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Protein methylation inhibits Na^+–Ca^{2+} exchange activity in cardiac sarcolemmal vesicles

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We have examined the effect of membrane methylation on the Na^+–Ca^{2+} exchange activity of canine cardiac sarcolemmal vesicles using *S*-adenosyl-L-methionine as methyl donor. Methylation leads to approximately 40% inhibition of the initial rate of Na_i^+-dependent Ca^{2+} uptake. The inhibition is due to a lowering of the V_{max} for the reaction. The inhibition is not due to an effect on membrane permeability and is blocked by *S*-adenosyl-L-homocysteine, an inhibitor of methylation reactions. The following experiments indicated that inhibition of Na^+–Ca^{2+} exchange was due to methylation of membrane protein and not due to methylated phosphatidylethanolamine (PE) compounds (i.e., phosphatidyl-*N*-monomethylethanolamine (PMME) or phosphatidyl-*N*,*N'*-dimethylethanolamine (PDME)): (1) We solubilized sarcolemma and reconstituted activity into vesicles containing no PE. The inhibition by *S*-adenosyl-L-methionine was not diminished in this environment. (2) We reconstituted sarcolemma into vesicles containing PMME or PDME. These methylated lipid components had no effect on Na^+–Ca^{2+} exchange activity. (3) We verified that many membrane proteins, probably including the exchanger, become methylated.

Introduction

Transsarcolemmal movements of Ca^{2+} are an important factor in cardiac muscle contraction, and the sarcolemmal Na^+–Ca^{2+} exchanger is one mechanism by which Ca^{2+} may rapidly enter and leave myocardial cells. However, the extent of involvement of Na^+–Ca^{2+} exchange in myocardial excitation-contraction coupling is controversial. One way to learn more about Na^+–Ca^{2+} exchange is by using isolated sarcolemmal vesicles, and this system has now been used extensively [1,2]. Several interventions modulate the Na^+–Ca^{2+} exchange activity of cardiac sarcolemmal vesicles. For example, Na_i^+-dependent Ca^{2+} uptake is stimulated by an inside-positive membrane potential [3,4], high pH [5], proteinase treatment [6], phospholipase D treatment [7], various anionic amphiphiles [8–10], intravesicular Ca^{2+} [11], or redox modification [12].

Recently, Panagia et al. [13] reported that methylation of sarcolemmal vesicles, using *S*-

Abbreviations: PMME, phosphatidyl-*N*-monomethylethanolamine; PDME, phosphatidyl-*N*,*N'*-dimethylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; MOPS, 4-morpholinepropanesulfonic acid.

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adenosyl-L-methionine as methyl donor, inhibited Na^+–Ca^{2+} exchange activity. The inhibition was attributed to two products of phosphatidylethanolamine (PE) methylation, phosphatidyl-*N*-monomethylethanolamine (PMME) and phosphatidyl-*N*,*N'*-dimethylethanolamine (PDME). The concept that phospholipid methylation is an important modulator of cellular processes was initially proposed by Hirata and Axelrod [14]. This model, however, has been challenged [15], partly because of the small quantity of methylated intermediates which can be detected.

Plasma membranes contain methyl transferases responsible for the stepwise synthesis of PC from PE and for the methylation of membrane proteins. There are many examples of methylation with *S*-adenosyl-L-methionine resulting in altered membrane function. In some cases, effects are assumed to be due to lipid methylation [16–19]. In other cases, it is acknowledged that effects could be due to either lipid or protein methylation [20–22]. Protein methylation has also been implicated as a step in the processing of damaged membrane proteins [23]. We investigated the effects of sarcolemmal methylation on Na^+–Ca^{2+} exchange activity. We conclude that inhibition of Na^+–Ca^{2+} exchange activity is due to methylation of sarcolemmal protein and is not due to lipid methylation as has been reported [13].

Materials and Methods

Materials. Phospholipids (phosphatidylcholine (PC) (egg), phosphatidylethanolamine (PE) (egg), phosphatidylserine (PS) (bovine brain)), were from Avanti Polar Lipids; asolectin from Associated Concentrates; cholesterol, PMME, and PDME from Sigma; Bio-Beads SM2 from Bio-Rad; and TLC plates (silica gel G1) from Merck. All chemicals were of at least reagent grade.

Sarcolemmal isolation. Highly purified sarcolemmal vesicles were isolated from canine ventricles as described previously [24] with minor modifications [10].

Solubilization / reconstitution. Sarcolemmal vesicles were solubilized and Na^+–Ca^{2+} exchange activity was reconstituted using either the Triton X-100/Bio-Bead or the cholate/dilution method as described in detail elsewhere [25,26]. Some specific conditions are given in the text below.

Labelling of membranes. Membranes were labelled by incubation with *S*-adenosyl-L-[*Me*-^{3}H]methionine (^{3}H-SAM, 2 μCi/ml) for 30 min at 37 °C. Unincorporated ^{3}H-SAM was then removed by precipitating the vesicles with cold 10% trichloroacetic acid. Lipids were extracted by the method of Bligh and Dyer [27]. Protein, which aggregated at the aqueous/organic interface, was collected and re-extracted by the Bligh-Dyer method. The extent of methylation of the lipid and protein phases was then determined by liquid scintillation techniques.

SDS-PAGE. Methylated proteins were separated on a 10% Laemmli gel. Lanes were then cut into 1–2 mm pieces and solubilized with 600 μl of Soluene (Packard) at 100 °C for one hour. ScintA (10 ml, Packard) was added for counting. Apparent molecular weights were determined with Sigma (SDS-7B) prestained markers.

Na^+–Ca^{2+} exchange. Na^+–Ca^{2+} exchange was measured as Na_i^+-dependent Ca^{2+} uptake as previously described in detail [10,25,26]. Briefly, 0.005 ml of Na^+ (140 mM)-loaded vesicles were rapidly diluted to 0.25 ml with 140 mM KCl, 1.25 μCi/ml $^{45}Ca^{2+}$, variable $^{40}CaCl_2$, 0.4 μM valinomycin, and 10 mM Mops-Tris (pH 7.4) at 37 °C. The Ca^{2+} uptake reaction was then automatically quenched by the addition of EGTA at a preset time and rapidly filtered. All points were obtained in duplicate. A blank was subtracted for each point for which the vesicles were diluted into a Ca^{2+} uptake medium containing NaCl rather than KCl. This corrects for small amounts ($< 5\%$) of Na^+-gradient independent Ca^{2+} uptake. Unless otherwise stated, the [Ca^{2+}] was 10 μM and the time of uptake was 1.5 s.

Results

Fig. 1 shows that preincubation of cardiac sarcolemmal vesicles with *S*-adenosyl-L-methionine inhibits Na_i^+-dependent Ca^{2+} uptake. Such an effect has also been reported by others [13]. Maximal effects of each *S*-adenosyl-L-methionine concentration occurred within 30 min of preincubation. Under these conditions, half maximal inhibition of Na^+–Ca^{2+} exchange required about 50 μM *S*-adenosyl-L-methionine. Maximal inhibi-

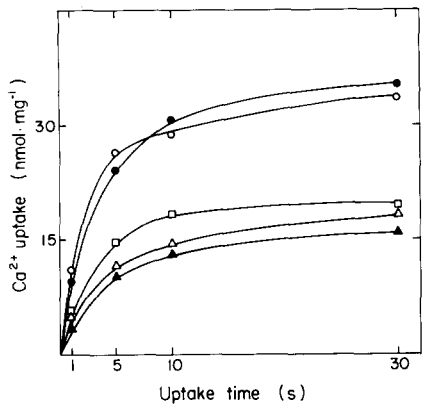

Fig. 1. Time course of Na_i^+-dependent Ca^{2+} uptake. Sarcolemmal vesicles were preincubated at 37 °C for 30 min in the absence or presence of *S*-adenosyl-L-methionine (SAM) without or with *S*-adenosyl-L-homocysteine (SAH). Control (○), 50 μM SAM (□), 100 μM SAM (△), 200 μM SAM (▲), 50 μM SAM + 200 μM SAH (●).

tion was usually 35–50%. An excess of *S*-adenosyl-L-homocysteine, a competitive inhibitor of methylation reactions, completely blocked the effects of *S*-adenosyl-L-methionine (Fig. 1). Effects of *S*-adenosyl-L-methionine required a preincubation period; the presence of *S*-adenosyl-L-methionine in only the Ca^{2+} uptake media had no effect on Na^+–Ca^{2+} exchange. The inhibition of Na^+–Ca^{2+} exchange activity by methylation was primarily due to a lowering of the V_{max}. As shown in Fig. 2, only a small effect on the apparent K_m (Ca^{2+}) was observed. The effects of methylation

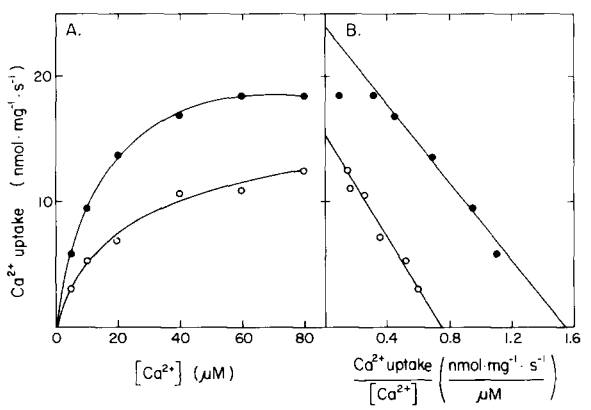

Fig. 2. (A) The initial rate of Na^+–Ca^{2+} exchange activity as a function of [Ca^{2+}]. Control (●) and 100 μM SAM-treated (○) vesicles. (B) Eadie-Hofstee plot of the same data. K_m(Ca^{2+}) values were 19.8 and 15.6 μM, respectively. SAM, *S*-adenosyl-L-methionine.

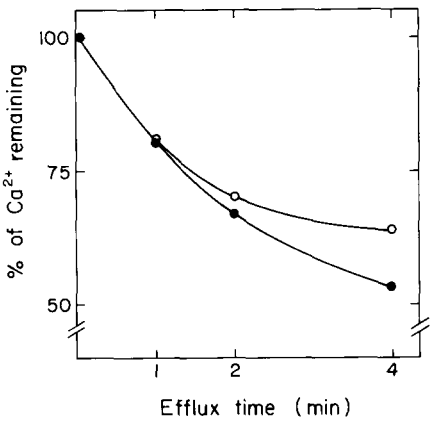

Fig. 3. Passive Ca^{2+} efflux after *S*-adenosyl-L-methionine (SAM) treatment. Sarcolemmal vesicles were first pre-incubated in the absence (●) and presence (○) of 100 μM SAM. The vesicles were then loaded with Ca^{2+} by Na_i^+-dependent Ca^{2+} uptake for 1 min in 10 μM Ca^{2+}. The Ca^{2+}-loaded vesicles were diluted into 140 mM KCl, 10 mM Mops-Tris (pH 7.4), 1 mM EGTA to initiate Ca^{2+} efflux. Vesicles were filtered at various times and analyzed for intravesicular Ca^{2+}. The initial Ca^{2+} loads were 39.0 and 29.1 nmol/mg for the control and treated vesicles, respectively.

on Na^+–Ca^{2+} exchange are not due to an effect on membrane permeability properties. Fig. 3 demonstrates that the passive leakage of Ca^{2+} from sarcolemmal vesicles is unchanged after methylation.

The effects of methylation with SAM may be due to methylation of PE to PMME or PDME. To test this possibility, we began solubilization/reconstitution experiments in which we could control the membrane lipid environment. Sarcolemma was solubilized and reconstituted by the Triton X-100/Bio-Bead method. Sarcolemmal proteins were reconstituted into either asolectin or a mixture of PC/PS/cholesterol (30 : 50 : 20, w/w). The latter mixture was chosen to avoid the presence of any lipids (i.e., PE) which can be methylated by *S*-adenosyl-L-methionine in the final proteoliposomes. Asolectin, a crude mixture of soybean lipids, contains about 34% PE [28]. The reconstituted vesicles were then preincubated with *S*-adenosyl-L-methionine (Table I). As shown, the inhibitory effects of methylation are independent of the presence of PE. This suggests that it is protein methylation and not PE methylation which is responsible for inhibition.

TABLE I

Na^+–Ca^{2+} EXCHANGE IN RECONSTITUTED VESICLES AS A FUNCTION OF THE *S*-ADENOSYL-L-METHIONINE CONCENTRATION

Native sarcolemmal vesicles were solubilized and reconstituted with asolectin or PC/PS/cholesterol (30:50:20, w/w). The reconstituted vesicles were incubated with the indicated concentration of *S*-adenosyl-L-methionine ([SAM]) for 30 min at 37°C prior to the Na^+–Ca^{2+} exchange reaction.

[SAM] (μM)	Lipid in reconstitution medium			
	asolectin		PC/PS/cholesterol	
	nmol/mg per s	% control	nmol/mg per s	% control
0	17.4	100	15.0	100
10	16.3	93.7	12.9	86.0
20	16.0	92.0	12.5	83.3
30	15.6	89.7	12.0	80.0
40	13.7	78.7	10.1	67.3
50	12.5	71.8	9.3	62.0
100	9.8	56.3	8.0	53.3

A caveat is that some endogenous sarcolemmal PE will still be present after the solubilization/reconstitution procedure. PE comprises about 28% of sarcolemmal phospholipids [29]. In the Triton X-100/Bio-Bead procedure, endogenous phospholipids are diluted about 13-fold by the phospholipids in the solubilization mixture. Thus, the vesicles reconstituted with PC/PS/cholesterol actually contain about 2% PE. To further reduce the level of PE, we reconstituted solubilized sarcolemma by the cholate/dilution method using 30-fold excess of exogenous phospholipid (PC/PS/cholesterol). The exchange activity of these reconstituted vesicles could still be inhibited by methylation. The initial rate of Na_i^+-dependent Ca^{2+} uptake decreased from 6.2 ± 1.5 to 3.1 ± 0.4 nmol/mg protein per s after a 30 min preincubation with 50 μM *S*-adenosyl-L-methionine.

To further directly test the hypothesis that PMME and PDME, two products of PE methylation, inhibit Na^+–Ca^{2+} exchange activity, we again used solubilization/reconstitution techniques. Sarcolemmal vesicles were solubilized and reconstituted into PC/PS/cholesterol vesicles or into vesicles in which a fraction of the PC was replaced with PE, PMME, or PDME (Table II). The effects of PMME and PDME on Na^+–Ca^{2+} exchange were no different than the effect of PE itself. Thus, it is unlikely that inhibition of Na^+–Ca^{2+} exchange by membrane methylation is due to production of PMME or PDME from PE.

TABLE II

Na^+–Ca^{2+} EXCHANGE IN RECONSTITUTED VESICLES: EFFECTS OF PMME AND PDME

Native vesicles were solubilized and reconstituted in the presence of various lipid mixtures as described in Materials and Methods. $n = 4$.

	Na_i^+-dependent Ca^{2+} uptake (nmol/mg protein per s)
PC/PS/cholesterol (4:4:2)	20.9 ± 2.5
PC/PE/PS/cholesterol (3:1:4:2)	14.6 ± 3.9
PC/PMME/PS/cholesterol (3:1:4:2)	13.9 ± 0.8
PC/PDME/PS/cholesterol (3:1:4:2)	13.0 ± 1.3

The distribution of methylation products between the membrane lipids and proteins was determined. As shown in Table III, 64% of the label was incorporated into membrane lipid and 36% into protein of the native sarcolemmal vesicles. Excess *S*-adenosyl-L-homocysteine almost completely blocked methylation as expected. Sarcolemmal vesicles were also solubilized and recon-

TABLE III

DEGREE OF METHYLATION OF MEMBRANE COMPONENTS

Vesicles were incubated for 30 min at 37°C with [^{3}H]SAM. The reaction was stopped by the addition of 10% trichloroacetic acid, and lipids and proteins separated as described in Materials and Methods. SAM, *S*-adenosyl-L-methionine; SAH, *S*-adenosyl-L-homocysteine.

	Incorporation of [^{3}H]methyl groups (cpm)	
	lipid	protein
Native sarcolemmal vesicles	2464	1411
Native sarcolemmal vesicles + 200 μM SAH	45	155
Reconstituted sarcolemmal vesicles (PC/PS/cholesterol, 30:50:20, w/w)	160	1694

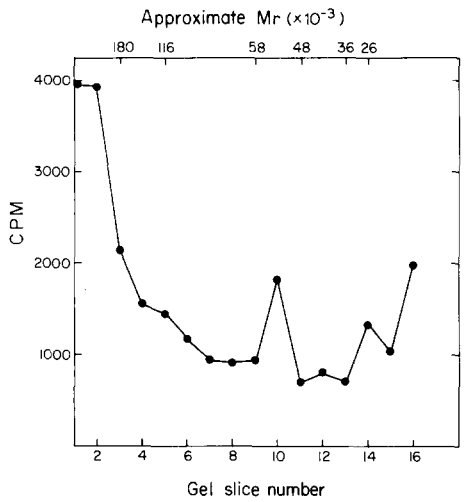

Fig. 4. Distribution of methylated proteins in cardiac sarcolemma. (See Materials and Methods for details.) Slice number 1 is stacking gel and slice number 16 is dye front.

stituted into proteoliposomes containing no PE and labelled with ^{3}H-SAM. In this case, significant labelling of only the membrane proteins occurred (Table III). In one experiment, methylated sarcolemmal lipids were separated by TLC. About 50% of the label was found in PC and 50% in a spot corresponding to PMME and PDME.

We also determined the distribution of label among sarcolemmal proteins. Fig. 4 shows that methylation was not confined to a few discrete protein bands. Methylated proteins were present over the entire molecular weight range.

Discussion

We confirm the observations of Panagia et al. [13] that methylation inhibits the Na^+–Ca^{2+} exchange activity of cardiac sarcolemmal vesicles. Panagia et al. [13] attributed the inhibition to methylation of PE to PMME, PDME, or PC. In this model, changes in lipid domains would influence exchange activity. This model would seem plausible in that Na^+–Ca^{2+} exchange activity is sensitive to membrane lipid components [7–10] and others have also attributed membrane effects to methylation of PE (see for example, Refs. 16–19). However, the quantity of membrane lipid which becomes methylated is relatively small [15,19]. For example, Panagia et al. [19] find that about 3 pmol of methyl groups per mg protein become incorporated into sarcolemmal lipids at 10 μM *S*-adenosyl-L-methionine. Since the phospholipid content of sarcolemma is about 2 μmol/mg [29], only about 1 in 10^6 phospholipids is affected. As extraordinarily sensitive interactions would be required, we explored an alternative hypothesis.

Incubation of membranes with *S*-adenosyl-L-methionine also causes proteins to become methylated, and we examined whether the inhibition of Na^+–Ca^{2+} exchange activity could be due to protein methylation. In the first set of experiments, we solubilized the sarcolemmal vesicles, and then reconstituted the membrane proteins into an excess of either asolectin (which contains about 34% PE) or PC/PS/cholesterol, i.e., a mixture containing no PE. *S*-Adenosyl-L-methionine was equally effective in inhibiting the Na^+–Ca^{2+} exchange activity of these two types of reconstituted vesicles (Table I). Thus, inhibition by *S*-adenosyl-L-methionine does not seem to require the presence of PE. Table III confirms that almost no lipid is methylated in the vesicles reconstituted with PC/PS/cholesterol.

The lipid methylation hypothesis requires that at least one of the products of PE methylation (PMME, PDME, or PC) is a potent inhibitor of Na^+–Ca^{2+} exchange activity. Table II shows that replacing PE with either PMME or PDME in reconstituted sarcolemmal vesicles has no effect on Na^+–Ca^{2+} exchange. Activity is actually higher, not lower, when the PE is replaced with PC.

The data strongly suggest that the inhibition of Na^+–Ca^{2+} exchange activity by *S*-adenosyl-L-methionine is not due to methylation of PE. The implication is that methylation of membrane proteins is responsible for the inhibition. Using ^{3}H-SAM, we determined that significant methylation of proteins was occurring with both the native and reconstituted sarcolemmal vesicles (Table III). SDS-PAGE (Fig. 4) demonstrated that many sarcolemmal proteins become methylated. We speculate that the Na^+–Ca^{2+} exchange protein is one of these proteins, and that direct methylation of the exchanger is responsible for inhibition. The physiological significance of protein methylation in eukaryotes is uncertain, but methylation may be important in protein repair or recycling during the aging process [23].

Many cases in which effects of membrane methylation are attributed to lipid methylation may in fact be due to protein methylation. Solubilization/reconstitution techniques, as used here, may be a useful general tool for resolving these possibilities.

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