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ACTIVATION OF Ca^{2+}-STIMULATED ATPase BY PHOSPHOLIPID N-METHYLATION IN CARDIAC SARCOPLASMIC RETICULUM

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Incubation of cardiac sarcoplasmic reticulum (SR) in the presence of S-adenosyl-L-methionine, a methyl donor for the enzymatic N-methylation of phosphatidylethanolamine, increased Ca^{2+}-stimulated ATPase activity. The increase in Ca^{2+}-ATPase activity was not due to changes in the affinity for Ca^{2+} and was prevented by methyl acetimidate, an inhibitor of phospholipid N-methylation. The results suggest a possible regulatory role of phospholipid N-methylation in SR Ca^{2+}-pump mechanism.

The critical dependence of cardiac function on Ca^{2+} is now well known and the sarcoplasmic reticulum (SR) is considered to play an important role in modulating both Ca^{2+} release and Ca^{2+} uptake activities in the myocardium (1). The active transport of Ca^{2+} in SR is primarily achieved by Ca^{2+}-stimulated Mg^{2+} dependent ATPase (Ca^{2+}-ATPase) which is embedded in an annulus of phospholipids (2). Although the effects of phospholipids on the ATPase activity and Ca^{2+} transport are related to their apolar character for providing hydrophobic environment (2), the specifity of any given phospholipid in the function of Ca^{2+}-pump has not been fully elucidated. Recently, the synthesis of phosphatidylcholine (PC) by methylation of the amino moiety of phosphatidylethanolamine (PE) has been implicated in the regulation of several membrane-related events (3,4). We have identified three methyltransferase active sites which convert PE to PC in cardiac subcellular membranes in a step-wise manner (5-7). The N-methylation of phospholipids has been shown to enhance Ca^{2+} fluxes in the erythrocyte (8) and kidney cortex basolateral membranes (9). In the

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present study, we report the effects of phospholipid N-methylation on the Ca^{2+}-ATPase activity in purified cardiac SR membranes.

MATERIALS AND METHODS

Cardiac microsomes containing predominantly SR vesicles were isolated by differential centrifugation according to the method described earlier (10). The final pellet was washed twice and suspended in 0.25 M sucrose, 20 mM Tris-HCl (pH 6.8). The SR preparation was examined by electron microscopy, as well as for different marker enzymes (10) and it was found to possess minimal contamination by other subcellular organelles. Cardiac SR membranes (30 µg/ml) were preincubated at 37°C in a medium containing 100 mM KCl, 5 mM $MgCl_2$ and 20 mM Tris-HCl (pH 6.8) with varying concentrations of S-adenosyl-L-methionine (AdoMet) for 10 min and then the total (Mg^{2+} + Ca^{2+}) and Mg^{2+} ATPase activities were determined for 5 min by measuring the hydrolysis of ATP (5 mM) in the presence and absence of 10 µM free Ca^{2+}, respectively. Free Ca^{2+} concentration was maintained by the addition of ethylene glycol-bis-(β-aminoethylether)- N, N'-tetraacetate (EGTA), and the free Ca^{2+} concentrations present were calculated as described previously (10). When Mg^{2+} ATPase was measured 0.2 mM EGTA was also added in the incubation medium. The Ca^{2+}-stimulated ATPase activity reported here is the difference between the total and Mg^{2+} ATPase activities.

Assay of phospholipid N-methylation was carried out at 37°C in an incubation medium similar to that used for ATPase assay. Both ATP and Ca^{2+} were included in the medium. The reaction was initiated by adding [^{3}H]-AdoMet and terminated 10 min later with the addition of 3 ml of chloroform:methanol: 2 N HCl (6:3:1, by vol) followed by 2 ml of 0.1 M KCl in 50% methanol. The incorporation of [^{3}H] methyl groups into SR membrane was measured as described previously (5,6). To fractionate and quantitate various [^{3}H] methyl-labelled phospholipids of the membrane, chromatogram on silica gel 60 F-254 was run at a room temperature in a solvent system containing propionic acid : n-propyl alcohol : chloroform : water (2:2:1:1, by vol). Appropriate standards (phosphatidylcholine, monomethyl-, dimethyl- and phosphatidylethanolamine) were run concomitantly. The lipid spots were visualized by exposure to iodine vapours, scraped and radioactivity counted. Corrections were made for the quenching due to silica gel, in all the data shown here. Radioactivity recovered from the thin layer plate accounted for approximately 80% of the total radioactivity present in the chloroform phase.

S-adenosyl-L-methionine was purified by ion-exchange chromatography as described earlier (5,6). Results were expressed as the mean ± S.E. Statistical analysis was carried out by Student's t test and a P level less than 0.05 was taken to reflect a significant difference between control and experimental values.

RESULTS

To determine whether phospholipid N-methylation alters Ca^{2+}-ATPase activity, SR membranes obtained from rat heart were preincubated for 10 min at 37°C, pH 6.8, with varying concentrations of S-adenosyl-L-methionine (AdoMet; 0.1, 10 and 150 µM). These three concentrations of AdoMet were selected because we have found (5-7) that three catalytic sites for methyltransferase operate at these concentrations. Table 1 shows that Ca^{2+}-ATPase activity was enhanced by 22% and 58% by 10 and 150 µM AdoMet, respectively;

Table 1. Effect of varying concentrations of the methyl donor S-adenosyl-L-methionine (AdoMet) on ATPase activities and phospholipid N-methylation in the rat heart sarcoplasmic reticulum (SR)

AdoMet (μM)	Mg^{2+} ATPase	Ca^{2+}-stimulated ATPase	[^{3}H] Methyl Incorporation (pmol/mg protein/15 min)			
	(nmol Pi/mg protein/5 min)		Total	PMME	PDME	PC
0	10840 ± 750	1150 ± 100	-	-	-	-
0.1	11286 ± 584	1050 ± 170	0.41 ± 0.02	0.19 ± 0.01	0.08 ± 0.003	0.07 ± 0.001
10	10854 ± 888	1406 ± 140	3.6 ± 0.03	0.61 ± 0.03	1.4 ± 0.09	0.92 ± 0.06
150	10952 ± 804	1812 ± 250*	14.8 ± 0.16	2.6 ± 0.43	3.1 ± 0.60	6.1 ± 0.31

Assays were carried out as described under Materials and Methods. Each value represents mean ± S.E. of four experiments done in triplicate. *P < 0.05.PMME: Phosphatidyl N-monomethylethanolamine; PDME: Phosphatidyl-N, N-dimethylethanolamine; PC: Phosphatidylcholine.

however, Mg^{2+} ATPase activity remained unaltered inspite of the increased phospholipid methylation of cardiac SR membranes. The ATP-supported Ca^{2+} uptake activity of SR in the presence of 5 mM oxalate (10) was increased by about 45% upon methylation with 150 μM AdoMet (data not shown). The activation of Ca^{2+}-ATPase did not appear to be species dependent since about 50% stimulation of this enzyme activity was evident when SR membranes from canine heart were preincubated for 3 min with (5215 nmol Pi/mg/5 min) or without (3524 nmol Pi/mg/5 min) 150 μM AdoMet. The maximal stimulation of Ca^{2+}-ATPase took place at a concentration of AdoMet that synthesized PC and comparatively smaller amounts of intermediate products such as monomethyl-, and dimethyl-phosphatidylethanolamine (Table 1).

The effect of methyl acetimidate, a specific inhibitor of phospholipid N-methylation that blocks the amino group of PE molecule (11), was examined on cardiac SR ATPase activity. Membranes were preincubated with 150 μM AdoMet after methyl acetimidate pretreatment and the results are shown in Fig. 1. Methyl acetimidate markedly reduced phospholipid N-methylation and prevented the AdoMet-induced increase in Ca^{2+}-ATPase activity. Similar results were obtained with S-adenosyl-L-homocysteine, another inhibitor of phospholipid N-methylation (5,6).

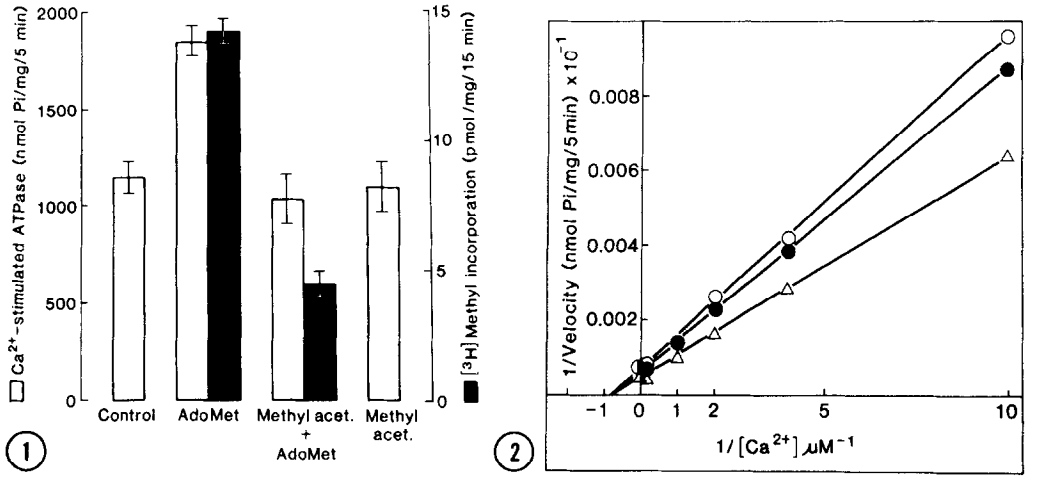

Fig. 1. Inhibition of Ca^{2+}-ATPase activation by methyl acetimidate hydrochloride. Cardiac SR membranes were incubated with methyl acetimidate (8 mg/mg protein) at 37°C for 15 min. The treated vesicles were centrifuged at 40,000 g for 45 min, resuspended in 0.25 M sucrose, 20 mM Tris-HCl (pH 6.8) and used for Ca^{2+}-ATPase and phospholipid N-methylation assays as described in Table I. Control membranes follow the same protocol without methyl acetimidate and AdoMet treatment.

Fig. 2. Effect of varying Ca^{2+} concentrations on Ca^{2+}-ATPase with or without AdoMet. The double reciprocal plots indicate that the apparent K_a value (1.4 μM) for control (○) and membranes methylated by 10 μM (●) or 150 μM (△) AdoMet does not change, although Vmax values greatly differ. Vmax values are 1220, 1480 and 2060 nmol Pi/mg/5 min for control, 10 μM and 150 μM AdoMet, respectively.

In order to determine if the observed increase in SR Ca^{2+}-ATPase activity was accompanied by changes in the affinity for Ca^{2+}, the ATPase activity was measured at different concentrations of free Ca^{2+} (10^{-7} - 10^{-5}M) following preincubation with 10 or 150 μM AdoMet (Fig. 2). The double reciprocal plot of the data showed that the affinity for Ca^{2+} (K_a=1.4 μM) was unaltered by the phospholipid methylation occurring at 10 and 150 μM AdoMet and the increased activity of Ca^{2+}-ATPase was due to changes in the maximal rate of reaction (Vmax).

DISCUSSION

Membrane phosphorylation by cAMP-dependent protein kinase and calmodulin-dependent protein kinase has now been well documented to stimulate Ca^{2+}-ATPase and

Ca^{2+}-uptake activities in cardiac SR (12-14). It has also been shown that membrane phosphorylation by phospholipid-dependent protein kinase (kinase C) is associated with the stimulation of Ca^{2+}-pump mechanism (15,16). In the present study membrane phospholipid N-methylation was found capable of increasing Ca^{2+}-ATPase activity and this suggests the activation of Ca^{2+} transport across the SR membrane. In fact, our preliminary observations regarding the stimulatory effect of AdoMet on SR Ca^{2+} uptake support this view. Although the mechanism by which phospholipid N-methylation enhances ATPase activity is not known at present, our results are consistent with the observations of other investigators (17) that the SR enzyme activity in the presence of PC is higher in comparison to that in the presence of PE. Recently, Boyle et al. (18) have reported the effect of *in vivo* changes in phospholipid composition on the function of lipid-dependent liver microsomal enzymes. Furthermore, both Ca^{2+} transport and Ca^{2+}-ATPase activities were significantly altered in mice which were maintained on a choline deficient diet and treated with methylation inhibitors. All these data suggest that Ca^{2+} transport system in different tissues including heart is highly sensitive to the phospholipid composition of the microsomal membrane in which it is embedded.

The possible interactions of different protein kinases and phospholipid methyltransferases in the membrane can be seen to be intimately involved in the regulation of Ca^{2+} transport system. In this regard there is evidence indicating a cyclic AMP-dependent mechanism for the activation of phospholipid N-methylation in rat hepatocytes and Leydig cells (19,20). On the other hand, cholera toxin and NaF, which increased the activity of adenylate cyclase without the involvement of the β-adrenergic receptor, had no effect on phospholipid methylation (21). Hence, the effect of cAMP on phospholipid methylation seems controversial and extensive studies are required to make any meaningful conclusions. In addition, the regulation of phospholipid N-methylation by Ca^{2+} appears to be mediated by calmodulin dependent protein kinase in rat liver microsomes (22).

In view of a close relationship between SR Ca^{2+} transport activity and muscle relaxation, the observed augmentation in SR Ca^{2+}-ATPase activity by phospholipid N-methylation can be seen to enhance the rate of cardiac relaxation. The signficance of this finding is evident from the fact that catecholamines, which are known to increase SR Ca^{2+} transport activity and shorten the diastole (1) have also been reported to stimulate membrane phospholipid N-methylation in myocardium (23). Likewise, stimulation of SR Ca^{2+} transport (24) and augmentation of membrane phospholipid N-methylation (25) have been observed in early stages of myocardial hypertrophy which are associated with heart hyperfunction (26). A depression in membrane phospholipid N-methylation (6), a decrease in SR Ca^{2+} transport and the inability of cardiac muscle to relax (10) have been observed in diabetic cardiomyopathy. These findings suggest that membrane phospholipid N-methylation may play an important role in the regulation of membrane Ca^{2+} transport and cardiac function under physiological and pathophysiological conditions.

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