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Stimulation of Ca^{2+}-pump in rat heart sarcolemma by phosphatidylethanolamine *N*-methylation

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Incubation of purified cardiac sarcolemmal vesicles (SL) in the presence of *S*-adenosyl-L-methionine, a methyl donor for the enzymatic *N*-methylation of phosphatidylethanolamine (PE), increased the Ca^{2+}-stimulated ATPase and ATP-dependent Ca^{2+} accumulation activities. Quantitative analysis of the methylated phospholipids revealed that maximal increase of Ca^{2+}-pump activities was associated with predominant synthesis and intramembranal accumulation of phosphatidyl-*N*,*N*-dimethylethanolamine. The stimulation of SL Ca^{2+}-pump activities was prevented by inhibitors of PE *N*-methylation such as *S*-adenosyl-L-homocysteine and methyl acetimidate hydrochloride. The results suggest a possible role of PE *N*-methylation in the regulation of Ca^{2+}-transport across the heart SL membrane.

Cardiac sarcolemma (SL) contains an outwardly directed, ATP-dependent Ca^{2+} transport system that participates in extruding Ca^{2+} from the cell during the relaxation phase of the cardiac cycle [1]. Sarcolemmal Ca^{2+}-stimulated, Mg^{2+}-dependent ATPase (Ca^{2+}-stimulated ATPase) and ATP-dependent Ca^{2+} accumulation activities, which are functional expression of the pump mechanism [2], have been shown to increase upon phosphorylating the membranes with cyclic AMP-dependent protein kinase [3] and calmodulin [2]. In fact, it has been demonstrated that heart sarcolemmal Ca^{2+}-pump activities are regulated by a phosphorylation-dephosphorylation cycle [4]. Since the natural domain of the Ca^{2+} transport system is the hydrophobic region of the membrane [5], it is conceivable that this system may be sensitive to changes in lipid composition and physical properties of its microenvironment. In this regard, it should be noted that acidic phospholipids and polyunsaturated fatty acids have been reported to stimulate the SL Ca^{2+}-pump activities [6,7]. Although phospholipid methylation of membranes from different tissues has been shown to activate Ca^{2+}-pump mechanisms [8,9], no such information on heart sarcolemma in available in the literature. It is pointed out that the N-methylating reaction consists of a sequential addition of three methyl groups from the physiological donor *S*-adenosyl-L-methionine (AdoMet) to the amino moiety of an intramembranal phosphatidylethanolamine (PE) molecule. This biological process, associated with the final synthesis of phosphatidylcholine (PC) via the intermediate formation of mono- and dimethyl derivatives, has

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been implicated in regulating several membrane-related events [10]. Recently, we have described the characteristics of three active catalytic sites of the phospholipid methyltransferase system in heart sarcolemma [11]. In the present study, we report the effect of PE *N*-methylation on the Ca^{2+}-pump activities in purified cardiac SL vesicles.

Sarcolemma-enriched membrane vesicles were isolated from rat (male Sprague-Dawley) ventricles by the method of Pitts [2]. In this preparation, the activity of $(Na^+ + K^+)$-ATPase, a well known marker for sarcolemma, was 24.3 ± 1.8 μmol P_i/mg per h; this activity represented 12–14-fold increase in purity over homogenate values determined in the presence of 1 mM ouabain. Cytochrome *c* oxidase and rotenone-insensitive NADPH-cytochrome *c* reductase activities in these sarcolemmal vesicles revealed minimal contamination by mitochondria and microsomes (approx. 3–5%). Unlike sarcoplasmic reticular membrane, the SL Ca^{2+}-stimulated enzyme showed negligible hydrolysis of *p*-nitrophenylphosphate, indicating that the Ca^{2+}-pump activity in the SL vesicles was not due to contamination by microsomes. For the Ca^{2+}-stimulated ATPase assay, cardiac SL vesicles (25 μg protein) were preincubated at 37°C in a medium (pH 7.4) containing 160 mM KCl, 5 mM $MgCl_2$, 5 mM NaN_3 and 20 mM Mops (4-morpholinepropanesulfonic acid) with varying concentrations of AdoMet (Sigma Chemical Co.) 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 (2 mM) in the presence and absence of 1 μM free Ca^{2+}, respectively. The concentration of free Ca^{2+} was maintained by adding EGTA (ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetate) and was calculated as described previously [13]. 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 total and Mg^{2+}-ATPase activities. ATP-dependent Ca^{2+} accumulation of SL vesicles was determined as previously described [14]. Assay of phospholipid *N*-methylation was carried out at 37°C in an incubation medium similar to that used for the ATPase activities. Both ATP and Ca^{2+} were included in the medium. After 2 min preincubation of the SL membranes (0.5 mg protein), the reaction was initiated by adding *S*-adenosyl-L-[*methyl*-^{3}H]methionine ([^{3}H]-AdoMet, New England Nuclear) and terminated 15 min later with the addition of 3 ml of chloroform/methanol/2 M HCl (6 : 3 : 1, by vol). The detailed procedure for the measurement of total and intermediate methylated phospholipids has been reported earlier [11]. AdoMet was purified as indicated in Ref. 11. The results were analyzed statistically by the Student's '*t*'-test and *P* values < 0.05 were considered to reflect significant differences.

To determine whether phosphatidylethanolamine *N*-methylation has any influence on Ca^{2+}-pump, cardiac sarcolemma was incubated with 0.1, 10 and 150 μM AdoMet under conditions for assaying Ca^{2+}-stimulated ATPase and ATP-dependent Ca^{2+} accumulation activities. These concentrations were selected because we have demonstrated that three catalytic sites for sarcolemmal phosphatidylethanolamine methyltransferase op-

TABLE I

TOTAL AND INTERMEDIATE LIPID PRODUCTS FORMED AFTER SARCOLEMMAL PE *N*-METHYLATION AT VARYING CONCENTRATIONS OF [^{3}H]AdoMet

Assays were carried out as indicated in the text. Values are averages of three experiments. [^{3}H]AdoMet, *S*-adenosyl-L-[*methyl*-^{3}H]methionine; PMME, phosphatidyl-*N*-monomethylethanolamine; PDME, phosphatidyl-*N*,*N*-dimethylethanolamine; PC, phosphatidylcholine.

[^{3}H]AdoMet (μM)	pmol [^{3}H]methyl groups incorporated/mg per 15 min			
	Total	PMME	PDME	PC
0.1	0.19	0.084	0.039	0.031
10	2.74	0.49	1.01	0.63
150	16.10	2.29	3.44	6.94

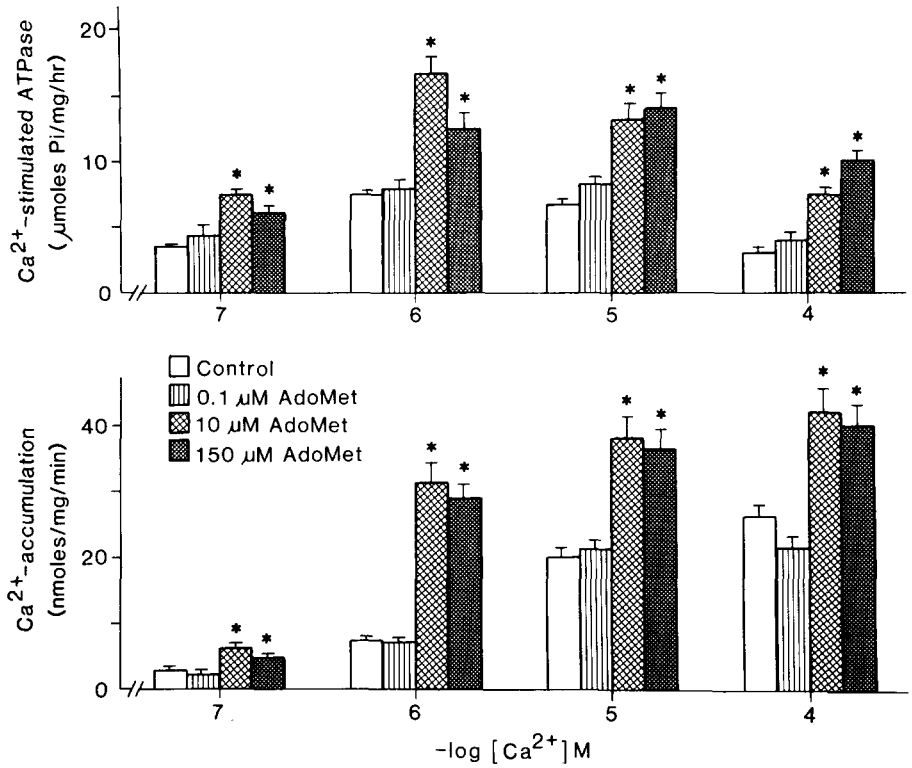

Fig. 1. Activation of heart sarcolemmal Ca^{2+}-pump activities by AdoMet. Assays for Ca^{2+}-stimulated ATPase and ATP-dependent Ca^{2+} accumulation activities were carried out as indicated in the text. Different concentrations of free Ca^{2+} were employed in the presence or absence of 0.1, 10, 150 μM AdoMet. Values are means ± S.E. of four experiments done in triplicate. * Significantly ($P < 0.05$) different from control.

erate at 0.1, 10 and 150 μM AdoMet [11]. Total and intermediate methylated lipid products formed after methylating SL membranes in the above assay conditions are shown in Table I. The activity of three catalytic sites involved in the methylation process is manifested by the major synthesis of specific phospholipid molecules, namely phosphatidyl-*N*-monomethylethanolamine (PMME), phosphatidyl-*N*,*N*-dimethylethanolamine (PDME) and PC at 0.1 μM (site I), 10 μM (site II) and 150 μM (site III) AdoMet, respectively. Results in Fig. 1 indicate that Ca^{2+}-stimulated ATPase activity was significantly enhanced at 10 and 150 μM AdoMet; this effect was evident when different concentrations of free Ca^{2+} were used in the assay media. It may be noted that 100 μM Ca^{2+} inhibited the Ca^{2+}-stimulated ATPase activity in all the preparations; a similar effect was also observed with the purified enzyme from calf heart [6]. No changes in Mg^{2+}-ATPase activity were evident upon methylating membranes; Mg^{2+}-ATPase values were 169 ± 11, 170 ± 12, 169 ± 13 and 173 ± 10 μmol P_i/mg per h for controls and membranes methylated at 0.1, 10 and 150 μM AdoMet, respectively ($n = 4$). A significant increase of ATP-dependent Ca^{2+} accumulation activity was also found at 10 and 150 μM AdoMet in the presence of different concentrations of free Ca^{2+} (Fig. 1). It is of interest that, under optimal concentrations of free Ca^{2+}, both Ca^{2+}-stimulated ATPase and ATP-dependent Ca^{2+} accumulation were maximally enhanced at 10 μM AdoMet which is associated with the synthesis of PDME mainly and small amounts of other methylated products such as PMME and PC.

In one set of experiments, *S*-adenosyl-L-homocysteine (AdoHcy, Sigma Chemical Co.), a potent inhibitor of PE *N*-methylation in cardiac sarcolemma [11], was added upon methylation of the membranes with 10 μM AdoMet. Table II indicates that the activation of both Ca^{2+}-stimulated ATPase and ATP-dependent Ca^{2+} accumulation

TABLE II

PREVENTION OF SARCOLEMMAL Ca^{2+}-PUMP ACTIVATION BY INHIBITORS OF THE PE *N*-METHYLATION PROCESS

Sarcolemmal Ca^{2+}-stimulated ATPase and ATP-dependent Ca^{2+} accumulation activities were assayed at 1 μM and 10 μM free Ca^{2+}, respectively. Methylation of the membranes was performed at 10 μM AdoMet, with or without 100 μM *S*-adenosyl-L-homocysteine (AdoHcy), in an incubation medium similar to that used for ATPase activities. Methyl acetimidate pretreatment (16 mg/ml SL protein) was carried out at 37°C for 15 min; the treated vesicles were sedimented, resuspended in 160 mM KCl/20 mM Mops (pH 7.4) and subsequently incubated with 10 μM AdoMet. Control preparations (Ca^{2+}-stimulated ATPase: 7.2 μmol P_i/mg per h; ATP-dependent Ca^{2+} accumulation: 21.2 nmol/mg per min) followed the same protocol without AdoMet, AdoHcy or methyl acetimidate treatment. Other conditions are indicated in the text. Values are averages of two experiments done in triplicate which agreed within 10%.

Experimental conditions	% of control	
	Ca^{2+}-stimulated ATPase	ATP-dependent Ca^{2+} accumulation
Control	100	100
Methylation (AdoMet)	215.2	184.4
Methylation (AdoMet + AdoHcy)	102.3	94.4
Methyl acetimidate pretreatment and methylation (AdoMet)	93.8	96.1

was abolished when the membranes were methylated in the presence of AdoHcy. In another set of experiments, methyl acetimidate hydrochloride (Pierce Chemical Co.), which blocks the amino group of intramembranal PE molecule and thereby prevents its subsequent methylation [15], was used. It can be seen (Table II) that incubation of the SL vesicles with 10 μM AdoMet after pretreatment with methyl acetimidate had negligible effect on Ca^{2+}-stimulated ATPase and ATP-dependent Ca^{2+} accumulation activities. Separate experiments showed that both AdoHcy and methyl acetimidate had no effect of their own on the Ca^{2+}-transport activities. These results indicate that the stimulation of Ca^{2+}-pump activities by AdoMet may be due to PE N-methylation of the SL membranes.

The maximal enhancement (about 100% stimulation) of Ca^{2+}-stimulated ATPase and ATP-dependent Ca^{2+} accumulation activities was associated with the synthesis of PDME predominantly, whereas the other two methyl derivatives, PMME and PC, were formed in relatively small amounts. On the other hand, PE *N*-methylation in erythrocyte [8] and kidney cortex basolateral [9] membranes has been shown to activate the ATP-dependent Ca^{2+} pump by 40% and 15%, respectively. Furthermore, unlike cardiac sarcolemma, erythrocyte membrane ATPase stimulation was accompanied by the major synthesis of PMME [8]. These conflicting observations may be due to tissue-specificity of the Ca^{2+} transport system with respect to its organization in the membrane. Nonetheless, activation of the heart sarcolemmal Ca^{2+}-pump by PE N-methylation seems specific in nature since the activity of Mg^{2+}-ATPase was not affected under the experimental conditions employed in this study.

Although the sarcolemmal Ca^{2+}-pump is considered to be intimately involved in the efflux of Ca^{2+} from the cell for the relaxation of myocardium [1,7], the relative contribution of this Ca^{2+} transporting system with respect to the other mechanisms present in sarcolemma, sarcoplasmic reticulum and mitochondria has not yet been established. From a functional viewpoint, however, the observed activation of the sarcolemmal Ca^{2+}-pump activity by PE *N*-methylation can be seen to promote Ca^{2+} efflux and thus enhance the rate of cardiac relaxation. Such a behaviour of SL Ca^{2+}-pump would be synergistic with that of the sarcoplasmic reticular Ca^{2+}-ATPase, which has been shown to be activated by PE *N*-methylation [16]. Activation of the SL Ca^{2+}-pump by PE *N*-methylation can also be seen to prevent the occurrence of intracellular Ca^{2+} overload, which is considered to represent an important mechanism in the pathogenesis of cardiac contractile failure (1). Since sarcolemmal phospholipid *N*-methylation was altered under some pathological conditions such as diabetic [11] and adriamycin-induced [17]

cardiomyopathy, it is likely that changes in the PE *N*-methylation activities are involved in the modification of Ca^{2+} transport mechanisms in the diseased myocardium.

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