

Alterations in Phospholipid *N*-Methylation of Cardiac Subcellular Membranes Due to Experimentally Induced Diabetes in Rats

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Abstract

Phosphatidylethanolamine *N*-methylation was examined in cardiac subcellular membranes after inducing chronic experimental diabetes in rats (65 mg streptozotocin/kg, i.v.). The incorporation of radiolabeled methyl groups from *S*-adenosyl-L-methionine in diabetic sarcolemma was significantly depressed at all three catalytic sites (I, II, and III) of the methyltransferase system. An increase in methyl group incorporation was evident at site I without any changes at sites II and III in diabetic sarcoplasmic reticulum and mitochondria. Similar changes were also seen for the individual *N*-methylated lipids (monomethyl-, dimethylphosphatidylethanolamine, and phosphatidylcholine) specifically formed at each catalytic site in all cardiac membranes from diabetic animals. These alterations in *N*-methylation were reversible by a 14-d insulin therapy to the diabetic animals. In the presence of 10 μ M ATP and 0.1 μ M Ca^{2+} , *N*-methylation was maximally activated at site I in both control and diabetic sarcolemma and sarcoplasmic reticulum, but not in mitochondria. Incubation of cardiac membranes with *S*-adenosyl-L-methionine showed that Ca^{2+} -stimulated ATPase activities in both sarcolemma and sarcoplasmic reticulum were augmented; however, the activation of diabetic sarcolemma was lesser and that of diabetic sarcoplasmic reticulum was greater in comparison with the control preparations. These results identify alterations in phosphatidylethanolamine *N*-methylation in subcellular membranes from diabetic heart, and it is suggested that these defects may be crucial in the development of cardiac dysfunction in chronic diabetes. (*J. Clin. Invest.* 1990. 86:777-784.) Key words: cardiac subcellular membranes • diabetic cardiomyopathy • phosphatidylethanolamine *N*-methylation • rat myocardium

Introduction

Phospholipid *N*-methylation reaction consists of the sequential addition of three methyl groups from the physiological

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Received for publication 25 May 1989 and in revised form 27 March 1990.

1. *Abbreviations used in this paper:* AdoMet, *S*-adenosyl-L-methionine; [^3H]AdoMet, *S*-adenosyl-L-[methyl- ^3H]methionine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PMME, phosphatidyl-*N*-monomethylethanolamine; PDME, phosphatidyl-*N,N*-dimethylethanolamine; SL, sarcolemma; SR, sarcoplasmic reticulum.

J. Clin. Invest.

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0021-9738/90/09/0777/08 \$2.00

Volume 86, September 1990, 777-784

donor *S*-adenosyl-L-methionine (AdoMet)¹ to the amino moiety of an intramembranal phosphatidylethanolamine (PE) molecule (1-10). The final synthesis of phosphatidylcholine (PC) is preceded by the formation of intermediates, phosphatidyl-*N*-monomethylethanolamine (PMME) and phosphatidyl-*N,N*-dimethylethanolamine (PDME), in the presence of the membrane-bound methyltransferase system. Previous studies in our laboratory using rat heart subcellular membranes have shown the existence of three methyltransferase catalytic sites (I, II, and III) for PE *N*-methylation, each exhibiting different kinetic parameters, pH profile, sensitivity to divalent cations, and pathobiological behavior (1, 2, 7, 9). These three sites can be identified at 0.055 μ M (site I), 10 μ M (site II), and 150 μ M (site III) concentrations of AdoMet (1, 2, 7). Under optimal conditions, predominant synthesis of specific *N*-methylated phospholipids, namely PMME, PDME, and PC, was found to occur in cardiac membranes at sites I, II, and III, respectively (2, 7).

The phospholipid *N*-methylation has been suggested to be important in changing several membrane associated functions (3) including the control of Ca^{2+} fluxes in the myocardium (4-6). Furthermore, a defect in PE *N*-methylation has been demonstrated in cardiac hypertrophy (7), alcoholic cardiomyopathy (8), and catecholamine-induced cardiomyopathy (9). Although alterations in PE *N*-methylation have also been shown in cardiac sarcolemma (heavy fraction) obtained from diabetic rats (10), no information concerning changes in PE *N*-methylation activity in cardiac sarcoplasmic reticulum (SR) and mitochondria due to this disease is available in the literature. The present study, therefore, was undertaken to investigate the PE *N*-methylation activity in SR and mitochondria from animals with streptozotocin-induced diabetes; sarcolemmal (SL) membrane (light fraction) was used for the purpose of comparison. In addition, this study examined whether changes in PE *N*-methylation activity in different subcellular membranes were reversible upon treatment of diabetic animals with insulin. It may be pointed out that chronic diabetes due to streptozotocin has been shown to result in a cardiomyopathy associated with defects in cardiac ultrastructure, function, and metabolism (10-14). Because PE *N*-methylation has been shown to increase the Ca^{2+} -pump activities in heart SL and SR (4, 5), the effects of PE *N*-methylation on the SL and SR Ca^{2+} -stimulated ATPase activities in control and diabetic hearts were also studied.

Methods

Diabetic animal model. Male Sprague-Dawley rats weighing \sim 175 g each were made diabetic with a single intravenous injection of streptozotocin (65 mg/kg body wt) delivered in a citrate-buffered vehicle. Age-matched control animals received citrate buffer only. All rats were provided commercial food and water *ad libitum* until they were killed by decapitation at 8 wk. Some diabetic animals were given subcutaneous injections of 3 U protamine zinc insulin per day for the last 2 wk

before they were killed at 8 wk. Hearts were removed, atria and any large vessels were carefully trimmed, and the ventricular tissue was processed for the isolation of subcellular membranes. Blood samples were taken at the time of sacrifice and analyzed for plasma insulin concentration by standard radioimmunoassay technique (Amersham Corp., Arlington Heights, IL) and for glucose levels by means of a glucose reagent kit (Sigma Chemical Co., St. Louis, MO). The above experimental protocol is similar to that employed elsewhere for establishing the presence of diabetic cardiomyopathy (10–14).

Preparation of cardiac subcellular membranes. Purified light sarcoplasmic preparation was isolated from pools of two to three hearts according to the method described by Pitts (15). Membrane fraction enriched with sarcoplasmic reticulum (microsomal fraction) was isolated according to the method of Harigaya and Schwartz (16) as described elsewhere (14). The mitochondrial fraction was isolated by the method of Sordahl et al. (17). All these procedures were carried out at 0–5°C, and the membrane activities were assayed immediately after completion of the isolation protocol. To determine the purity of membranes, the activities of marker enzymes such as ouabain-sensitive Na⁺,K⁺-ATPase (12), cytochrome *c* oxidase (14), rotenone-insensitive NADPH cytochrome *c* reductase, and K⁺-EDTA ATPase (14) were measured in all the membrane preparations.

PE N-methylation assay. Phospholipid methyltransferase activity was assayed by measuring the incorporation of [³H]methyl groups into membrane phospholipids in the presence of *S*-adenosyl-L-[methyl-³H]methionine ([³H]AdoMet) as described earlier (1, 2). Assays were performed with 0.5 mg of membrane protein in 0.5 ml of reaction medium under optimal conditions for the three catalytic sites involved in the methyltransferase reactions, as indicated previously (1, 2). Unless otherwise mentioned, incubation for catalytic site I was carried out in the presence of 1 mM MgCl₂, 0.055 μM [³H]AdoMet (80.6 Ci/mmol) at pH 8.0 (50 mM Tris-glycylglycine buffer). For the catalytic sites II and III, incubation was performed without MgCl₂ using 10 μM [³H]AdoMet (200 μCi/mmol), pH 7.0 (50 mM imidazole buffer), and 150 μM [³H]AdoMet (200 μCi/mmol), pH 10.0 (50 mM sodium hydroxide-glycine buffer), respectively. After a preincubation period of 10 min at 37°C, the reaction was initiated by adding [³H]AdoMet and was terminated 30 min later with the addition of 3 ml of chloroform/methanol/2 N HCl (6:3:1, by volume), followed by washing three times with 2 ml of 0.1 M KCl in 50% methanol. In addition to the measurement of total methyl group incorporation, the *N*-methylated phospholipids (PMME, PDME, and PC) were separated by thin-layer chromatography and the methyl group incorporation in these lipid products was determined (1, 2).

Determination of Ca²⁺-stimulated ATPase and Mg²⁺ ATPase activities. Cardiac sarcoplasmic and sarcoplasmic reticular membranes (30 μg/ml) were preincubated at 37°C in a medium containing 100 mM KCl, 5 mM MgCl₂, 5 mM NaN₃, and 20 mM Tris-HCl (pH 7.4 for SL, pH 6.8 for SR) with varying concentrations of AdoMet for 5 min; thereafter, total (Mg²⁺ and Ca²⁺) and Mg²⁺ ATPase activities were determined for 5 min by measuring the hydrolysis of ATP (4 mM) in the presence and absence of free Ca²⁺ (1 μM for SL, 10 μM for SR), respectively. Different concentrations of free Ca²⁺ used with SL and SR preparations were selected for the optimal conditions for these membrane systems. Free Ca²⁺ concentration was maintained by the addition of EGTA and was calculated as described previously (14). When Mg²⁺ ATPase was measured, 0.2 mM EGTA was also added in the incubation medium. The Ca²⁺-stimulated ATPase activity reported here is the difference between the total and Mg²⁺ ATPase activities. Mitochondrial membrane was also preincubated at 37°C in a medium containing 4 mM MgCl₂, 1 mM EDTA, and 50 mM Tris-HCl (pH 7.4) with varying concentrations of AdoMet for 5 min and then Mg²⁺ ATPase activity was determined for 5 min by measuring the hydrolysis of ATP (4 mM). It should be pointed out that heart mitochondria, unlike SL and SR, do not exhibit Ca²⁺-stimulated ATPase activity. Furthermore, SL Ca²⁺-stimulated ATPase is much more sensitive to low concentrations of vanadate in comparison with the SR Ca²⁺-stimulated ATPase (18, 19). In this regard, 2 μM vanadate was

found to inhibit the Ca²⁺-stimulated ATPase activities in SL and SR by 65% and 5%, respectively. The sarcoplasmic reticular vesicles, unlike the SL vesicles, did not show any Na⁺-Ca²⁺ exchange activity (6).

Statistics. Results are presented as a mean±SE. The statistical differences between mean values for two groups were evaluated by the Student's *t* test. For comparing more than two groups, multiple analysis of variance was carried out and Duncan's new multiple-range test was used to determine differences between the means within the population; a *P* value < 0.05 was considered statistically significant (20).

Results

The experimental animals showed, 8 wk after the injection of streptozotocin, elevated levels of plasma glucose and decreased levels of plasma insulin. These diabetic animals exhibited depressed body weight and heart weight in relation to control animals but showed a higher heart weight/body weight ratio (Table I). Such characteristics of the diabetic animals are similar to those reported earlier (10–14). Insulin-treated diabetic rats showed normalized plasma glucose, insulin levels, and other parameters except that the body weight was still decreased in this group. The protein yields for SL, SR, and mitochondria from experimental hearts were similar to those for the control preparations (Table I). Subcellular membranes prepared from the hearts were examined (*n* = 4) for the marker enzyme activities in order to determine the possible extent of cross contamination. The SL vesicles prepared by discontinuous sucrose density gradient (15) from control hearts exhibited a 16-fold enrichment in the SL marker Na⁺,K⁺ ATPase activity (23.5±1.6 μmol P_i/mg per h) in comparison to the homogenate value (1.46±0.35 μmol P_i/mg per h). Approximately 20% of the SL Na⁺ pump was inhibited by 1 mM ouabain; however, a mild treatment of the vesicles with 0.2 mg of sodium deoxycholate/mg protein (21) to eliminate the membrane barrier resulted in complete inhibition of the enzyme by ouabain. Thus, the asymmetric properties of Na⁺,K⁺ ATPase suggest the occurrence of inside-out oriented vesicles

Table I. General Characteristics of the Control and Experimental Rats

	Control	Diabetic	Insulin-treated diabetic
Body wt (g)	443±14	267±17*	370±9*
Ventricular wt (g)	1.25±0.06	0.87±0.09*	1.09±0.10
Ventricular/body wt ratio (mg/g)	2.82±0.08	3.25±0.07*	2.97±0.06
Plasma glucose (mg/dl)	150±15	480±18*	120±20
Plasma insulin (μU/ml)	32±3	14±2*	28±2
Subcellular membrane protein yield (mg/g)			
SL	1.01±0.02	0.96±0.02	0.98±0.03
Mitochondria	8.10±0.93	7.21±0.78	8.02±0.91
SR	1.62±0.21	1.47±0.24	1.60±0.52

Values are means±SE of 9–11 experiments. Diabetes was induced by an intravenous injection of 65 mg/kg streptozotocin and the animals were killed 8 wk later. For the insulin-treated group, 6-wk diabetic animals were given 3 U insulin per day subcutaneously and the animals were killed 2 wk later.

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

in this SL fraction, and that is confirmed by the presence of an active Ca^{2+} pump (4). The cytochrome *c* oxidase (mitochondrial marker) and rotenone-insensitive NADPH cytochrome *c* reductase (SR marker) activities in the SL fraction were ~ 0.5 – 0.7 -fold of the corresponding values in the heart homogenate (97 ± 10 nmol cytochrome *c*/mg per min and 5.6 ± 0.7 nmol cytochrome *c* reduced/mg per min, respectively), whereas the K^+ -EDTA ATPase activity (myofibrillar marker) was not detectable. Cytochrome *c* oxidase and rotenone-insensitive NADPH cytochrome *c* reductase were about 4.5- and 8.6-fold in mitochondrial and SR preparations over the corresponding homogenate value, respectively. After mild deoxycholate treatment (21) both mitochondria and SR fractions showed a low ouabain-sensitive Na^+ , K^+ ATPase activity (1.5 – 2.0 $\mu\text{mol P}_i$ /mg per h), suggesting a small degree of SL contamination in these fractions. The values for purity factors were similar in control and experimental preparations and support the contention that the cardiac membranes under study had only minimal but equal amount of contamination from other subcellular fragments.

In a previous study (10), using heavy SL membranes prepared by the hypotonic shock/LiBr treatment method, we have reported a depressed PE *N*-methylation activity at all three sites in diabetic hearts. To test whether the changes in methylation activities shown earlier were not due to any artifacts associated with the method of SL preparation, PE *N*-methylation was carried out in light SL prepared by the sucrose-density gradient method (15). The data in Table II indicate that all three catalytic sites were depressed in diabetic preparation and thus, the present results confirm our earlier findings. Furthermore, treatment of diabetic animals with insulin was found to normalize the observed changes in the SL PE *N*-methylation activities (Table II). Since the weight loss occurring in diabetes (10) may be responsible for changes in phospholipid *N*-methylation, weight-matched control rats

Table II. PE *N*-Methyltransferase Activity in SL Isolated by the Sucrose-Density Gradient Method from Control, Diabetic and Insulin-treated Diabetic Rat Hearts

Experimental groups	Catalytic site		
	I	II	III
	<i>pmol [³H]methyl groups incorporated/ mg protein per 30 min</i>		
Control	0.64 ± 0.04	6.6 ± 0.3	137 ± 6
Weight-matched control	0.70 ± 0.06	6.1 ± 0.5	142 ± 10
Weight- and age-matched control	0.61 ± 0.05	6.9 ± 0.6	130 ± 12
Diabetic	$0.42 \pm 0.03^*$	$4.1 \pm 0.2^*$	$81 \pm 5^*$
Insulin-treated diabetic	0.58 ± 0.02	6.2 ± 0.17	128 ± 4

Each value represents a mean \pm SE of four to six experiments. The incubations were performed for 30 min in the presence of 0.055, 10, and 150 μM [^3H]AdoMet for catalytic sites I, II, and III, respectively. * Significantly ($P < 0.05$) different from control. Control refers to age-matched normal rats whereas weight-matched control represents younger normal rats in comparison to the diabetic group. The weight- and age-matched control group refers to age-matched normal rats fed restricted diet for 8 wk to achieve weight similar to that for the diabetic group.

(body wt 271 ± 12 g; ventricular wt 0.84 ± 0.04 g; $n = 4$), which were younger in age, were employed (22). In addition, a group of animals maintained on food and caloric restricted diet for 8 wk (22) was used as weight- and age-matched controls (body wt 281 ± 26 g; ventricular wt 0.89 ± 0.08 g; $n = 6$). The results in Table II indicate no difference in the SL phospholipid *N*-methylation activities among the age-matched, weight-matched, and weight- and age-matched control groups employed in this study.

To examine the enzymatic *N*-methylation of PE in other subcellular membranes of control and diabetic animals, SR and mitochondrial membranes from each group were incubated for different time intervals, and the incorporation of [^3H]methyl groups into total methylated phospholipids at catalytic sites I, II, and III was studied (Figs. 1 and 2). The activity curves for the three sites, studied under optimal conditions in SR and mitochondria, indicate that the methylation activity for site I, unlike sites II and III, was increased in diabetic heart. Since catalytic site I in heart SR and mitochondria was sensitive to the diabetic condition, PE *N*-methylation activity in these preparations was studied in the presence of different concentrations of [^3H]AdoMet (0.022– 0.22 μM) and the results are presented in Fig. 3. At all the concentrations of AdoMet employed both diabetic SR and mitochondrial membranes had significantly higher activity of PE *N*-methylation than control preparations. Treatment of the diabetic animals with insulin for 2 wk reversed the changes towards the control values. The weight-matched as well as weight- and age-matched control animals showed normal activities (Table III).

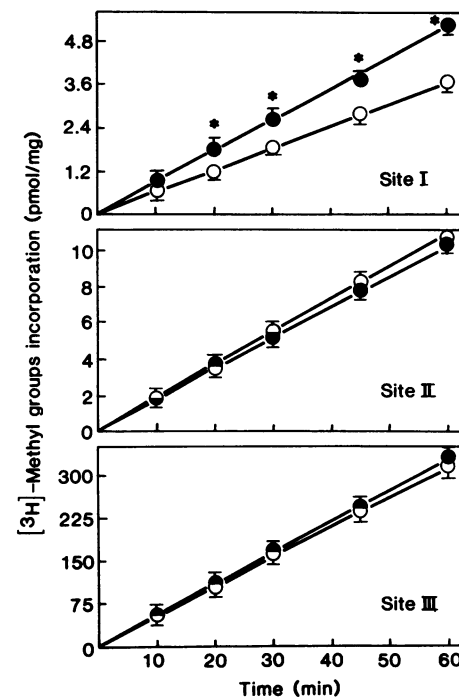


Figure 1. Time course study for the incorporation of [^3H]methyl groups from [^3H]AdoMet into sarcoplasmic reticular phospholipids for control (o) and diabetic (●) rat hearts. The incubations were performed in the presence of 0.055, 10, and 150 μM [^3H]AdoMet at catalytic sites I, II, and III, respectively. Results represent the means \pm SE of four experiments. *Significantly ($P < 0.05$) different from control.

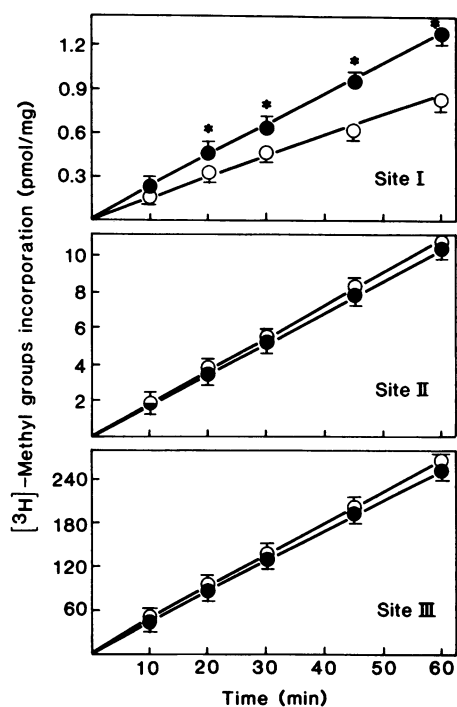


Figure 2. Time course study for the incorporation of [^3H]methyl groups from [^3H]AdoMet into mitochondrial phospholipids at sites I, II, and III for control (○) and diabetic (●) rat hearts. All conditions were same as in Fig. 1 and results are mean \pm SE of four experiments. *Significantly ($P < 0.05$) different from control.

Table IV shows the formation of *N*-methylated phospholipids at site I PE *N*-methylation in SL, SR, and mitochondria from control and diabetic hearts. It should be noted that PMME was the major lipid intermediate whereas PDME and PC were formed in lesser quantities in all the membranes studied. In comparison with the respective control membranes, the formation of these lipid products was significantly lower in

diabetic SL, but it was higher in diabetic SR and mitochondria. From Table IV it is also apparent that SR and mitochondrial activation of the first methyltransferase step, which is rate-limiting (23), results in an increased intramembranal availability of methylated precursors for the second and consequently the third step, with an overall increased synthesis of all the three methylated lipids under these assay conditions. Treatment of diabetic animals with insulin for a period of 2 wk was found to normalize the diabetes-induced changes in these phospholipid intermediates (data not shown) as has been reported for skeletal muscle microsomes (24). On the other hand, under conditions typical for assaying catalytic sites II and III, no difference in the *N*-methylated products (PMME, PDME, and PC) was found between control and diabetic mitochondrial as well as SR preparations. The values ($n = 3$) for PMME, PDME, and PC were reduced by $34\pm 3\%$, $39\pm 2\%$, and $33\pm 4\%$ in diabetic SL at site II and by $38\pm 3\%$, $32\pm 4\%$, and $44\pm 4\%$ at site III, respectively, but were normalized by the 2-wk insulin treatment.

Physiological concentrations of Ca^{2+} in the presence of micromolar ATP are known to stimulate PE *N*-methylation in the liver (25) and skeletal muscle microsomes (24). It has been shown that PE methyl transfer reaction starts on the cytoplasmic side of the membranes where cytosolic AdoMet is available to donate methyl groups (3). Therefore, in the beating heart cell, phospholipid methylation can be seen to occur in the presence of cytosolic levels of free Ca^{2+} ranging from $\sim 10^{-7}$ M during rest to 10^{-5} M in response to excitation (26). Fig. 4 shows the effect of different concentrations of free Ca^{2+} (10^{-7} to 10^{-4} M) plus $10 \mu\text{M}$ ATP on the PE *N*-methylation activity at $0.055 \mu\text{M}$ AdoMet (site I, where PMME is specifically synthesized) in cardiac SL, SR, and mitochondria from control and diabetic hearts. The addition of Ca^{2+} stimulated SL and SR methylation in both control and diabetic preparations; the activation was maximal at 10^{-7} M Ca^{2+} and then declined toward basal values at higher Ca^{2+} concentrations. On the other hand, in both control and diabetic mitochondria, a significant ($P < 0.05$) inhibition of PE *N*-methylation was observed at 10^{-4} M Ca^{2+} . It may be noted that at each Ca^{2+}

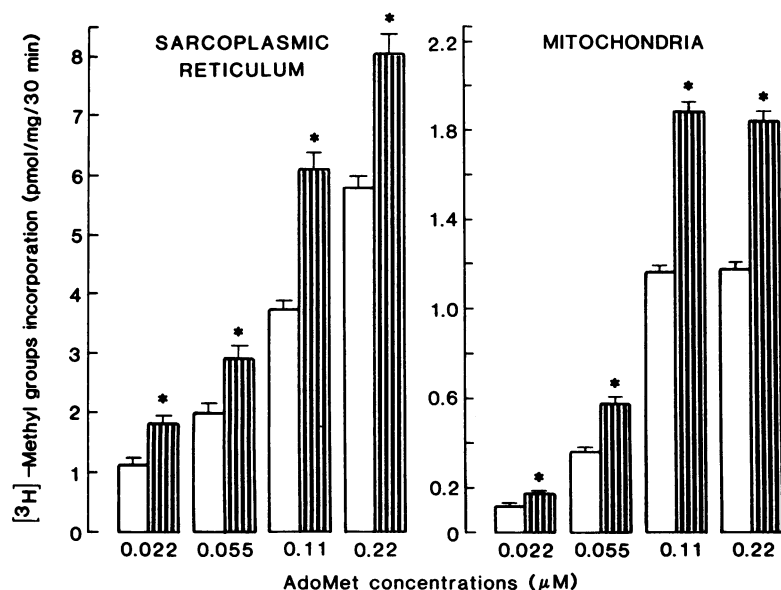


Figure 3. Site I incorporation of [^3H]methyl groups into sarcoplasmic reticular and mitochondrial phospholipids from control (open bars) and diabetic (striped bars) rat hearts at different [^3H]AdoMet concentrations. Results represent the means \pm SE of four experiments. *Significantly ($P < 0.05$) different from control.

Table III. Phospholipid N-Methylation Activity at Catalytic Site I in SR and Mitochondrial Preparations from Control, Diabetic and Insulin-treated Diabetic Rat Hearts

Experimental group	SR	Mitochondria
	<i>pmol [³H]methyl groups incorporated/ mg protein per 30 min</i>	
Control	1.8±0.07	0.32±0.02
Weight-matched control	2.0±0.11	0.30±0.02
Weight- and age-matched control	1.7±0.10	0.37±0.03
Diabetic	3.1±0.22*	0.61±0.05*
Insulin-treated diabetic	2.0±0.14	0.36±0.02

Each value represents a mean±SE of four to six experiments. The incubation was performed for 30 min in the presence of 0.055 μM [³H]AdoMet for catalytic site I.

* Significantly (*P* < 0.05) different from control. Control, weight-matched control, and weight- and age-matched control groups are same as described in Table II.

concentration, the diabetic hearts exhibited lower methylation activity in SL but higher activities in both SR and mitochondria (Fig. 4). The SL, SR, and mitochondrial membranes obtained from insulin-treated diabetic hearts showed site I methyltransferase activities comparable to those of control hearts in the presence of 10⁻⁷ and 10⁻⁴ M Ca²⁺. In this regard, the values (*n* = 3) for insulin-treated diabetic membranes were: 0.61±0.05, 1.2±0.09, and 0.68±0.04 for SL; 1.7±0.16, 2.3±0.19, and 1.8±0.13 for SR; 0.36±0.03, 0.39±0.04, and 0.24±0.03 pmol [³H]methyl groups incorporated/mg protein per 30 min for mitochondria, at 0, 10⁻⁷, and 10⁻⁴ M Ca²⁺ concentrations, respectively.

As reported in previous studies (14, 19), the basal activities of both SL and SR Ca²⁺ pumps were found to be markedly depressed in diabetes (Table V). The effects of incubation of cardiac SL and SR in the presence of AdoMet on Ca²⁺-stimulated ATPase are also shown in Table V. Membranes obtained from control and diabetic hearts were preincubated for 5 min at 37°C with varying concentrations of AdoMet (0.1, 10, and 150 μM) as a methyl donor for the intramembranal synthesis of N-methylated phospholipids by the methyltransferase system (4, 5). Under these conditions, stimulation of both SL and SR Ca²⁺ pumps has been shown to occur at 10 and 150 μM AdoMet concentrations which are known to induce a predominant synthesis and intramembranal accumulation of PDME

and PC, respectively (4, 5). At 10 μM AdoMet, Ca²⁺-stimulated ATPase was enhanced by 200% in control and 150% in diabetic SL membrane; a similar trend was also observed at 150 μM AdoMet reflecting the decreased methyltransferase activity of the diabetic SL. Nevertheless, the stimulation by 10 and 150 μM AdoMet was able to enhance the depressed Ca²⁺ pump activity of the diabetic SL to values similar to the basal (no AdoMet) values for control SL (Table V). The Ca²⁺-stimulated ATPase activity in the SR preparations was also increased by incubation with AdoMet; however, owing to its higher methyltransferase activity, the diabetic membrane exhibited a higher AdoMet-dependent stimulation of the Ca²⁺ pump in comparison to control preparations (Table V). In particular, upon methylating the diabetic SR membrane with 10 or 150 μM AdoMet the depressed Ca²⁺ pump activity became higher or equal, respectively, in comparison to the basal Ca²⁺ pump activity in control membranes. It may be noted also that the Ca²⁺ pump in control SR was maximally stimulated at 150 μM AdoMet (high intramembranal levels of PC), whereas maximal stimulation occurred at 10 μM AdoMet (high intramembranal PDME) in diabetic SR. This finding may suggest that, unlike the control SR membranes, PDME molecules in diabetic SR may be of greater functional significance. The Mg²⁺ ATPase activity of the cardiac subcellular membranes was not affected by diabetes (Table VI). In this regard, the values (*n* = 3–4) for control preparations were 155±5.3, 124±3.1, and 82.8±2.2, whereas those for diabetic preparations were 129±6.2, 102±5.1, and 72.3±3.4 μmol P_i/mg per h for SL, SR, and mitochondria, respectively. These activity values remained unaltered upon incubation of the control and diabetic membranes with different (0.1, 10, 150 μM) concentrations of AdoMet.

Discussion

The results of this study indicate that the rate-limiting step of the methylation pathway (catalytic site I) was increased in the 8-wk diabetic SR and mitochondria. In contrast, previous studies (10) have reported a decrease of all three sites in heavy sarcolemma from the 8-wk diabetic hearts, which finding is confirmed by using the light SL preparation in this study. It is unlikely that these changes in PE N-methylation were due to differential contamination in the subcellular fractions because the marker enzyme activities in SL, SR, and mitochondria showed equal but negligible cross-contamination in both control and experimental preparations. In fact the increase of PE N-methylation in diabetic mitochondria and SR at site I was

Table IV. Incorporation of [³H]Methyl Groups into N-Methylated Phospholipids of Different Subcellular Membranes at Catalytic Site I for Control and Diabetic Rat Hearts

	SL		SR		Mitochondria	
	Control	Diabetic	Control	Diabetic	Control	Diabetic
	<i>pmol [³H]methyl groups incorporated/mg protein per 30 min</i>					
PMME	0.22±0.02	0.15±0.01*	0.50±0.02	0.78±0.05*	0.15±0.01	0.21±0.01*
PDME	0.12±0.01	0.08±0.005*	0.39±0.01	0.60±0.02*	0.10±0.005	0.15±0.01*
PC	0.09±0.01	0.06±0.003*	0.37±0.02	0.62±0.02*	0.07±0.004	0.11±0.005*

Each value represents a mean±SE of three experiments. * Significantly (*P* < 0.05) different from respective control value.

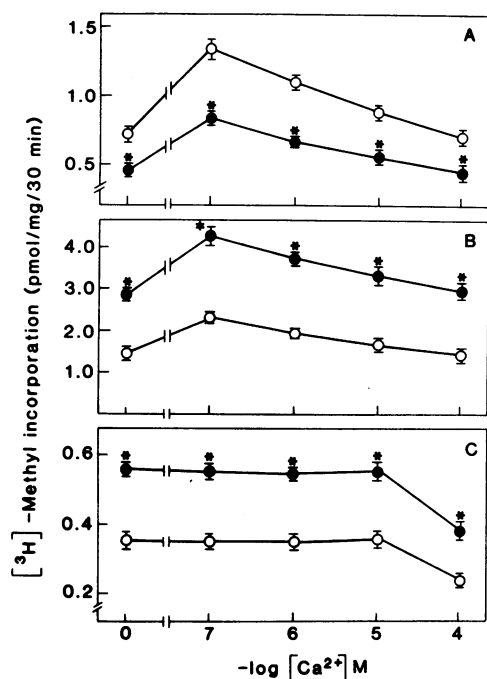


Figure 4. Effect of different Ca^{2+} concentrations on (A) SL, (B) SR, and (C) mitochondrial phospholipid *N*-methylation in control (○) and diabetic (●) rat hearts. The enzyme activity was assayed in the presence of free Ca^{2+} , 10 μM ATP, and 0.055 μM [^3H]AdoMet. Values are means \pm SE of four experiments. *Significantly ($P < 0.05$) different from control.

specific in nature because sites II and III in these fractions were unaltered. Since streptozotocin-induced diabetes in rats is associated with loss of body weight, it can be argued that the observed changes in cardiac phospholipid methylation are due to the catabolic effect of diabetes. Because our experiments with weight-matched as well as weight- and age-matched control animals showed values for the phospholipid methylation

Table V. Effects of Varying Concentrations of AdoMet on Ca^{2+} -stimulated ATPase in SL and SR Membranes from Control and Diabetic Rat Hearts

AdoMet μM	SL		SR	
	Control	Diabetic	Control	Diabetic
None	9.4 \pm 0.6	5.6 \pm 0.4	10.6 \pm 0.6	6.6 \pm 0.5
0.1	11.3 \pm 1.2 (120 \pm 10)	5.5 \pm 1.0 (97 \pm 5)	11.0 \pm 0.7 (104 \pm 3)	7.2 \pm 0.3 (117 \pm 8)
10	19.9 \pm 1.2* (211 \pm 8)	8.9 \pm 0.6* (164 \pm 8)	13.0 \pm 0.6* (123 \pm 4)	14.9 \pm 0.8* (237 \pm 14)
150	16.2 \pm 0.9* (174 \pm 9)	8.6 \pm 1.3* (154 \pm 6)	16.6 \pm 1.2* (157 \pm 7)	10.7 \pm 0.7* (167 \pm 13)

Values are means \pm SE of four experiments. Numbers in parenthesis are the percentage of respective values in the absence of AdoMet (none).

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

similar to those for the age-matched control animals, it appears that the observed diabetes-induced changes are not due to any catabolic influence. Moreover, such changes appear to be secondary to diabetes per se because treatment of the diabetic animals with insulin for 2 wk reversed the values towards the control level. In this regard, the possibility that hypothyroidism associated with the diabetic model (14, 22, 27) could contribute to this membrane lesion cannot be excluded on the basis of the present results. However, it should be noted that experimental protocols which normalize plasma thyroid hormone levels in diabetes failed to normalize cardiac dysfunction, as well as other abnormalities in contractile proteins and sarcoplasmic reticular calcium transport (14, 27, 28). Indeed, the exact mechanism(s) responsible for the observed changes in membrane phospholipid methylation is (are) unknown at present, but it seems to be one of the many metabolic derangements in diabetes (29). Abnormal plasma levels of insulin, glucose, ketone bodies and lipids could be contributing factors, including the high plasma levels of free fatty acids (30) which are known to affect both the structure and function of cardiac membranes (31). At any rate, it is evident that at the same stage of diabetes, the behavior of the methylation system in SR and mitochondrial differs from that in SL both in terms of the number of catalytic sites affected and the type of alterations. Differences were observed also in catecholamine-induced cardiomyopathy where mitochondrial PE *N*-methylation was unaltered but parallel alterations were observed in the SL and SR *N*-methylation activities (9). Thus, the differential behavior of SL, SR and mitochondrial PE *N*-methylation activities in different disease states of the heart indicates that these activities may have a distinct role in the myocardial cell function.

As reported earlier, both SL and SR Ca^{2+} pump systems were found to be depressed in diabetic heart (14, 19). From a functional viewpoint, a decrease in both Ca^{2+} sequestration from the cytoplasm by the SR Ca^{2+} pump and Ca^{2+} extrusion from the cell by the SL Ca^{2+} pump can be seen to increase the cytosolic Ca^{2+} level, whereas the opposite occurs when these pumps activities are stimulated. We have shown that a direct relationship exists between PE *N*-methylation and Ca^{2+} pump activities in the normal heart, because the activation of membrane methylation was accompanied by the enhancement of both SL and SR Ca^{2+} pumps (4, 5). The simultaneous assessment of Ca^{2+} pump and methylation activities in the diabetic heart has now revealed that the above relationship was evident in SL only since both methylation and Ca^{2+} pump were depressed. Furthermore, the stimulation of the SL Ca^{2+} pump in diabetic heart was less than the control upon in vitro addition of AdoMet. This may suggest that a decrease in methylation activity in diabetic SL may contribute to depressing the SL Ca^{2+} pump. Although the Ca^{2+} pump was depressed also in diabetic SR, the AdoMet-induced stimulation was higher in diabetic than in control SR owing to the elevated methylation rate of the diabetic membranes. The lack of correlation between Ca^{2+} pump and methylation in diabetic SR in the absence of AdoMet may be due to the interference of other regulatory factors influencing the Ca^{2+} pump activity in SR membranes (5, 26), and these may include a down-regulation of the Ca^{2+} ATPase gene. However, it remains to be seen whether or not the cytosolic AdoMet concentrations are low in diabetic cardiomyopathy because this may contribute to lowering the intramembranal *N*-methylated phospholipids for affecting the Ca^{2+} pump. Nonetheless, it should be noted that diet supple-

Table VI. Effects of Varying Concentrations of AdoMet on Mg^{2+} ATPase in Subcellular Membranes from Control and Diabetic Rat Hearts

AdoMet μM	SL		SR		Mitochondria	
	Control	Diabetic	Control	Diabetic	Control	Diabetic
None	155±5.3	129±6.2	124±3.1	102±5.1	82.8±2.2	72.3±3.4
0.1	154±6.2	128±6.1	123±3.6	102±4.6	80.4±2.1	70.9±2.1
10	150±6.8	128±6.5	123±5.0	100±5.0	77.7±3.2	71.7±2.9
150	152±6.9	128±6.3	122±4.6	99±5.8	78.4±1.0	72.2±3.0

Values are means±SE of three to four experiments done in duplicate.

mentation of methionine, a precursor for the methyl donor AdoMet, was shown to improve the cardiac performance in diabetic rats (32).

Our results indicate that micromolar ATP plus physiological concentrations of Ca^{2+} stimulated the activity of methyltransferase site I in both control and diabetic SL and SR, whereas only an inhibitory effect by 10^{-4} μM Ca^{2+} was seen in mitochondria. In this regard, it may be noted that cardiac mitochondria are known to possess a large capacity of Ca^{2+} accumulation, and their function is depressed under conditions of Ca^{2+} overload (26). The Ca^{2+} plus ATP-induced effect on phospholipid methylation was also seen in liver and skeletal muscle microsomes, where it was suggested to be mediated by a calmodulin-dependent regulatory system (24, 25). Whether or not calmodulin intrinsic to the heart SL and SR membranes is essential in conferring Ca^{2+} sensitivity to the *N*-methyltransferase system remains to be elucidated in future studies. However, the maximal activation with micromolar ATP seems to suggest the involvement of a phosphorylation mechanism.

Acknowledgments

This work was supported by a grant to Dr. Panagia from the Medical Research Council, Canada. Dr. Y. Taira was a postdoctoral fellow and Dr. P. K. Ganguly was a scholar of the Heart and Stroke Foundation of Canada.

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