exchanger. The Na^{+}-Ca^{2+} exchanger is an electrogenic pump that has a stoichiometry of 3 to 2, i.e. 3 Na^{+} to 2 Ca^{2+}. This pump possesses two modes of function, the

forward mode and the reverse mode (33,34,35). The reverse mode is functional during contraction, and depends on the increase in cytosolic Na^+ concentration due to the inward Na^+ current during cardiac depolarization. In its reverse mode, therefore, the Na^+-Ca^{2+} exchanger couples the inward movement of 2 Ca^{2+} with the extrusion of 3 Na^+ (36,37,38,39). The forward mode is functional during relaxation and will be discussed later.

4. Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum

The Ca^{2+} ions that enter the cytosol can both trigger contraction directly and cause further release of Ca^{2+} from the sarcoplasmic reticulum, by the calcium-induced Ca^{2+}-release mechanism. The SR calcium release may also be triggered by the wave of depolarization transmitted to it via the sarcolemmal invaginations known as T-tubules (40,41).

All the above mentioned mechanisms function in increasing the cytosolic concentration of Ca^{2+} from its resting to the activating level. This increased concentration of Ca^{2+} leads to contraction upon the formation of the acto-myosin complex as a consequence of the interaction of the cation with the troponin and tropomyosin complex. Calcium at these concentrations, also stimulates mitochondrial oxidative phosphorylation as well as glycolysis and lipolysis and therefore increases ATP output which is required for the contraction process (40).

The process of relaxation requires that the Ca^{2+} concentration of the cytosol be lowered back to its resting level. Several

mechanisms are involved in lowering the cytoplasmic concentration of calcium, both at the level of the sarcolemma and intracellular organelles.

At the SL level is present an outwardly directed ATP-dependent Ca^{2+}-transporting system which is functional in extruding Ca^{2+} during relaxation. In "in vitro systems", the pump exhibits two functional expressions, the Ca^{2+}-stimulated-Mg^{2+}-dependent ATPase and ATP-dependent Ca^{2+} accumulation (42,43). Extrusion of Ca^{2+} from the cytoplasm is also effected by the forward mode of the sarcolemmal Na^{+}-Ca^{2+} exchanger, where the Na^{+}-gradient across the membrane provides the driving force (40). At the level of the intracellular organelles the Ca^{2+}-stimulated-Mg^{2+}-dependent ATPase of the sarcoplasmic reticulum reduces the cytoplasmic concentration of Ca^{2+} via an energy-dependent pump mechanism (41). The function of the mitochondria in reducing the cytosolic Ca^{2+}-concentration in physiological conditions is uncertain. It is more clearly understood to serve as a calcium sink in Ca^{2+} overload situations (42).

Thus, it is obvious that the above mentioned mechanisms function not only in changing the intracellular calcium concentration during contraction and relaxation but also in preventing Ca^{2+} overloading of the cell regardless of the enormous concentration gradient present. The latter is true, especially, at the level of the sarcolemma.

Effect of PE N-methylation on the different Ca^{2+} regulatory systems

Since the natural domain of the Ca^{2+} transport systems is the hydrophobic region of the membrane, it is reasonable to assume that these systems may be sensitive to changes in the physical properties of their microenvironment, which is made of lipids (44). As previously mentioned, both Ca^{2+}-binding and Ca^{2+}-ATPase are implicated in the entry of Ca^{2+} into the myocardium (45). PE-N-methylation was seen to reduce low affinity Ca^{2+}-binding to SL membranes (45). Methylation also decreased the Ca^{2+} dependent-ATPase. Consistent with the above was the finding that Na^{+}-dependent Ca^{2+}-uptake was decreased upon methylation of SL vesicles (35). It seems that as far as the Na^{+}-Ca^{2+} exchanger is concerned, PDME affords a greater inhibitory effect than any of the other N-methylated phospholipids (35).

As stated above, cardiac sarcolemma also contains an outwardly directed calcium pump (42,43). It was reported that polyunsaturated fatty acids and acidic phospholipids are able to stimulate the Ca^{2+}-pump. Work done on the effect of PE-N-methylation on SL Ca^{2+}-pump activity has shown a stimulation of the pump (44). Under optimal concentration of free Ca^{2+}, both Ca^{2+} stimulated ATPase and ATP-dependent Ca^{2+} accumulation, which are functional expressions of the pump, were maximally enhanced by the predominant formation of PDME inside the membrane (44). At the intracellular level, the Ca^{2+}-stimulated-Mg^{2+}-dependent ATPase (Ca^{2+} stimulated-ATPase) of the sarcoplasmic reticulum was also seen to be activated by the

enzymatic methylation of membranal PE (46). This therefore results in enhanced sequestration of Ca^{2+} by the SR membrane.

In all the above mentioned cases the respective changes observed upon PE-N-methylation were abolished in the presence of methylation inhibitors like for example S-adenosylhomocysteine (AdoHcy) (35,44,45,46). AdoHcy is a product of the AdoMet-dependent methyltransferase reaction and is a potent inhibitor of PE N-methylation. Pretreatment with methylacetimidate.HCl, an amino-group blocking agent and hence a potent inhibitor of PE N-methylation, also prevented the observed methylation-induced changes in Ca^{2+} systems (35,44,46). From the above observation, on the effect of PE N-methylation on the various Ca^{2+}-transport systems, it appears that PE N-methylation tends to work towards preventing Ca^{2+}-overload of the myocytes. By decreasing both the level of the putative Ca^{2+}-gating mechanism(SL Ca^{2+} -ATPase) and Ca^{2+}-superficial stores, PE N-methylation controls Ca^{2+} influx into the myocardial cells. The same result is also achieved by virtue of its inhibitory effect on the Na^{+}-dependent Ca^{2+} uptake activity of the Na^{+}-Ca^{2+} exchanger. On the other hand, PE N-methylation stimulates both the SL and the SR Ca^{2+}-pump activities (44,47), which remove Ca^{2+} from the cytosol. Thus by stimulation of these two Ca^{2+}-pumps, PE N-methylation not only enhances the rate of cardiac relaxation and successfully prevents the occurrence of intracellular Ca^{2+}-overload but also enhances force of contraction due to the fact that increased SR Ca^{2+} sequestration enhances Ca^{2+} released during contraction.

While "in vitro" membrane effects are observed as a consequence of PE N-methylation, one may argue whether the methylation pathway has a significant activity under pathophysiological conditions and whether such activity could be of sufficient magnitude to affect membrane-related functions. Some of the important cellular events, which were found to be modulated "in vivo" by the methylation process, have been recently reviewed (48). It is also known that membrane lipids are organized into domains (49) and provide both structural integrity and a suitable microenvironment for the normal functioning of membrane proteins(50-56). Therefore, if a given protein resides in a specific lipid microdomain, alterations of that domain may trigger protein dysfunction. An alteration could be induced even by small changes of the total membrane lipids, because such changes may become quantitatively relevant if they are restricted to specific microdomains (57). Thus, it is possible that the N-methylation pathway, which accounts only for a small modification of the hearts membrane phospholipids, could modify the localized lipid environment of certain Ca^{2+} transport systems, thereby influencing their function (48).

In summary, we can suggest that the transmethylation reaction may be one of the modulators of various intramembranal Ca^{2+}-regulatory systems, which is effective in protecting the cardiomyocytes against excessive accumulation of Ca^{2+}.

Status of PE N-methylation in pathophysiological conditions.

So far our discussion has shown that enzymatic N-methylation of intramembrane PE influences Ca^{2+}-transport across both myocardial sarcolemma and sarcoplasmic reticulum. The overall effect of the alterations seems to be geared towards reducing the cytosolic Ca^{2+} concentration and preventing overload. Thus it is quite logical to assume that PE N-methylation may play a role in cardiomyopathic states which involve altered Ca^{2+} fluxes and therefore will itself show abnormalities in its activity in the different cardiomyopathic states.

PE N-methylation activity showed time-related alterations during the development of isoproterenol-induced cardiomyopathy (58). These changes were biphasic, being stimulated at 1 and 3hrs after isoproterenol administration and depressed at 24 hrs. They were observed at site I (rate-limiting step) of SL and SR N-methyltransferase, whereas no change was seen in mitochondria. Sites II and III were not modified by isoproterenol treatment (58). Catecholamine induced cardiomyopathy is a model of heart disease that is associated with abnormalities in the membrane regulation of Ca^{2+} movements. Injection of isoproterenol in a single high dose has been shown to induce Ca^{2+}-overload leading to myocardial cell damage by 24 hr post-administration (59). As previously discussed, a direct relationship exists between N-methylation and Ca^{2+}-pump activities of the heart, in that activation of membrane methylation is accompanied by enhancement of both SL and SR Ca^{2+}-pumps (60,61). Thus it is relevant to note that over the time-course of 24 hr

post-isoproterenol injection, time-related changes of microsomal Ca^{2+}-pump were seen (58) which parallel those in PE N-methylation.

A similar selective activation of catalytic site I was also seen at the initial stages of streptozotocin-induced diabetic cardiomyopathy (62). In diabetic animals an initial stimulation of catalytic site I was followed by a depression at all three sites(62). The initial stimulation may be due to the elevated levels of plasma catecholamines (Christensen, 1974) which has been shown to induce a ß-adrenoceptor-dependent increase in N-methylation activity in normal myocardium (Taira et al. 1990). On the other hand, the depression of N-methylation observed at the late stages of diabetes may be due to a defect in the adrenergic receptor mechanism since the number of ß-adrenoceptor in cardiac muscle was shown to be reduced in chronic diabetes (Heyliger et al. 1982). The fact that sarcolemmal Ca^{2+}-ATPase is stimulated in chronic diabetes (Dhalla et al. 1984), and depressed by N-methylation, can suggest that the former may be a consequence of the depressed N-methylation activity in diabetic animals. The correlation, however, is not maintained in the case of low affinity Ca^{2+} binding which is reported as decreased in chronic diabetes (Pierce et al. 1983); in fact, since it is also decreased by N-methylation (Panagia et al. 1983), its reduction during diabetes cannot be attributed to the decreased transmethylation activity. Absence or presence of correlation between changes in PE N-methylation and low affinity Ca^{2+} binding or Ca^{2+}-ATPase,

respectively, was noticed in SL membranes isolated from hypertrophied hearts (63).

Two facts should be noted at this point, based on the observations stated thus far. Firstly, the observed lack of correlation between the changes in methyltransferase activity and those of some Ca^{2+}-related transport systems, whereas direct correlation exists in other instances, may be attributed to the fact that PE-N-methylation is probably only one of the several mechanisms which are associated with altering cellular Ca^{2+}-transport systems during the development of different cardiac abnormalities. Secondly, the differential behaviour of sarcolemmal, microsomal and mitochondrial PE N-methyltransferase activities in different disease states suggests that these activities may play different roles in myocardial cell function.

Abnormalities in cardiac PE N-methylation were also seen in genetically determined cardiomyopathy (UM-X7.1 strain of Syrian hamster) where intracellular Ca^{2+}-overload seems to be the primary cause of cell death (64).

From the discussion thus far, it becomes obvious that PE N-methylation effects fairly specific and well defined influences on the Ca^{2+}-regulatory systems of the myocardial cell at various subcellular levels. It affords these changes by altering the lipid microenvironment around these Ca^{2+}-regulatory proteins thus altering the lipid-protein interaction, which leads to a change in the protein function (2). The activity of the various Ca^{2+}-regulatory systems seems to be altered such that they function to lower

cytosolic Ca^{2+}-concentration and hence prevent overload (35,44,45,46). It was also observed that the status of PE N-methylation was shifted from its normal activity under some pathophysiological conditions, where myocardial Ca^{2+}-overload was significant (47,58,62,63,64). Direct correlation was also observed in these different disease states between changes in PE N-methylation and some of the Ca^{2+}-regulatory activities, although others failed to show such correlation. These observations led us to the aim of the project.

Since PE N-methylation appears to function towards lowering the cytosolic level of Ca^{2+}, and preventing the occurrence of intracellular Ca^{2+}-overload, then it should be affected during actual overload conditions. Thus we proceeded to study the status of the myocardial PE N-methyltransferase system in a typical model of Ca^{2+}-overload, the calcium paradox.

The calcium paradox

Perfusion of the hearts with medium containing Ca^{2+} after a brief period of perfusion with Ca^{2+}-free medium results in an irreversible loss of contractile function (65,66). This phenomenon, referred to as "The Calcium Paradox" of the heart, is associated with development of contracture, ultrastructural damage, depletion of high energy phosphate stores, alteration in cation content, and leakage of intracellular constituents. All these alterations in hearts subjected to calcium paradox are attributed to the occurence of intracellular Ca^{2+}-overload (65,66).

1. Morphological consequences

During the calcium-free perfusion a separation between the surface coat and the external lamina of the glycocalyx, and a change in the orientation of the intramembranal particles on the P-faces of the sarcolemma has been observed resulting in blebbing (67,68,69). Other studies have shown a slight separation of the opposing faces of the intercalated disks (70,71,72) followed by dilation of the tubular system (66,73).

Ca^{2+} readmission after a period (5-10 min) of Ca^{2+}-free perfusion results in massive ultrastructural damage characterized by severe contracture, disruption of myofilaments, swelling of mitochondria with the formation of electron dense particles (66,74). These changes are not obvious under condition of low temperature (21°C).

2. Electrical and mechanical consequences

During calcium free perfusion (rabbit heart), the action potential duration lengthens, AV conduction time increases resulting in Wenckebach 2:1 AV block (73,75,76,77,78). The heart rate decreases until mechanical activity stops completely while electrical activity continues (electromechanical dissociation). This dissociation between the electrical and mechanical activity was first demonstrated by Mine et al. (79).

Within minutes of reperfusion with calcium containing solution, the cells depolarize to less than -50mV and action

potentials fail to develop. After 2 - 3 minutes of Ca^{2+}-repletion AV conduction abnormalities and ventricular arrhythmias develop. Ventricular electrical activity also disappears although the electrical activity of the atria is unaffected (75,76,80). This is demonstrated on the mechanical activity of the heart by the development of contracture after a brief time of uncoordinated mechanical activity (72,73,76,81).

3. Biochemical consequences

Calcium free perfusion of rat hearts up to even one hour did not cause any measurable release of creatine kinase (CK). Oxidative metabolism in mitochondria was however reduced (Robertson et al 1982). This may be attributed both to the inhibition of Ca^{2+}-dependent dehydrogenases and reduction of ATP turnover in the Ca^{2+}-depleted myocardium (82). Na^{+}-K^{+} activity in SL and calcium pump activity in SR after 10 min of Ca^{2+}-free perfusion, were reduced by 75% and 55% respectively (83,84).

Reintroduction of calcium leads to a massive loss of myoglobin and cytosolic enzymes such as CK, lactate dehydrogenase and pyruvate kinase (65,70,73,76,81,85,87). The tissue levels of Na^{+} and Ca^{2+} increase and those of K^{+} and Mg^{2+} decrease (73,88,89). Mitochondrial and lysosomal enzymes are however not lost during the calcium paradox insult (87). The calcium paradox phenomenon is accompanied by an marked decline in the level of high energy phosphates, followed by a concomitant increase in the quantity of their metabolites. The greatest change occurs at approximately 30

sec to one min from start of Ca^{2+}-repletion. After about 30 sec of Ca^{2+}-repletion, the observed levels of CP and ATP were 45% and 65% of control, respectively. At the same time an increase was seen in creatine (15%), ADP (85%) and AMP (2800%) (76,90,91).

Alterations observed in the subcellular Ca^{2+}-transport systems includes depressed SL Ca^{2+}-binding and Na^{+}-K^{+} ATPase activities (84). The Na^{+}-Ca^{2+} exchanger and the Ca^{2+}-pump activities of the SL membranes were depressed upon reperfusion although they were unaffected during Ca^{2+}-free perfusion period (92). The former showed a biphasic change i.e. an increase in activity after 1-2 min of repletion followed by a depression at 5-10 min. The latter showed a decrease at 2-10 min of repletion. The activity of the Ca^{2+}-stimulated ATPase of the SR was also depressed on repletion, although, contrary to other reports (84), it was unaffected during calcium-free perfusion.

4. Possible mechanism of Ca^{2+}-paradox

The different structures and routes involved in Ca^{2+} entry into the cardiomyocyte during Ca^{2+} paradox include the glycocalyx, the slow Ca^{2+} channels, the Na^{+}-Ca^{2+} exchanger, passive diffusion and abnormal sites of Ca^{2+} entry.

First of all the role of the glycocalyx in Ca^{2+} paradox condition is controversial (93,94,95,96). It has been reported that Ca^{2+}-free perfusion causes splitting of the glycocalyx and increased permeability of the sarcolemma to Ca^{2+}, following cell-cell separation (Singal et al 1979; Ashraf et al 1981; Ganote et

al. 1983). However, when the Ca^{2+}-paradox is evolved in the presence of barium, the glycocalyx remains intact but the cells become overloaded with calcium (95). Moreover, cobalt and manganese which are protective against calcium paradox do not prevent separation of the glycocalyx. Thus this route as a possibility leading to Ca^{2+} overload is quite uncertain.

The second possibility are the slow calcium channels. Reports have stated that Ca^{2+}-antagonists or calcium channel blockers do reduce the damage caused by calcium paradox. However, this viewpoint has been debated on the grounds that: (i) increased intracellular Ca^{2+} inactivates these channels; (ii) depletion of extracellular calcium converts these to sodium channels; (iii) the concentrations of antagonists used are not sufficient to prevent or reduce the gain in calcium that occurs upon calcium readmission. Thus the chances of calcium channels to be the route of entry for calcium during overload is very controversial. A more satisfactory possibility lies with the Na^{+}-Ca^{2+} exchanger (94,97).

The Na^{+}-Ca^{2+} exchanger has been strongly suggested as being a major candidate in calcium overload during calcium paradox (98). Makino et al. (1988) have reported that the activity of the Na^{+}-Ca^{2+} exchanger during Ca^{2+}-paradox is biphasic. It is stimulated during early periods of reperfusion but depressed during the latter stages (92). It was suggested that the early stimulation may represent an adaptive mechanism due to the reperfusion-induced influx of Ca^{2+} through the slow channels, whereas the delayed depression may contribute further to cellular Ca^{2+} overload. In

par with this observation is the fact that the intracellular concentration of Na^+ has been shown to increase on perfusion of the hearts with Ca^{2+}-free medium, and to play a critical role in the genesis of reperfusion induced Ca^{2+}-overload (89,98,99,100,101). Lowering of Na^+ in the perfusion medium during Ca^{2+}-free perfusion is protective against Ca^{2+}-paradox damage (102,103). This may be explained by the fact that the low Na^+ during Ca^{2+}-free perfusion prevents the marked increase in Na^+ and therefore when Ca^{2+} is reintroduced, the low intracellular Na^+ prevents overloading of Ca^{2+} in exchange for Na^+ via the Na^+-Ca^{2+} exchanger. Recently it was also reported that benzamil, which is a blocker for the Na^+-Ca^{2+} exchanger, is quite successful in preventing Ca^{2+}-overload and related cellular damage during calcium paradox (104). Thus, the above facts would indicate the Na^+-Ca^{2+} exchanger as a plausible candidate in causing Ca^{2+}-overload during paradox insult.

Other routes may involve damaged areas of intercalated discs or certain ion selective channels that may become distorted during Ca^{2+} depletion and allow Ca^{2+} to enter (105).

Together with the alterations of the Ca^{2+} influx system during the development of calcium paradox, the cells also become compromised in their capacity to remove calcium. Both ATP-dependent Ca^{2+}-uptake and Ca^{2+}-stimulated ATPase activities of the sarcolemmal Ca^{2+}-pump which is involved in the efflux of calcium from the myocardium (106), were decreased on 2-10 min of reperfusion of Ca^{2+}-depleted hearts(92). This alteration would favour the retention of Ca^{2+} in the cardiac cell and potentiate Ca^{2+}

overload. Moreover, calcium repletion following Ca^{2+}-deprivation was also reported to decrease the calcium pumping activity of the sarcoplasmic reticulum (107).

The above discussion shows that the calcium gain of the cells during calcium repletion is associated with not only an increased intake in calcium but also in part with a loss in the ability of cells to remove calcium from the cytosol.

Several interventions have been reported to prevent or attenuate the magnitude of calcium paradox damage. These include lowering the extracellular pH (108), reducing the sodium concentration (88,109), the presence of other divalent cations (barium, manganese, cobalt, magnesium) (95,96,110,111), and of pharmacological agents (dimethylsulfoxide, benzamil, taurine, phenothiazines) (80,104,112,113,114). The most well reported cases are however the protective effects of hypothermia (4°C), (70,73,91,99,100) and of Ca^{2+} antagonists (114) such as verapamil, nifedipine and diltiazem.

METHODS

Model of Ca^{2+} deprivation and Ca^{2+} paradox

Male Sprague-Dawley rats weighing between 250-300 g were sacrificed by decapitation and their hearts rapidly excised and placed in ice-cold normal saline. The hearts were then arranged for retrograde coronary perfusion at 37°C by a non-circulating Langendorff system with a modified Krebs-Henseleit (K-H) buffer solution (pH 7.4) of the following composition: NaCl, 120mM; $NaHCO_3$, 25 mM; KCl, 4.8 mM; KH_2PO_4, 1.2 mM; $MgSO_4$, 1.2 mM; $CaCl_2$, 1.2 mM; glucose, 8.6 mM. The coronary flow was maintained at approximately 10ml/min by a Masterflex peristaltic pump and the hearts paced electrically at a rate of 240 pulses per min and twice the threshold voltage. The hearts were all initially stabilized for 15 min and then Ca^{2+}-deprivation was induced for different time-periods using K-H in which $CaCl_2$ was replaced by 0.5 mM EGTA and the osmolarity maintained with sucrose. The times of Ca^{2+}-deprived perfusion were 1, 2.5, 5, 10 and 20 min followed by a 5 min reperfusion period. Experiments to see the effect of the duration of reperfusion were also done where the hearts were perfused with 5 min Ca^{2+} free solution and reperfusion time of 1, 2.5 and 5 min were used. All K-H solutions used were maintained at 37°C and gassed continuously with 95% O_2 and 5% CO_2.

Contractile force development was monitored by an isovolumetric pressure transducer using a Gilson polygraph.

Preparation of cardiac subcellular membranes

1. Heavy sarcolemmal fraction.

The heavy sarcolemmal fraction was isolated by the hypotonic shock-LiBr treatment method (Dhalla et al., 1981). For this purpose the ventricles were minced and homogenized in 20 vol of 10 mM Tris-HCl containing 1 mM EDTA (pH 7.4). The homogenate was passed through 4 layers of gauze and centrifuged at 1000 *g* for 10 min. The sediment was suspended in 20 vol of 10 mM Tris-HCl (pH 7.4), gently stirred for 15 min and centrifuged for 10 min at 1000 g. This procedure was repeated with 20 vol of 10 mM Tris-HCl (pH 8.0) and the sediment thus obtained was extracted for 45 min with 20 vol of 0.4 M LiBr in 10 mM Tris-HCl (pH 7.4). After centrifugation at 1000 *g* for 10 min, the pellet was washed with 20 vol of 10 mM Tris-HCl (pH 7.4) for 10 min and sedimented at 1000 *g* for 10 min. Further extraction was performed with 0.6 M KCl in 10 mM Tris-HCl (pH 8.0) for 15 min to remove the residual protein contaminants. This suspension was centrifuged for 10 min at 1000 *g*, the sediment was washed thoroughly with 10 vol of 10 mM Tris-HCl (pH 7.4) and suspended in 1 mM Tris-HCl (pH 7.0). All the preparative procedures were carried out at 0 - 4°C.

2. Light sarcolemmal fraction

For this purpose the ventricles were washed, minced and homogenized in 0.6 M sucrose, 10 mM imidazole/HCl, pH 7.0 (3.5 ml/g tissue) with a polytron PT - 20 (5 x 20 sec, setting 5). The resulting homogenate was centrifuged at 12,000 *g* 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 pH 7.4 (KCl/MOPS), the supernatant was centrifuged at 96,000 *g* 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 $Na_2P_2O_7$, and 0.1 M Tris-HCl, pH 8.3. After centrifugation at 95,000 *g* for 30 min using a Beckman swinging Bucket rotor, the band at the sucrose-buffer interface was taken and diluted with three volumes of KCl/MOPS solution. A final centrifugation at 96,000 *g* for 30 min resulted in a pellet rich in sarcolemma. This pellet was resuspended in 250 mM sucrose/10 mM histidine buffer. All the preparative procedures were carried out at 0 - 4°C. The isolation procedure is the same as that described by Pitts et al. (1979).

3. Microsomes

The ventricular tissue was first washed with ice-cold buffer (0.25 M sucrose, 20 mM Tris-HCl, 1 mM EDTA pH 7.0). The ventricular tissue was then blotted dry and weighed. The tissue was then placed in 10 vol of ice-cold homogenizing medium (10 mM $NaHCO_3$, 5 mM NaN_3, 15 mM Tris pH 6.8), cut up into small pieces and homogenized in a Waring blender for 45 sec (20 sec - 1 min rest - 25 sec). The homogenate was filtered through four layers of gauze, centrifuged at 10,000 *g* for 20 min, collected and recentrifuged at 40,000 *g* for 45 min. The pellet was suspended in 8 vol of 0.6 M KCl, 20 mM Tris-HCl (pH 6.8) and centrifuged at 40,000 *g* for 45 min. The resulting pellet containing mostly sarcoplasmic reticulum was suspended in approximately 1 - 1.5 ml of 0.25 M sucrose, 20 mM

Tris-HCl (pH 6.8). The isolation procedure was carried out according to the method of Harigaya and Schwartz at 0 - 4°C. The normal yield was approximately 1.48 mg membrane protein/g heart.

4. Mitochondria

The ventricular tissue was placed in ice-cold 0.25 M sucrose, 20 mM Tris-HCl, 1 mM EDTA (pH 7.0), allowed to chill slightly and then blotted dry and weighed. The tissue was then placed in 10 vol ice-cold 0.18M KCl, 10 mM Na_2EDTA, 0.5% albumin, pH 7.4 (KEA), cut into small pieces and then homogenized in a Waring blender for 20 sec. The homogenate was then filtered through gauze and centrifuged at 1000 *g* for 10 min. The supernatant was collected and centrifuged at 10,000 *g* for 20 min. The resulting mitochondrial pellet was further purified by resuspension in 10 vol KEA and centrifuged at 1000 *g* for 10 min. The supernatant was again collected and spun at 8000 *g* for 10 min. The mitochondrial pellet thus obtained was suspended in 2 ml of suspension medium (50 mM KCl, 20 mM Tris-HCl, pH 6.8). The entire isolation procedure was carried out at 0 - 4°C according to the method of Sordahl et al. Normal protein yield was approximately 2-3 mg/ml.

Assay for phospholipid N-methylation

Phospholipid N-methylation was assayed by measuring the incorporation of [^{3}H]-methyl groups into membrane phospholipids in the presence of S-adenosyl-L-[methyl-^{3}H]methionine ([^{3}H]-AdoMet). Assays were performed with 300 μg of protein in 0.5 ml total reaction medium at three different [^{3}H]-AdoMet concentrations,

namely 0.05 μM (pH 8.0, 50 mM Tris-glycylglycine, 1 mM $MgCl_2$), 10μM (pH 7.0, 50 mM imidazole), and 150 μM (pH 10, 50 mM NaOH-glycine). These three AdoMet concentrations fall into three functionally different ranges characteristic for the optimal activity of the three catalytic sites (I, II and III) involved in the specific synthesis of individual N-methylated phospholipids (PMME, PDME and PC).

After a preincubation period of 10 min at 37°C, the reaction was initiated by adding [^{3}H]-AdoMet at the three different concentrations mentioned above, and terminated after 30 min of incubation with the addition of 3 ml of chloroform/methanol/2N HCl (6:3:1, v/v). The tubes were shaken vigorously for 3 min using a vortex mixer. This was followed by the addition of 2 ml of 0.1 M KCl in 50% methanol and again the tubes were shaken vigorously for 1 min and then centrifuged at 2000 *g* for 10 min. The aqueous phase was aspirated and the chloroform phase washed twice with 2 ml of 0.1 M KCl in 50% methanol.

Separation of methylated phospholipids

To fractionate and quantitate the various [^{3}H]-methyl-labelled phospholipids of the membrane, 0.6 ml of the chloroform phase was evaporated almost to dryness under N_2 stream. The residue was immediately dissolved in 50 μl of chloroform/methanol (2:1, v/v) and quantitatively applied to silica gel 60 F -254 thin layer plates (0.25 mm thickness) under a light N_2 stream. The chloroform containing test tubes were carefully washed twice with 50 μl

chloroform/methanol mixture and each washing was again applied to the layer. The chromatogram was run at room temperature in a solvent system containing propionic acid/n-propyl alcohol/chloroform/water (2:2:1:1, v/v). After the solvent front had migrated approximately 15 cm, the plates were air dried at room temperature. The lipid spots were visualized by exposure to iodine vapours, scraped and the radioactivity was counted in 5 ml Ready-Solv HP. Corrections were made for quenching due to silica gel in all the data expressed. Summation of the radioactivity counts for all the methylated phospholipids (PMME + PDME + PC) was used to calculate the value (pmol/mg protein/30 min) for total N-methylated phospholipids.

Protein was determined by the method of Lowry et al. with bovine serum albumin as standard.

Analysis of the data

Each observation was done in duplicate on at least 4 - 6 different preparations and the mean ± S.E. were calculated. The level of significance of the difference between control and experimental samples was determined by the Student's *t* test. A P value < 0.05 was taken to reflect significance between control and experimental results.

RESULTS

Changes in phosphatidylethanolamine N-methyltransferase activity in heavy SL and SR of rat hearts during Ca^{2+} paradox

To investigate possible alterations of the PE N-methyltransferase during Ca^{2+} paradox, we studied this enzyme activity in two subcellular membrane systems, SL and SR, both at varying times of calcium readmission with a constant Ca^{2+}-free perfusion time of 5 min, and varying times of Ca^{2+}-free perfusion with a constant time of readmission (5 min). Table 1 describes the effect of Ca^{2+} paradox on the three catalytic sites of the sarcolemmal methyltransferase system at varying times of Ca^{2+} readmission and constant time (5 min) of Ca^{2+}-free perfusion. It is quite apparent that readmission of Ca^{2+} induced a time-dependent inhibition of the SL methyltransferase activity at catalytic site II starting at 1 min of reperfusion and being significantly different from control at 2.5 min. No significant change was observed at this site due to Ca^{2+}-free perfusion alone (0 min). Site II and III methyltransferase activities were found to be normal both upon Ca^{2+}-free and Ca^{2+}-readmission conditions. In the case of the SR (Table 2), readmission of Ca^{2+} induced a similar time dependent change in methyltransferase activity, which was limited to catalytic site I and was already significant at 1 min of readmission. SR catalytic sites II and III were unaffected. Differences in the site of N-methyltransferase alteration at various subcellular levels had been

Table 1. Phosphatidylethanolamine N-methyltransferase activity in the heavy sarcolemmal fraction isolated from rat hearts after 5 min of Ca^{2+}-free perfusion followed by different time-periods of Ca^{2+}-readmission.

Experimental Groups	CATALYTIC SITE		
	I	II	III
Control	0.680 ± 0.03	9.2 ± 0.2	148 ± 8
Duration of Ca^{2+}-readmission after 5 min Ca^{2+}-free perfusion			
0 min	0.748 ± 0.02	9.0 ± 0.1	148 ± 9
1 min	0.755 ± 0.04	7.3 ± 0.1	148 ± 9
2.5 min	0.755 ± 0.04	6.8 ± 0.4*	141 ± 13
5 min	0.680 ± 0.02	6.4 ± 0.1*	151 ± 9

Values are mean ± S.E of 4-6 experiments and are expressed as pmol [^{3}H] methyl-groups incorporated/mg protein/30 min. Perfusion with Ca^{2+}-free medium was carried out for 5 min; hearts were then perfused with medium containing 1.25 mM Ca^{2+} for the indicated time-periods. Hearts perfused with normal medium for comparable periods served as controls. Sarcolemmal membranes were isolated according to the LiBr hypnotic shock method. Phosphatidylethanolamine N-methyltransferase activities were measured under standard conditions for catalytic site I, II, and III as described in Methods. * Significantly ($p < 0.05$) different from control.

Table 2. Phosphatidylethanolamine N-methyltransferase activity in sarcoplasmic reticular membranes isolated from rat hearts after 5 min of Ca^{2+}-free perfusion followed by different time-periods of Ca^{2+}-readmission.

Experimental Groups	CATALYTIC SITE		
	I	II	III
Control	0.673 ± 0.02	7.0 ± 0.3	137 ± 11
Duration of Ca^{2+}-readmission after 5 min Ca^{2+}-free perfusion			
0 min	0.653 ± 0.04	6.6 ± 0.1	138 ± 14
1 min	0.538 ± 0.04*	7.4 ± 0.2	137 ± 7
2.5 min	0.464 ± 0.02*	7.1 ± 0.2	145 ± 9
5 min	0.377 ± 0.04*	6.3 ± 0.2	129 ± 4

Values are mean ± S.E of 4-6 experiments and are expressed as pmol [^{3}H]-methyl groups incorporated/mg protein/30 min. Perfusion with Ca^{2+}-free medium was carried out for 5 min; hearts were then perfused with medium containing 1.25 mM Ca^{2+} for the indicated time-periods. Hearts perfused with normal medium for comparable periods served as controls. SR membranes were isolated according to the Harigaya and Schwartz. Phosphatidylethanolamine N-methyltransferase activities were measured under standard conditions for catalytic site I, II, and III as described in Methods. * Significantly ($p < 0.05$) different from control.

Table 3. Incorporation of [^{3}H]-methyl groups into sarcolemmal N-methylated phospholipids from hearts at different time-periods of Ca^{2+}-free perfusion followed by 5 min of Ca^{2+}-readmission.

Experimental Groups	SITE I		SITE II		SITE III	
	pmol/mg/30 min	% control	pmol/mg/30 min	% control	pmol/mg/30 min	% control
Control	0.667 ± 0.04	100	8.3 ± 0.1	100	233 ± 5	100
Duration of Ca^{2+}-free perfusion before 5 min of Ca^{2+}-readmission						
1 min	0.667 ± 0.02	100	8.1 ± 0.1	98	128 ± 4	96
2.5 min	0.667 ± 0.02	100	6.8 ± 0.2*	82	141 ± 3	106
5 min	0.667 ± 0.02	100	5.7 ± 0.1*	69	137 ± 2	102
10 min	0.693 ± 0.04	104	6.5 ± 0.2*	79	137 ± 3	103
20 min	0.693 ± 0.08	104	6.5 ± 0.2*	79	136 ± 4	102

Values are means ± S.E. of 4-6 experiments and express the incorporation of [^{3}H]-methyl groups into N-methylated phospholipids (PMME + PDME + PC) under standared N-methyltransferase site I, II and III assay conditions. Perfusion with Ca^{2+}-free medium was carried out for the indicated times. Hearts were then perfused with medium containing 1.25 mM Ca^{2+} for 5 min. Hearts perfused with normal medium for comparable periods served as controls. Other details are as in legend of Table 1.

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

observed previously in the event of catecholamine-induced cardiomyopathy (58) and diabetic cardiomyopathy (62,115).

The effect of varying the time of Ca^{2+}-free perfusion keeping constant the time of readmission (5 min) was also investigated in the two membrane systems. Table 3 shows the effect of different times of Ca^{2+}-free perfusion on the heavy sarcolemmal N-methyltransferase enzyme system. As was pointed out above the site of inhibition is catalytic site II, and here a minimum of 2.5 min of calcium free perfusion is required to induce a significant decrease in the enzymatic activity after 5 min of Ca^{2+} readmission. However, increasing the time of Ca^{2+}-free perfusion even to 20 min did not modify the degree of inhibition from that produced at 2.5 min of Ca^{2+}-free perfusion. This would suggest that the inhibition of SL PE N-methyltransferase site II activity does not depend on the duration of Ca^{2+}-free perfusion. Prolongation of the Ca^{2+}-free time up to 20 min did not alter the catalytic properties of site I and III as detected at the end of Ca^{2+} repletion time (Table 3). In the case of the sarcoplasmic reticular system (Table 4) a shorter time of Ca^{2+}-free perfusion (1 min) was required to produce a significant inhibition of the site I activity after 5 min of Ca^{2+} readmission. SR site I alteration was independent of the duration of the Ca^{2+}-free time; up to 20 min of Ca^{2+}-free perfusion followed by 5 min of Ca^{2+} repletion did not affect site II and III activities (Table 4). These observations are quite consistent with previous studies on Ca^{2+}-paradox (17) which showed that maximal damage to the myocardium occurs during Ca^{2+}-repletion, with very few changes

Table 4. Incorporation of [^{3}H]-methyl groups into sarcoplasmic reticular N-methylated phospholipids from hearts at different time-periods of Ca^{2+}-free perfusion followed by 5 min of Ca^{2+}-readmission.

Experimental Groups	SITE I		SITE II		SITE III	
	pmol/mg/30 min	% control	pmol/mg/30 min	% control	pmol/mg/30 min	% control
Control	0.660 ± 0.03	100	6.4 ± 0.3	100	124 ± 6	100
Duration of Ca^{2+}-free perfusion before 5 min of Ca^{2+}-readmission						
1 min	0.370 ± 0.03*	56	6.4 ± 0.2	100	127 ± 9	102
2.5 min	0.436 ± 0.03*	66	6.7 ± 0.1	104	116 ± 3	93
5 min	0.370 ± 0.03*	56	5.8 ± 0.2	90	116 ± 4	93
10 min	0.403 ± 0.01*	61	5.7 ± 0.1	89	115 ± 5	93
20 min	0.442 ± 0.01*	67	6.9 ± 0.2	108	127 ± 14	102

Values are means ± S.E. of 4-6 experiments and express the incorporation of [^{3}H]-methyl groups into N-methylated phospholipids (PMME + PDME + PC). Perfusion with Ca^{2+}-free medium was carried out for the indicated times; Hearts were then perfused with medium containing 1.25 mM Ca^{2+} for 5 min. Hearts perfused with normal medium for comparable periods served as controls. Other details are as in legend of Table 1.

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

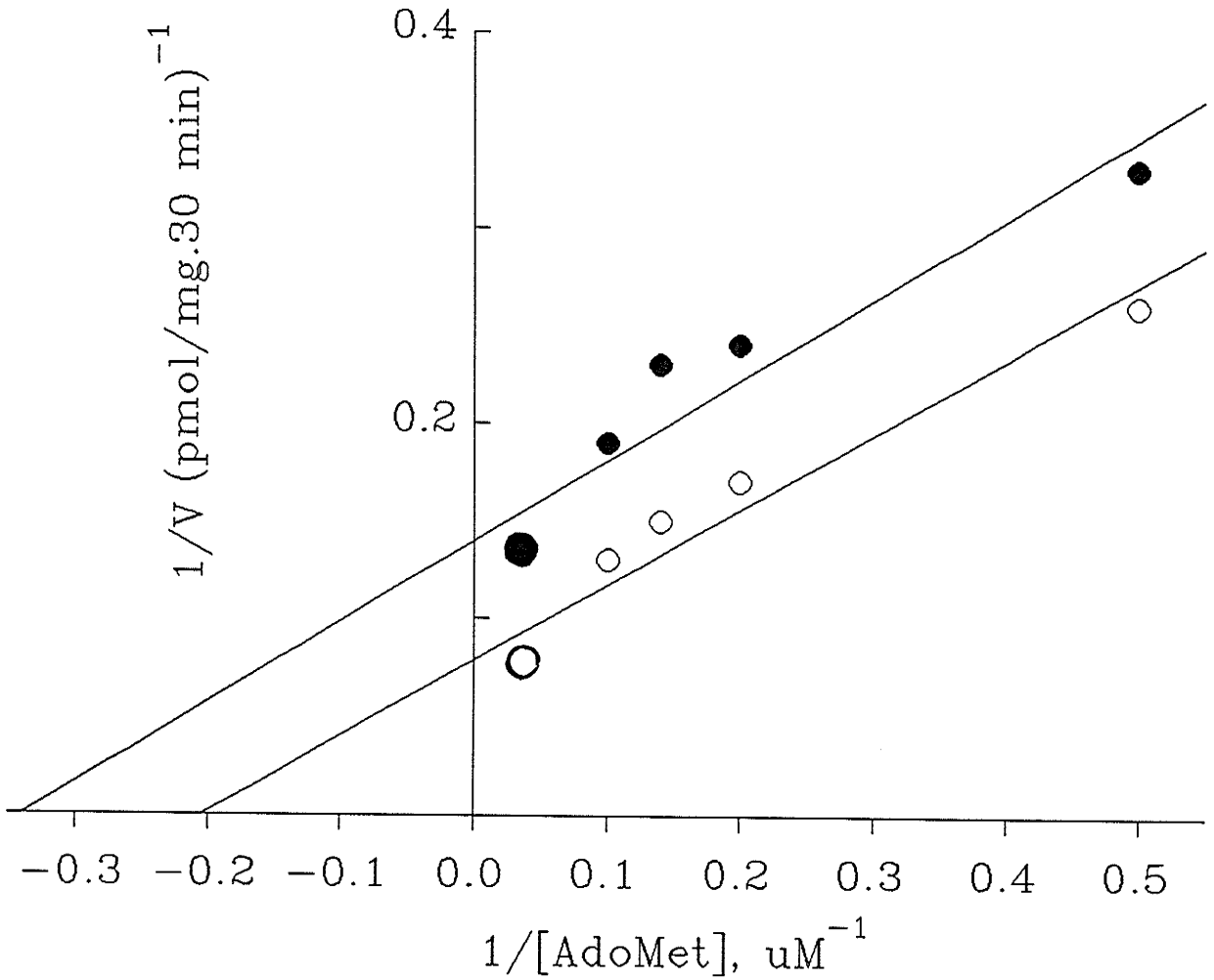

FIGURE II. Lineweaver-Burk plots of total [^{3}H] methyl groups incorporation into sarcolemmal (site II) N-methylated phospholipids for control and paradox hearts. [^{3}H] AdoMet concentrations used were 2-50 μM. K_m and V_{max} for control hearts were 4.5 uM and 12.5 pmol/mg/30min respectively. K_m and V_{max} for paradox hearts were 2.94 uM and 7.14 pmol/mg/30min respectively. Sarcolemmal fractions were isolated according to the procedure of Dhalla et al.(1981).

occuring after Ca^{2+} free perfusion alone.

Having established an inhibition of the SL and SR PE N-methyltransferase enzyme system during Ca^{2+} paradox, we proceeded to study the kinetic characteristics of this inhibition in control and paradox hearts at the standard time of Ca^{2+} paradox (5 min Ca^{2+}-free and 5 min of readmission). A kinetic analysis of the data in heavy SL fraction (Fig. 2) indicates that both the apparent affinity for AdoMet and the apparent V_{max} values for the paradox preparation were significantly altered from control values (uncompetitive inhibition). Quantitative alteration of K_m and V_{max} was 35% and 43% of control values respectively. In the case of the SR (Fig. 3), however, the apparent V_{max} for the paradox preparations was significantly lowered (42%) with K_m remaining unaltered (competitive inhibition). Both the K_m and V_{max} values observed in the SL and SR fractions of control hearts are consistent with those reported previously (12,62).

Since the incubation of rat heart subcellular membrane system with the methyl donor S-adenosyl-L-[methyl-^{3}H]methionine results both in N-methylation of PE and methylation of a heterogenous fraction of non-polar lipids in the membrane (117), we also examined the [^{3}H]-methyl incorporation into non-polar lipids during the phenomenon of Ca^{2+}-paradox.

[^{3}H]-methyl group incorporation into the SL and SR non-polar lipid fraction was studied at 5 min Ca^{2+}-free perfusion (0 min readmission) and at 5 min of readmission after 5 min of Ca^{2+}-free perfusion (Table 5). The [^{3}H]-methyl group incorporation into SL

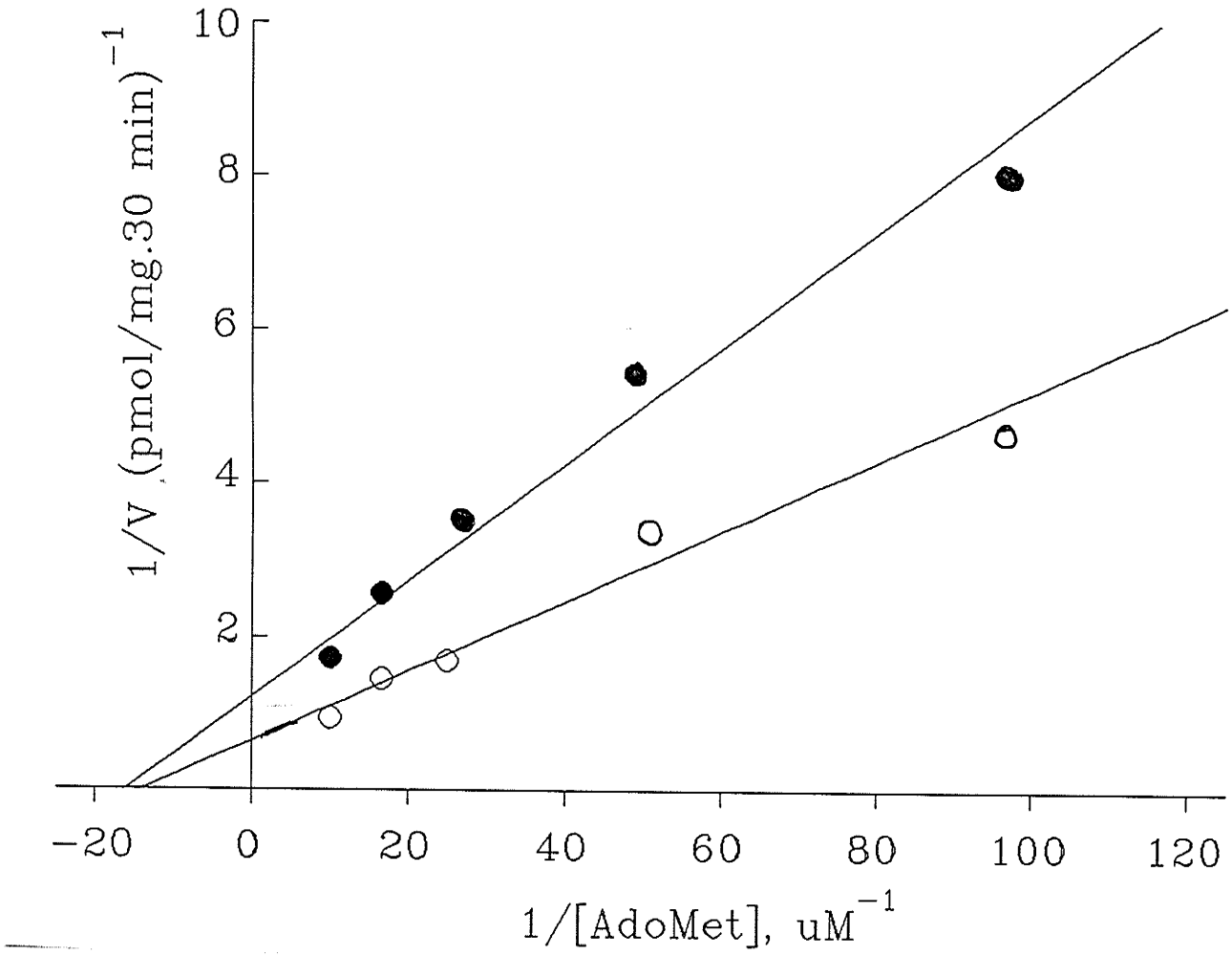

FIGURE III. Lineweaver-Burk plots of total [^{3}H]methyl groups incorporation into sarcoplasmic reticular (site I) N-methylated phospholipids for control and paradox hearts. [^{3}H] AdoMet concentrations used were 0.01-0.3 uM. K_m and V_{max} for control hearts were 0.07 uM and 1.43 pmol/mg/30 min respectively. K_m and V_{max} for paradox hearts were 0.066 uM and 0.83 pmol/mg/30min respectively. Sarcoplasmic reticular fractions were isolated according to the procedure of Harigaya and Schwartz.

non-polar lipids showed no significant difference from control values at either 0 min or 5 min of readmission. However, the trend at the lower concentrations of AdoMet (0.05 and 10 μM) was towards an increase after Ca^{2+}-free perfusion and a significant decrease in methylated non-polar lipids was seen after 5 min of Ca^{2+}-readmission when compared to that at the end of Ca^{2+}-free perfusion. On the other hand, the quantity of SR methylated non-polar lipids at 0.05 μM AdoMet increased significantly after Ca^{2+}-free perfusion (0 min readmission) and decreased significantly after 5 min of Ca^{2+}-readmission (Table 5); although the differences were not significant, this trend was also observed at higher concentrations of AdoMet.

The increase in methylated non-polar lipids seen at the end of Ca^{2+}-free perfusion is consistent with previous observation (117), that there is an increase in neutral lipids at the end of Ca^{2+}-free perfusion. No information is at present available regarding the level of neutral lipid on Ca^{2+}-readmission.

Effect of Ca^{2+} paradox on the transmethylation system of the mitochondria

The status of the mitochondrial PE N-methyltransferase system during Ca^{2+} paradox was also examined. At the mitochondrial level, the transmethylation reaction at the standard time of Ca^{2+} paradox (5 min Ca^{2+}-free and 5 min readmission) produced no significant alterations from control at any of the catalytic sites (Table 6). Thus, PE N-methyltransferase activity was totally unaffected at

Table 5. Comparison of AdoMet-dependent [^{3}H]-methyl incorporation into non-polar lipids of SL and Sr membranes isolated from rat hearts subjected to 5 min of Ca^{2+}-free perfusion without or with 5 min of Ca^{2+}-readmission.

Experimental Groups	Sarcolemma			Sarcoplasmic Reticulum		
	AdoMet			AdoMet		
	0.05 uM	10 uM	150 uM	0.05 uM	10 uM	150 uM
Control	0.295 ± 0.03	4.0 ± 0.3	91 ± 6	0.307 ± 0.03	3.1 ± 0.2	69 ± 4
Duration of Ca^{2+}-free perfusion before 5 min of Ca^{2+}-readmission						
0 min	0.360 ± 0.03	4.4 ± 0.1	99 ± 3	0.448 ± 0.02*	4.0 ± 0.5	72 ± 10
5 min	0.304 ± 0.061^	3.4 ± 0.2^	97 ± 6	0.108 ± 0.01*^	3.4 ± 0.3	69 ± 3

Values are means ± S.E. of 4-6 experiments and are expressed as pmol [^{3}H]-methyl groups incorporated/mg/30 min. Experiments were done under assay conditions for PE N-methyltransferase site I (0.05 uM AdoMet), II (10 uM), III (150 uM). Perfusion with Ca^{2+}-free medium was carried out for 5 min; thereafter, the hearts were not (0 min) or were (5 min) perfused with medium containing 1.25 mM Ca^{2+}. Hearts perfused with normal medium for comparable periods served as controls.

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

^ Significantly ($P < 0.05$) different from values at 0 min.

the level of the mitochondrion by the phenomenon of Ca^{2+}-paradox. Examination of the [^{3}H]-methyl incorporation into the non-polar lipid fraction of the mitochondrion also reveals no significant differences with respect to control at all the three concentrations of AdoMet (0.05 μM, 10 μM and 150 μM) studied.

Changes in PE N-methyltransferase activity in the light sarcolemmal fraction of rat hearts during Ca^{2+} paradox

In order to remove the possibility of artifacts in the findings related to the heavy sarcolemmal fraction we evaluated the status of PE N-methyltransferase during Ca^{2+}-paradox in light sarcolemmal fraction isolated by the method of Pitts et al. (1979). In this case 5 min of Ca^{2+}-free perfusion followed by 5 min of Ca^{2+}-readmission resulted in an inhibition by about 59% of [^{3}H]-methyl incorporation from the control value at catalytic site II (Table 7). Both the degree of inhibition and the site of inhibition are consistent with the findings related to the heavy SL fraction as shown above (Table 1). Catalytic sites I and III were unaffected, as it was also observed in the previous case. 5 min of Ca^{2+} free perfusion followed by 5 min of Ca^{2+}-readmission also caused a decrease in [^{3}H]-methyl incorporation into the non-polar lipid fraction at a concentration of AdoMet of 10 μM, also this result was similar to that of the heavy sarcolemmal fraction. Thus this proves that the changes seen in the SL PE N-methyltransferase system are due to the Ca^{2+}-paradox phenomenon rather than to an artifact of the method of SL isolation.

Table 6. Synthesis of methylated lipids in mitochondria isolated from rat hearts subjected to Ca^{2+}-paradox.

Catalytic Site	N-methylated PL		Non-polar lipids	
	Control	Paradox	Control	Paradox
	pmol/mg/30 min		pmol/mg/30 min	
I	0.678 ± 0.02	0.791 ± 0.02	0.486 ± 0.04	0.642 ± 0.15
II	10.3 ± 0.5	9.5 ± 0.6	4.4 ± 0.8	5.6 ± 0.9
III	172 ± 3	170 ± 3	78 ± 2	55 ± 8

Values are means ± S.E. of 4 experiments and are expressed as pmol [^{3}H]-methyl groups incorporated/mg protein/30 min. Perfusion with Ca^{2+}-free medium was carried out for 5 min; hearts were then perfused with medium containing 1.25 mM Ca^{2+} for 5 min. Hearts perfused with normal medium for comparable periods served as controls. N-methylated phospholipids (PMME + PDME + PC) and non-polar lipids were quantitated by thin layer chromotography after incubation of the membranes under standard N-methyltransferase site I, II, and III assay conditions.

Effect of sulfhydryl group modifiers and fatty acids on PE N-methyltransferase activity of cardiac SL and SR

Recent studies indicated that reagents that modify sulfur-containing amino acid residues in the N-methyltransferase system (119) as well as free fatty acids(116) influence the cardiac phospholipid methylation process. In an attempt to evidence the possible molecular mechanism responsible for the changes in cardiac PE N-methyltransferase described above, we verified, in separate experiments, the effect of sulfhydryl group modifying reagents (Table 8) and fatty acids (Table 9) on the paradox affected sites of SL (site II) and SR (site I) membranes.

The sulfhydryl group modifying reagent dithiothreitol (DTT) at a concentration of 5 mM caused a stimulation of the transmethylation reaction for both the control and paradox hearts, in the two subcellular membrane systems studied. In both cases the degree of stimulation of control and paradox hearts was quite similar. Stimulation of SR site I was much greater than that seen for the SL site II methyltransferase activity. The DTT-induced activation and its quantitative difference between site I and II are consistent with previous studies (119).

The other two sulfhydryl group modifying reagents, reduced glutathione (GSH) and oxidized glutathione (GSSG), at a concentration of 5 mM induced no significant change in sarcolemmal N-methyltransferase activity in both control and paradox hearts (Table 8).

Table 7. [^{3}H]-methyl incorporation into N-methylated and non-polar lipid products of the light sarcolemmal fraction isolated from rat hearts subjected to Ca^{2+}-paradox.

Catalytic Site	N-methylated PL			Non-polar lipids		
	Control	Paradox		Control	Paradox	
	pmol/mg/30 min	pmol/mg/30 min	% control	pmol/mg/30 min	pmol/mg/30 min	% control
I	0.655 ± 0.07	0.720 ± 0.03	100	1.511 ± 0.4	1.542 ± 0.5	102
II	6.4 ± 0.7	3.8 ± 0.6*	59	3.3 ± 0.1	1.9 ± 0.2*	57
III	76 ± 13	74 ± 15	98	55 ± 10	47 ± 10	86

Values are means ± S.E. of 4 experiments. Perfusion with Ca^{2+}-free medium was carried out for 5 min; hearts were then perfused with medium containing 1.25 mM Ca^{2+} for 5 min. Hearts perfused with normal medium for comparable periods served as controls. N-methylated phospholipids and non-polar lipids were quantitated by thin layer chromotography after incubation of the membranes under standard PE N-methyltransferase site I, II, and III assay conditions.

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

Table 8. Effect of sulfhydryl group modifying reagents on the sarcolemmal site II sarcoplasmic Reticular site I PE N-methyltransferase activity during Ca^{2+}-paradox.

Experimental Groups	PE N-methyltransferase activity			
	Control		Paradox	
	pmol/mg/30 min	Change (%)	pmol/mg/30 min	Change (%)
A. Sl (site II)				
No addition	10.5 ± 0.5	100	7.8 ± 0.3	100
5 mM DTT	28.9 ± 2.9*	+174	18.7 ± 0.7*	+139
5 mM GSH	9.4 ± 0.3	-11	8.5 ± 0.1	+9
5 mM GSSG	9.0 ± 0.5	-15	6.7 ± 0.1	-14
B. SR (site I)				
No addition	0.690 ± 0.04	100	0.435 ± 0.04	100
5 mM DTT	9.004 ± 0.70*	+1205	5.564 ± 0.45*	+1179
5 mM GSH	1.476 ± 0.02	+114	1.112 ± 0.04*	+155
5 mM GSSG	0.414 ± 0.03	-40	0.334 ± 0.01*	-23

Values are means ± S.E. of 4 experiments. Sarcolemmal (SL) and sarcoplasmic reticular (SR) membranes were isolated from control hearts and hearts subjected to Ca^{2+}-paradox (5 min Ca^{2+}-free + 5 min Ca^{2+} readmission. Preincubation of membranes without and with each modifier was for 10 min at 37°C. Methylation was performed for 30 min in the same assay medium in the presence of 10 uM (site II) and 0.055 uM (site I) [^{3}H] AdoMet, respectively. DTT = dithiothreitol; GSH = reduced glutathione; GSSG = oxidized glutathione.

* Significantly ($P < 0.05$) different from values obtained in the absence of modifiers.

The DTT, GSH and GSSG - induced effects on SR site I methylation were in keeping with previous findings (119) and were not different in control and paradox membranes (Table 8).

In separate experiments the effect of two free fatty acids, oleic acid and arachidonic acid, whose intracellular concentration was shown to be increased at the end of Ca^{2+}-free period (117), was studied on sarcolemmal (site II) and sarcoplasmic reticular (site I) methyltransferase activity (Table 9). In the case of sarcolemma, both oleic acid and arachidonic acid produced a slight but significant inhibition of the transmethylation reaction in control hearts in contrast to paradox hearts where both free fatty acids caused a significant stimulation. In the case of sarcoplasmic reticulum the effect of both free fatty acids were significantly inhibitory to the PE N-methyl transferase activity of control and paradox hearts.

Table 9. Effect of free fatty acids on the sarcolemmal site II and sarcoplasmic reticular site I methyltransferase activity in Ca^{2+}-paradox.

Experimental Groups	PE N-methyltransferase activity			
	Control		Paradox	
	pmol/mg/30 min	Change (%)	pmol/mg/30 min	Change (%)
A. Sl (site II)				
No addition	8.9 ± 0.1	100	6.7 ± 0.04	100
+ Oleic acid	7.4 ± 0.3*	-16	8.4 ± 0.4*	+24
+ Arachidonic acid	7.9 ± 0.2*	-11	10.2 ± 0.13*	+51
B. SR (site I)				
No addition	0.675 ± 0.03	100	0.385 ± 0.01	100
+ Oleic acid	1.142 ± 0.01*	-71	0.119 ± 0.01*	-69
+ Arachidonic acid	0.526 ± 0.02*	-22	0.339 ± 0.02*	-12

Values are means ± S.E. of 4 experiments. Sarcolemmal (SL) and sarcoplasmic reticular (SR) membranes were isolated from control hearts and hearts subjected to Ca^{2+}-paradox (5 min Ca^{2+}-free + 5 min readmission. Preincubation of membranes without and with each fatty acid was for 10 min at 37°C. Methylation was performed for 30 min in the same assay medium in the presence of 10 uM (site II) and 0.055 uM (site I) [^{3}H] AdoMet, respectively.

* Significantly ($P < 0.05$) different from values obtained in the absence of fatty acid.

DISCUSSION

The present study focusses on the effect of calcium overload on the cardiac PE N-methyltransferase (PNMT) enzyme system, during the typical Ca^{2+} overload situation of the calcium paradox phenomenon. Extrapolating from the argument presented in the statement of the problem, and from previous studies which investigated alterations in PNMT during Ca^{2+} overload-related cardiac pathologies (58,62,63), one would expect an inhibition of PNMT activity during Ca^{2+} overload. This result was exactly what was observed in this study. The PE N-methyltransferase enzyme was studied in all the three subcellular membrane fractions in which it was previously shown to exist (118) and under varying durations of Ca^{2+}-free perfusion and Ca^{2+} readmission. The results show that at the sarcolemmal level the Ca^{2+} paradox damage on PE N-methylation is reperfusion-induced, while no alterations occur during the duration of calcium-free perfusion. This fact is suggested quite clearly by the data in Tables 1 and 3 which shows firstly that Ca^{2+}-free perfusion alone has no effect on PNMT (Table 1), and secondly that reperfusion-induced damage is independent of the time of Ca^{2+}-free perfusion (Table 3). However, the inhibition occuring upon Ca^{2+}-readmission is time dependent, i.e. the degree of inhibition of the SL PNMT system is progressively larger with increasing time of Ca^{2+}-readmission. These results, which indicate damage of an enzyme system produced on reperfusion with Ca^{2+} containing solution

while no change are seen on perfusion with Ca^{2+}-free solution only, are consistent with previous studies (17) showing that major damage during Ca^{2+}-paradox occurs during Ca^{2+}-repletion with very few changes occuring during the Ca^{2+}-free perfusion period. It may be pointed out at this juncture that, regardless of the fact that damage due to calcium paradox becomes apparent on Ca^{2+}-readmission, it may be that the myocardial cellular system becomes susceptible to this damage during the period of Ca^{2+}-free perfusion. In short, the Ca^{2+}-free perfusion sets the stage for the various damages (in this case to the PNMT system) produced on reperfusion with calcium containing solution.

The catalytic site that exhibits the significant change in activity at the SL level is catalytic site II of the PNMT system which is responsible for the second methyltransfer reaction synthesizing PDME.

Examination of PNMT system at a second subcellular level where it was previously found to exist(118), the sarcoplasmic reticulum, showed a behavioral pattern very similar to that seen at the level of the sarcolemma, although several points of difference were also noted (Tables 2 and 4). The sarcoplasmic reticular PNMT system, like the sarcolemmal system also exibited an inhibition of its activity during Ca^{2+}-paradox that occured on Ca^{2+}-repletion rather than during Ca^{2+}-free perfusion. This is quite consistent with previous studies(17) as was pointed out earlier in the discussion. Also in this case, like in the case of SL, the degree of inhibition is dependent on the time of Ca^{2+}-repletion (Table 2) and independent

of the time of Ca^{2+}-free perfusion (Table 4). In both subcellular membranes maximal PNMT inhibition occured at 5 min of Ca^{2+}-repletion after 5 min of Ca^{2+}-free perfusion. The PNMT system at the two subcellular levels differ in two respects, however: firstly, the catalytic site that is affected in the SR is site I, while sites II and III are completely unaffected (Table 2 and 4). This is in contrast to the sarcolemmal system where it is the catalytic site II that is altered while site I and III remain unaffected. These results are not inconsistent with previous studies (115) which have shown that not only is site I the most susceptible among the SR methyltransferase sites (47,115), but that in the same cardiomyopathic state the site of attack for SL and SR can be different (site I for SR and site II for SL) (115). The second point of difference observed is the minimum time of Ca^{2+}-free perfusion required to produce the activation which becomes apparent on Ca^{2+}-readmission. In the case of SR the minimum time of Ca^{2+}-free perfusion required to produce a significant inhibition of the PNMT system during Ca^{2+}-readmission is 1 min (Table 4) against 2.5 min in the case of SL (Table 2), a non significant deactivation being observed in the latter system after 1 min of Ca^{2+}-free perfusion. This second point of difference may be an indicator of different levels of protection the cardiomyocyte affords itself. It may be the case that by possessing a sarcolemmal PNMT system which depresses the Ca^{2+} influx-related mechanisms and is of greater resistance to Ca^{2+}-overload, the cardiomyocyte may in effect be attempting to protect itself as long as it is possible from the

suddenly increased concentration of calcium in the extracellular space due to the Ca^{2+}-readmission. Hypothetically, one may speculate that the sarcoplasmic reticular and the sarcolemmal PNMT systems may be related in Ca^{2+} paradox as follows: during Ca^{2+}-free perfusion it is well known that electromechanical dissociation occurs (17), i.e. electrical activity continues while mechanical activity ceases completely. By virtue of this fact $[Na^+]_i$ increases, as Na^+ is brought in during depolarization but is not removed that effectively, in fact the Na^+-K^+ ATPase is inhibited during calcium-free perfusion (84) and the Na^+-Ca^{2+} exchanger is unable to exchange Na^+ for extracellular Ca^{2+}, as this cation is absent. Upon Ca^{2+}-readmission, the Na^+-Ca^{2+} exchanger may now become highly effective in removing the Na^+ and thus increasing the cytosolic calcium concentration. Indeed, recent studies have suggested that the entry of Ca^{2+} into the cardiac cell via Na^+-Ca^{2+} exchange is a causative factor for the Ca^{2+} paradox damage (120,121). Since high $[Ca^{2+}]$ has been shown to inhibit PNMT (11) this initial rise in calcium on calcium readmission may be sufficient to produce a significant inhibition of the sarcoplasmic reticular PNMT system. Alteration to this system in the SR, in turn, may be effective in inhibiting the SR Ca^{2+}-pump activity. Thus, although this has not been tested directly in this study, a very good correlation has been observed between the changes in the SR Ca^{2+}-pump seen previously (107), and the present data on alteration in the SR PNMT system, with respect to the duration of calcium readmission. Although transmethylation may not be the only system causing the

observed changes in the SR calcium pump during calcium paradox, it may be one of the mechanisms responsible for the well documented changes in the SR Ca^{2+}-pump activity (107) which will cause a decreased sequestration of calcium from the cytosol, leading to a more aggravated calcium overload situation. Since the data show that the SL PENMT system is more resistent and therefore requires a more aggravated overload situation, this increased level of cytosolic calcium caused by continued SR Ca^{2+} dumping but decreased sequestration, may now be able to effect an inhibition to the sarcolemmal PNMT system. The inhibition thus produced, will cause the PE N-methylation-related changes of some SL Ca^{2+}-transport systems such that the calcium overload situation is aggravated even further. This speculation can be directly supported by previous findings which show that the SL Na^{+}-dependent Ca^{2+} uptake and Ca^{2+}-pump activities are inhibited and stimulated upon methylation, respectively (35,44,45,46). Further, during Ca^{2+}-readmission, the Na^{+}-dependent Ca^{2+} uptake was found to be activated (up to 2 min) while the Ca^{2+}-pump was depressed (92). This is consistent with the SL transmethylation reaction being inhibited at the same stage of paradox, as it is shown in this study; of further note the SL Ca^{2+} regulatory system are altered to a direction which potentiates overload. Another point of view that may explain the results is that the two subcellular methyltransferase systems may be differentially sensitive to $[Na^{+}]$ itself. This can therefore explain the different lengths of Ca^{2+}-free period being required to damage the two subcellular enzyme systems, the sarcolemmal PNMT

system being more resistant than the sarcoplasmic reticular one. From the above points, PE N-methylation may looked upon as both a cause and a consequence of Ca^{2+}-overload, according to the time sequence (i.e. time of Ca^{2+}-free and time of Ca^{2+} repletion) at which it is being studied. From the time course experiments done with SR and SL in this and in previous studies, we derived the standard calcium paradox time of 5 min of Ca^{2+}-free and 5 min of Ca^{2+} readmission that, mechanically, induced a marked rise in resting tension without any recovery of contractile force. This standard paradox time was used to study the third subcellular organelle where the PNMT has been reported to be localized the mitochondria. In these organelles the enzyme activity was totally unaffected (Table 6) at all of the three catalytic sites. So it seems that the mitochondrial PNMT system is resistant to the paradox insult.

Having established the inhibitory effect of the calcium paradox on the PNMT system, we proceeded to investigate the possible causes of this disfunction. The standard paradox time of 5 min Ca^{2+}-free perfusion before 5 min of Ca^{2+}-readmission, and the specific sites affected in the two different subcellular systems (site I for SR and site II for SL) were considered in these series of experiments. First we investigated the kinetic parametres of the SL and SR PNMT system of control and paradox hearts. The PNMT enzyme of the two subcellular membranes exibited a completely different kinetic behavior in paradox damaged hearts. An uncompetitive type of inhibition was observed in the heavy sarcolemmal fraction (site II), where a decrease in the apparent K_m (35%, indicating an

increase in affinity of the enzyme for AdoMet) was accompanied by a decrease in the apparent V_{max} (43%). In this regard it may be that the paradox-induced damage is associated with a decreased density of available catalytic sites that supersedes the increased affinity of the existing sites for the methyl donor. This may explain the overall depression of the SL site II methyltransferase reaction.

In the SR case, the inhibition is non-competitive, i.e., although the apparent V_{max} of the paradox hearts is lowered (42%), as in SL, the apparent K_m remains unaltered. This may be due to some factor preventing the association of the methyl donor with the catalytic site of the enzyme, thereby compromising the conversion of the phospholipid substrate to the methylated end-product.

Thus, it may be hypothesized that the SL and SR PNMT enzyme systems may be modulated by different mechanisms, as is obvious from their dissimilar kinetic profiles. Thus the next step in our analysis was to investigate the possible molecular mechanisms involved in the paradox-induced PNMT inhibition, based on the fact that free fatty acids (117) and sulfhydryl group modifying agents (119) were found to modulate the cardiac PMNT system.

With regard to the possible role of free fatty acids in the Ca^{2+}-paradox-related PNMT inhibition, it was suggested (116) that changes in phospholipase activities during certain patho-physiological conditions, e.g. diabetic and catecholamine-induced cardiomyopathy, may tend to cause disturbances in free fatty acid levels leading to the reported decreases in the SL PE-N-methylation

activity (58,62). In fact, definite and significant increases in free fatty acid level have been reported in pathophysiological conditions such as diabetes, certain dietary conditions and stress (122,123,124). Also evidence for fatty acid inhibition of PE N-methylation in liver membranes has been reported (125). In vitro experiments were therefore conducted to further investigate the effect of free fatty acids on PNMT activity by including them in the assay mixture. Two fatty acids which were found to be increased during Ca^{2+}-free perfusion (117), oleic acid and arachidonic acid, were used. In SL and SR membranes isolated from control hearts, both oleic acid and arachidonic acid were inhibitory to the PNMT system (Table 9). This is consistent with results obtained by Vetter et al. (116). In the case of the paradox hearts, both oleic and arachidonic acids were inhibitory to the PNMT system of the sarcoplasmic reticulum but stimulatory to that of the sarcolemma (Table 9). The latter effect and its possible consequences on the function of SL methyltransferase during Ca^{2+} paradox are difficult to explain at present and require further studies. However, it may be speculated that, since the free fatty acids are stimulatory to the SL PNMT system in the paradox condition, the apparent depression of the SL PNMT may be due partly to the reperfusion-induced decrease in membranal free fatty acid levels. The finding that SR and SL PNMT systems from paradox heart do not react in a similar manner to free fatty acids may be explained by the fact that the two different subcellular systems seem to be modulated by different factors or mechanisms

(126). This is not difficult to envision since, even in the present study, the two system have exibited many points of difference e.g. in the catalytic site susceptible to damage, kinetic profiles and in the resistance to calcium paradox damage. Our data showed also an increase in [^{3}H]-methyl group incorporation into a heterogeneous fraction of non-polar lipids of the membranes compared to control values at the end of Ca^{2+}-free perfusion, while a decrease was observed at the end of 5 min repletion. This was particularly evident in the affected site of the sarcoplasmic reticulum (site I) although a similar trend, but less significantly so, was seen in the case of the sarcolemma (site II). The nature of the methylated non-polar lipids was not examined in this study and is now under investigation. However, enzymatic methylation of fatty acids has been described for various tissues and the formed fatty acid methyl esters have been identified as a major component of the membrane non-polar lipid fraction (117). Therefore, the observed changes in non-polar lipid methylation during Ca^{2+} paradox might reflect changes in the formation of fatty acid methyl esters due to the different availability of free fatty acid substrates of the membrane (116). This would be consistent with data reported by Van der Vusse et al. (117) on the rise of cellular free fatty acids at the end of Ca^{2+}-free perfusion. No data are available in the literature regarding the status of free fatty acids at the end of Ca^{2+}-repletion to support the above interpretatiion of oour results on non-polar lipid methylation at this stage.

The second aspect we investigated was at the level of the enzyme protein itself. In this respect we examined the effect of sulfhydryl group modifying reagents, dithiothreitol (DTT) as well as reduced glutathione (GSH) and oxidized glutathione (GSSG) which are the glutathione forms in the cell (table 8). In the case of the hearts subjected to paradox similar results were observed as in the case of the control hearts, the degrees of stimulation and inhibition being around a similar range. Thus, from these series of experiments we can conclude that although the N-methyltransferase protein itself may be damaged during Ca^{2+}-overload, the damage most likely does not occur at the level of the sulfhydryl groups. However, in order to prove or disprove this point as the case may be, further experiments are required which should focus on the effects of including DTT in the perfusion medium in both Ca^{2+}-free and Ca^{2+}-containing buffers. Moreover, the possibility that an imbalance of the cellular thiol equilibrium (127) during Ca^{2+} paradox rather than Ca^{2+} itself be the cause of the observed inhibitions appears unlikely; since, if this were the case, the PNMT systems of control and paradox hearts would have reacted differentially to the thiol modifying reagents.

Thus, in summary, one can state that the cardiac PE N-methyltransferase enzyme system is definitely related to the Ca^{2+}-overload condition as it is expressed during Ca^{2+}-paradox, may be both at the level of being a cause and a consequence of this phenomenon. The molecular mechanism(s) by which it is affected are uncertain at this point in time, although one can venture to

state that free fatty acids may be one of the possibilities, especially at the level of the SL. Although the protein itself may be altered in some way, our data so far do not support the involvement of sulfhydryl groups in this alteration.

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