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Role For Sulfur-Containing Groups in the Na^+-Ca^{2+} Exchange of Cardiac Sarcolemmal Vesicles

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Summary. Different amino acid residues in cardiac sarcolemmal vesicles were modified by incubation with various chemical reagents. The effects of these modifications on sarcolemmal Na^+-Ca^{2+} exchange were examined. Dithiothreitol, an agent that maintains sulfur-containing residues in a reduced state, caused a time- and concentration-dependent decrease in Na^+-Ca^{2+} exchange. The treatment with dithiothreitol resulted in a decrease in V_{max} values but did not alter the K_m for Ca^{2+} for the Na^{2+}-Ca^{2+} exchange reaction. If Na^+ replaced K^+ as the ion present during the modification of sarcolemmal membranes with dithiothreitol, there was substantially less of an inhibitor effect on Na^+-Ca^{2+} exchange. Similar results were obtained with reduced glutathione, a reagent that also maintains sulfur-containing residues in a reduced state. Two sulfhydryl modifying reagents, methylmethanethiosulfonate and N′-ethylmaleimide, were capable of altering Na^+-Ca^{2+} exchange, and the type of ion present during modification significantly affected the extent of this alteration. Almost all of the chemical reagents investigated that modified other amino acid resides (carboxyl, lysyl, histidyl, tyrosyl, tryptophanyl, arginyl and hydroxyl) had the capacity to alter Na^+-Ca^{2+} exchange after preincubation with the sarcolemmal membrane vesicles. However, the sulfur residue-modifying reagents were the only compounds to exhibit significant differences in their action on Na^+-Ca^{2+} exchange, depending on whether Na^+ or K^+ was present in the preincubation modification medium. The tryptophan modifier, N-bromosuccinimide, was the sole reagent that elicited a substantial increase in membrane permeability. The evidence is consistent with the hypothesis that sulfur-containing residues interact with a Na^+-binding site for Na^+-Ca^{2+} exchange in cardiac sarcolemmal vesicles.

Key Words Na^+-Ca^{2+} exchange · sarcolemmal membrane · heart · sulfhydryl groups · amino acid modification

Introduction

An exchange of Na^+ ions for Ca^{2+} ions has been shown to occur across cardiac sarcolemmal membranes (Reeves & Sutko, 1979; Philipson & Nishimoto, 1980; Philipson, 1985; Reeves, 1985). Considerable evidence supports its involvement in force generation under certain conditions such as glycosidic challenge (Langer, 1982). However, the contribution of this transport system to force generation under control conditions is controversial (Langer, 1982).

Despite an involvement in myocardial function, little is known about the molecular nature of the Na^+-Ca^{2+} exchange protein. Some information exists concerning the characteristics of the antiporter Na^+ and Ca^{2+} binding sites (*see* Philipson, 1985; Reeves, 1985), but little is known concerning the molecular nature of these sites. On the basis of the response of Na^+-Ca^{2+} exchange to varying pH, Philipson and coworkers (Phlipson, Bersohn & Nishimoto, 1982) suggested that histidine residues may be important in the exchange reaction. However, no study has directly examined this hypothesis.

The purpose of the present investigation was to examine the extent of involvement of specific sarcolemmal amino acid residues in Na^+-Ca^{2+} exchange. Of particular emphasis was the identification of any residue which may affect Na^+-Ca^{2+} exchange due to a localization near the active site of exchange transport. The study used several group-specific chemical-modifying reagents. We report that chemical modification of several amino acid residues could significantly alter cardiac sarcolemmal Na^+-Ca^{2+} exchange activity. However, the presence of Na^+ during modification could influence the effects on Na^+-Ca^{2+} exchange only in the case of sulfur-modifying reagents.

Materials and Methods

Sarcolemmal membrane vesicles were isolated from canine ventricular tissue as described previously (Frank, Philipson & Beydler, 1984). These membranes exhibit high activities of marker enzymes associated with the plasma membrane fraction. K^+-*p*-nitrophenyl phosphatase activity was 27.0 ± 2.1 μmol/mg/hr, which represented a 67.0-fold increase over initial tissue homogenate values (n = 8). (Na^+,K^+)-ATPase activity was 17.2 ± 1.8 and 99.9 ± 9.9 μmol P_i/mg/hr in the absence and presence of 12.5

Table 1. Effect of dithiothreitol treatment of sarcolemmal membranes on kinetic parameters of Na^+-Ca^{2+} exchange[a]

Ion in modification medium	Dithiothreitol	K_m (mM)	V_{max} (nmol/mg/sec)
K^+	−	26.5 ± 4.1	10.5 ± 1.0
K^+	+	26.7 ± 6.9	4.3 ± 0.6
Na^+	−	27.3 ± 4.2	19.3 ± 0.9
Na^+	+	27.4 ± 3.8	11.5 ± 1.0

[a] Sarcolemmal vesicles were preincubated in 140 mM NaCl or KCl, 10 mM MOPS (pH 7.4) for 30 min at 37°C in the absence or presence of 5 mM dithiothreitol. As described in Materials and Methods, these vesicles were then diluted into Na^+-containing medium to facilitate passive Na^+ uptake, then diluted again into media appropriate to measure Na^+-dependent Ca^{2+} uptake. $[Ca^{2+}]$ of the extravesicular medium was varied. Assay incubation time was 1.5 sec, and 0.4 μM valinomycin was present. Data represent mean ± SE of 3–5 experiments.

μg/ml alamethecin (kindly donated by Dr. J. Grady, Upjohn) (n = 8). This membrane preparation exhibits relatively little contamination from other organelles as evidenced by electron microscopic analysis (Frank et al., 1984) and marker enzyme activities (Pierce & Philipson, 1985). Final sarcolemmal membrane pellets were resuspended in 10 mM MOPS[1] (pH 7.4) containing either 140 mM KCl or NaCl. A typical protocol for the modification of amino acid residues in the sarcolemmal vesicles was as follows: (i) Twelve μl of sarcolemmal vesicles in 140 mM KCl, 10 mM MOPS (pH 7.4) (25-50 μg of protein) were added to 3 μl of a medium containing 140 mM KCl, 250 μM EDTA ± the amino acid modifying reagent. If ethanol was used to dissolve the reagent, a similar concentration of ethanol was added to the control sample. The [ethanol] never exceeded 1%. The vesicles were incubated at 37°C. Preliminary experiments were carried out to determine the duration of incubation sufficient to insure chemical modification; (ii) Fifteen μl of a medium containing 140 mM NaCl, 50 μM EDTA was then added to the vesicular suspension to facilitate passive loading of Na^+ into the vesicles; (iii) After a 10-min incubation at 37°C, a 5-μl aliquot was removed and diluted into the 250-μl medium described below for the measurement of Na^+-Ca^{2+} exchange. The Na^+-loading protocol described in step *ii* is not long enough to fully equilibrate the intravesicular volume with Na^+. When these vesicles were measured for Na_i^+-dependent Ca^{2+} uptake, this resulted in lower V_{max} values for Na^+-Ca^{2+} exchange without altering the K_m for Ca^{2+} (*see* Table 1).

To study the influence of the substrate Na^+ on the effects of the modifying reagent on Na^+-Ca^{2+} exchange, the protocol was identical to that described above except KCl used in step *i* was replaced by a similar concentration of NaCl. The sarcolemmal vesicles used had been previously suspended in 140 mM NaCl, 10 mM MOPS (pH 7.4). In comparing K^+ vesicles with Na^+ vesicles, paired sarcolemmal membranes obtained from the same ventricular sample were used.

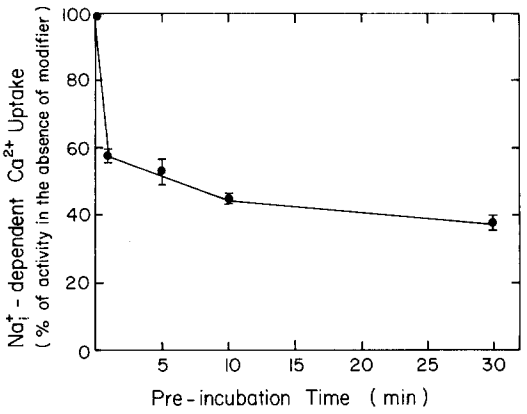

Fig. 1. Na^+-Ca^{2+} exchange in sarcolemmal vesicles after modification by dithiothreitol for varying preincubation times. Concentration of dithiothreitol in the preincubation medium was 5 mM. Modification took place in a medium which contained K^+ as the only cation (*see* Materials and Methods). Na_i^+-dependent Ca^{2+} uptake reaction was for 1.5 sec as described in Materials and Methods. Values are presented as a % of activity in sarcolemmal vesicles in the absence of modifier (2.6 ± 0.2 nmol Ca^{2+}/mg of protein/sec) (n = 4)

Na^+-Ca^{2+} exchange was measured using protocols described in detail previously (Philipson, 1984). In all experiments, Na^+-Ca^{2+} exchange was measured as Na_i^+-dependent Ca^{2+} uptake. The reaction medium (250 μl) contained 140 mM KCl, 10 μM $^{45}CaCl_2$, 0.4 μM valinomycin, 10 mM MOPS (pH 7.4) and 4–8 μg sarcolemmal protein. The exchange reaction took place at 37°C for 1.5 sec and was quenched by the addition of 0.03 ml of 140 mM KCl, 1 mM $LaCl_3$. Vesicles were harvested on Sartorius filters, washed with 2 × 3 ml of 140 mM KCl, 0.1 mM $LaCl_3$, dried, and suspended in scintillant cocktail to measure radioactivity.

To measure passive Ca^{2+} efflux, 5 μl of sarcolemmal vesicles suspended at a protein concentration of 3–4 mg/ml in 140 mM KCl, 10 mM MOPS, (pH 7.4) were diluted into 250 μl of a medium containing 140 mM KCl, 10 mM MOPS (pH 7.4), 1 mM $CaCl_2$, 1.25 μCi ^{45}Ca. Vesicles were allowed to equilibrate for 60 min at 0–5°C and then for an additional 30 min at 37°C. A 2.5 or 5 μl aliquot of modifying reagent (or water or ethanol) was added, and incubated at 37°C for varying times. To obtain a measurement of the total Ca^{2+} load in the sarcolemmal vesicles, a 220 μl aliquot was removed, filtered and washed with 2 × 3 ml of 140 mM KCl, 0.1 mM $LaCl_3$. To measure passive efflux, 3.0 ml of 140 mM KCl, 100 μM EDTA was added after the modification step. After 2 min, a 1-ml aliquot was removed, filtered, and washed as above.

To measure passive Na^+ efflux, about 100 μg of sarcolemma was preincubated for 60 min at 4°C in 140 mM KCl, 3 mM $^{22}NaCl$, 10 mM MOPS (pH 7.4) to allow passive loading of isotopic Na^+. Then 140 mM KCl and EDTA ± dithiothreitol were added (final concentrations: 0.03 and 5.0 mM, respectively), and

[1] The abbreviations used are: MOPS, 4-morpholinepropanesulfonic acid; EDTA, ethylene diamine tetraacetic acid; MMTS, methylmethanethiosulfonate; NEM, N'-ethylmaleimide; CMC, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; DCCD, N,N'-dicyclohexylcarbodiimide; NBD-Cl, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole; NBS, N-bromosuccinimide; FITC, fluoroscein isothiocyanate; PITC, phenyl isothiocyanate; EAA, ethyl acetimidate; DEP, diethylpyrocarbonate; NTCB, 2-nitro-5-thiocyanobenzoic acid; pCMB, *p*-hydroxymercuribenzoate; *p*CMBS, *p*-chloromercuriphenylsulfonic acid; DTNB, 5,5'dithiobis (2-nitrobenzoic acid).

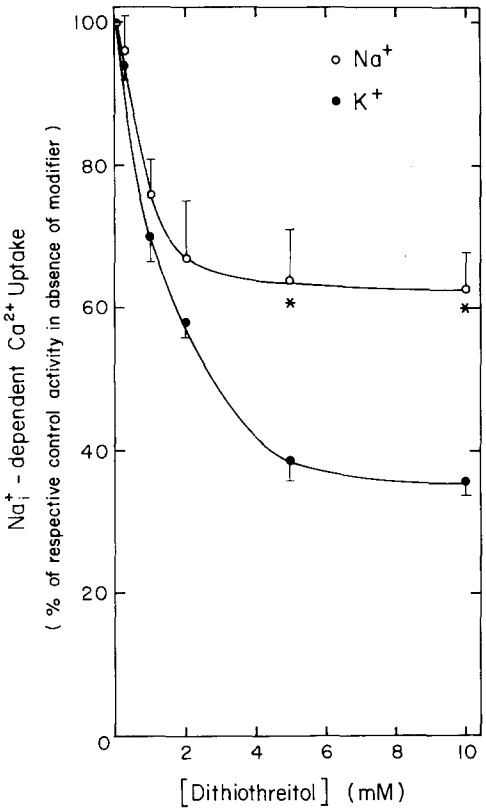

Fig. 2. Modification of Na^+-Ca^{2+} exchange activity by varying concentrations of dithiothreitol in K^+ or Na^+ preincubation media. Preincubation of the vesicles with dithiothreitol was for 30 min at 37°C. Na_i^+-dependent Ca^{2+} uptake in the absence of modifier was 2.3 ± 0.3 and 5.1 ± 0.9 nmol Ca^{2+}/mg/sec for vesicles initially suspended in K^+ and Na^+ media, respectively. K^+-vesicles were subsequently loaded with Na^+ after modification, prior to the Na^+-Ca^{2+} exchange reaction (*see* Materials and Methods). $^*P < 0.05$ between Na^+ and K^+ vesicles as detected by paired *t*-test analysis. ($n = 3$)

samples were incubated at 37°C for a further 30 min. Aliquots of this mixture were diluted 20-fold into 140 mM KCl, 100 μM EGTA, 10 mM MOPS, pH 7.4 medium to initiate passive efflux, and vesicles were harvested at selected times.

Data are expressed as means ± SE.

All reagents were purchased from Sigma Chemical, St. Louis, MO.

Results

When sarcolemmal vesicles were exposed to dithiothreitol the initial rate of Na^+-Ca^{2+} exchange was significantly inhibited. Inhibition was 40% after 1 min exposure to dithiothreitol, and increasing the exposure time to the modifier resulted in a further 20% inhibition of Na^+-Ca^{2+} exchange (Fig. 1).

The experiments shown in Fig. 1 were performed in the presence of 5 mM dithiothreitol in the modification medium. As indicated in Materials and

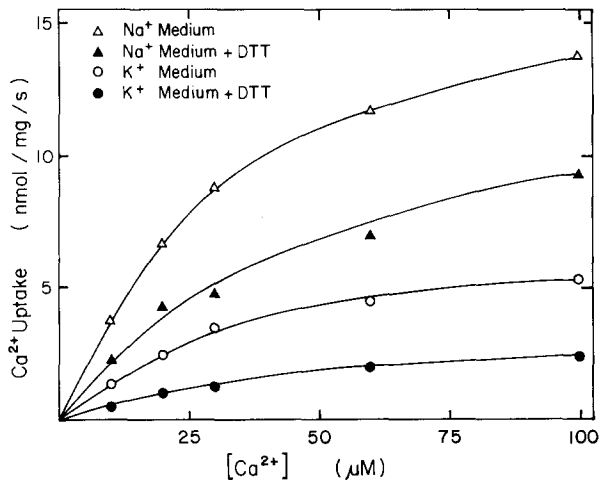

Fig. 3. [Ca^{2+}] dependence of sarcolemmal Na^+-Ca^{2+} exchange after dithiothreitol modification. Modification was carried out at 37°C for 30 min in the presence of 10 mM MOPS (pH 7.4) containing 140 mM NaCl or 140 mM KCl and 5 mM dithiothreitol (DTT), if present. Data depict results from a representative experiment

Methods, this dithiothreitol concentration was diluted 100-fold (50 μM final dithiothreitol) in the Na^+-Ca^{2+} exchange reaction medium. If 50 μM dithiothreitol was included in the Na^+-Ca^{2+} exchange reaction mixture without prior preincubation, it had no effect on Na^+-Ca^{2+} exchange. Therefore, the effects of dithiothreitol on Na^+-Ca^{2+} exchange were due to a modification of the membrane during the preincubation period.

The modification of the sarcolemmal membranes by dithiothreitol was done with K^+ as the sole cation present. Separate experiments determined if Na^+, a substrate of the Na^+-Ca^{2+} exchange reaction, could influence the modifying effect of dithiothreitol on Na^+-Ca^{2+} exchange. In addition, we investigated the concentration-dependent inhibition of Na^+-Ca^{2+} exchange by dithiothreitol. Vesicles were preincubated in the presence and absence of varying dithiothreitol concentrations for 30 min in a medium containing KCl or NaCl. As shown in Fig. 2, dithiothreitol inhibited Na^+-Ca^{2+} exchange in the presence of K^+ in the modification medium; a maximal inhibition of 64% was obtained at 10 mM dithiothreitol. In contrast, if Na^+ was present during the initial modification period with dithiothreitol, inhibition of Na^+-Ca^{2+} exchange was only 37% at 10 mM reagent concentration (Fig. 2).

The Ca^{2+} dependence of Na^+-Ca^{2+} exchange in control or dithiothreitol-modified vesicles was examined. In a representative experiment (Fig. 3), dithiothreitol again inhibited Na^+-Ca^{2+} exchange to a greater extent in K^+-containing medium than Na^+-containing medium. Varying the Ca^{2+} concentration in the Na^+-Ca^{2+} exchange reaction did not

Table 2. Alterations of sarcolemmal Na^+-Ca^{2+} exchange after preincubation of the vesicles with various amino acid modifiers in K^+- or Na^+-containing solutions[a]

Modifying reagent	Concentration (mM)	Incubation time (min)	Na^+-dependent Ca^{2+} uptake (% of control) after modification		
			in KCl	in NaCl	Δ
Sulfhydryl groups					
Dithiothreitol	5.0	30	39 ± 3	64 ± 7	−25[b]
Glutathione (red.)	4.0	30	59 ± 6	75 ± 6	−16[b]
MMTS	1.0	30	138 ± 9	110 ± 11	+28[b]
NEM	2.0	30	75 ± 7	54 ± 3	+21[b]
Carboxyl groups					
CMC	2.0	60	35 ± 5	43 ± 1	−8
EDC	5.0	60	50 ± 3	56 ± 5	−6
Tyrosine					
NBD-Cl	0.5	10	50 ± 1	56 ± 4	−6
Tryptophan					
NBS	2.0	30	46 ± 5	54 ± 3	−8
Arginine					
Phenylglyoxal	2.0	30	37 ± 2	44 ± 6	−7
Lysine					
FITC	0.1	4	94 ± 8	131 ± 9	−37[b]
PITC	0.1	4	98 ± 2	103 ± 4	−5
EAA	5.0	30	108 ± 4	108 ± 4	0
Histidine					
DEP	1.0	1	140 ± 2	143 ± 9	−3
Serine and threonine					
NTCB	2.0	60	74 ± 8	65 ± 2	+9

[a] Na^+-Ca^{2+} exchange was measured as the initial rate of Na_i^+-dependent Ca^{2+} uptake as described in Materials and Methods. Modification was carried out as described in Materials and Methods in the presence of the reagent for the incubation time indicated. In the case of experiments using the lysine modifiers, the pH was maintained at 10.25 during the modification procedure but the Na^+-Ca^{2+} exchange reaction was maintained at pH 7.4. In the DEP experiments all modification steps were carried out at 22°C except for the Na^+-Ca^{2+} exchange reaction, which was performed at 37°C. These alterations in the modification protocol were made to maintain the specificity of the reagent for the intended amino acid (for the lysine experiments) or to reduce degradation of the reagent in an aqueous medium (for the DEP experiments), as described elsewhere (Miles, 1977; Freedman, 1978).

[b] $P < 0.05$ between Na^+ and K^+ vesicles as detected by paired t-test analysis. ($n = 3$).

appear to alter this response. Although the V_{max} of Na^+-Ca^{2+} exchange was affected by dithiothreitol, the K_m for Ca^{2+} was not (Table 1). The inhibition of V_{max} by dithiothreitol was greater when K^+, rather than Na^+, was present in the modification medium. In the absence of dithiothreitol treatment, V_{max} was lower in K^+ vesicles than in Na^+ vesicles because these vesicles were not fully equilibrated with Na^+ (*see* Materials and Methods). K_m values, however, did not differ. The effects of lowered intravesicular Na^+ concentration on kinetic parameters of Na^+-Ca^{2+} exchange have been previously reported (Philipson & Nishimoto, 1982).

Dithiothreitol is a reagent known to maintain sulfhydryl residues in proteins in a reduced state (Bellomo, Mirabelli, Richelmi & Orrenius, 1983; Strauss, 1984). We examined whether another reagent which modifies these residues may exert a similar effect on Na^+-Ca^{2+} exchange in cardiac sarcolemmal vesicles. In addition, we examined whether Na^+ could alter the extent of alteration of Na^+-Ca^{2+} exchange activity induced by the modifying reagent. As shown in Table 2, reduced glutathione exhibited a similar effect as dithiothreitol on sarcolemmal Na^+-Ca^{2+} exchange. Both agents act by maintaining monothiols in the protein and reducing disulfides (Bellomo et al., 1983). As was the case with dithiothreitol, the presence of Na^+ in the modifying reaction medium with glutathione reduced the capacity of this reagent to inhibit Na^+-Ca^{2+} exchange.

Conversely, two reagents were examined which modify sulfhydryl residues in proteins (Smith & Kenyon, 1974; Smith, Maggio & Kenyan, 1975; Botts, Ue, Hozumi & Samet, 1979; Strauss, 1984). The reagents chosen were MMTS and NEM. These

reagents differ significantly in their structure and mode of modification (Smith & Kenyon, 1974; Smith et al., 1975; Botts et al., 1979; Strauss, 1984). As shown in Table 2, the effect of modification on Na^+-Ca^{2+} exchange differed strikingly between the two reagents. MMTS stimulated Na^+-Ca^{2+} exchange by almost 40%, whereas NEM inhibited Na^+-Ca^{2+} exchange. Similar to the results observed for the sulfhydryl-protecting reagents dithiothreitol and reduced glutathione, the extent of the alteration of Na^+-Ca^{2+} exchange after sulfhydryl modification differed considerably, depending on whether K^+ or Na^+ was present during the modification step. The effects of NEM were enhanced by a Na^+-modification medium, whereas the effects of MMTS were more evident after modification in a K^+ medium.

The alteration in sarcolemmal Na^+-Ca^{2+} exchange after MMTS modification was examined in greater detail in the experiments presented in Fig. 4. MMTS concentration was varied in the modification medium from 0.2–5 mM. When K^+ was present in the modification medium, Na^+-Ca^{2+} exchange was significantly stimulated at 1 and 2 mM MMTS. No stimulation was observed if Na^+ replaced K^+ in the modification medium.

Other reagents were employed to modify amino acid residues other than the the sulfur-containing groups. Preliminary experiments were carried out to determine conditions where Na^+-Ca^{2+} exchange would be inhibited by about 50% after modification with the reagents. This was done to maximize any differences which may be present when modification was accomplished in the presence of Na^+ or K^+ ions.

CMC and EDC are hydrophilic reagents that specifically modify carboxyl residues (Park, Kipnowski & Fanestil, 1983; Lotscher, DeJong & Capaldi, 1984). Treatment of the sarcolemma with these agents resulted in substantial inhibition of Na^+-Ca^{2+} exchange (Table 2). The hydrophobic carboxyl group modifier DCCD (Lotscher et al., 1984) only marginally inhibited (10%) Na^+-Ca^{2+} exchange (*data not shown*). The cation present in the modifying medium did not influence the extent of inhibition of Na^+-Ca^{2+} exchange by CMC or EDC.

Tyrosyl residues can be specifically modified by NBD-Cl (Ferguson, Lloyd & Radda 1974). Potent inhibition of Na^+-Ca^{2+} exchange by NBD-Cl was observed (Table 2). However, the presence of Na^+ or K^+ in the modifying medium did not alter the extent of this inhibition.

NBS modifies tryptophan residues (Bindslev & Wright, 1984). This reagent inhibited Na^+-Ca^{2+} exchange; however, the type of ion in the modification medium did not influence this inhibition (Table 2).

The possible involvement of arginine residues

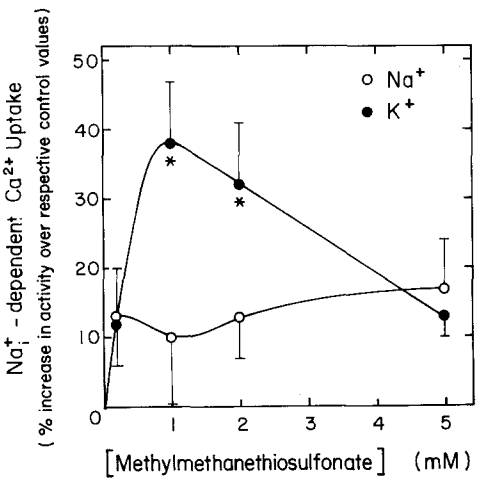

Fig. 4. Modification of Na^+-Ca^{2+} exchange activity by varying concentrations of MMTS in K^+ or Na^+ preincubation medium. Preincubation of the vesicles with the reagent was for 30 min at 37°C. Na^+-dependent Ca^{2+} uptake in the absence of modifier was 2.5 ± 0.5 and 6.1 ± 1.2 nmol/mg/sec in vesicles initially suspended in K^+ and Na^+ containing media, respectively. *See* legend to Fig. 2 and Materials and Methods for details. $^*P < 0.05$ between Na^+ and K^+ vesicles as detected by paired *t*-test analysis. ($n = 3$)

in Na^+-Ca^{2+} exchange was investigated with phenylglyoxal (Daemen & Riordan, 1974). Preincubation of sarcolemmel vesicles with phenylglyoxal resulted in an inhibition of Na^+-Ca^{2+} exchange, which again was not affected by the type of ion present (Table 2).

Lysine amino groups in proteins can be modified preferentially under basic conditions with the reagents FITC, PITC and EAA (Bindslev & Wright, 1984; Freedman, 1978). To insure specificity in modification, media during the preincubation and Na^+-loading steps were maintained at pH 10.25, although Na^+-Ca^{2+} exchange was carried out at pH 7.4. If K^+ was included in the preincubation medium, these reagents had no significant effect on Na^+-Ca^{2+} exchange (Table 2). If Na^+ replaced K^+ in the preincubation medium, FITC-modified membranes exhibited a stimulation of Na^+-Ca^{2+} exchange in comparison to control activities whereas PITC and EAA still had no effect on Na^+-Ca^{2+} exchange.

Histidine residues are specifically modified by DEP (Freedman, 1978). Concentrations of DEP were kept low to avoid side effects (Miles, 1977). Preincubation and loading media pH was maintained at 6.8 and temperature reduced to 22°C in order to slow the rate of degradation of DEP (Miles, 1977). DEP treatment of sarcolemmal membrane vesicles resulted in a small stimulation of

Table 3. Effect of modification of sarcolemmal amino acid residues on passive Ca^{2+} permeability[a]

Modifying reagent	Concentration (mM)	Incubation time (min)	Passive Ca^{2+} efflux	
			nmol/mg/2 min	% stimulation over control
None control	—	—	13.1 ± 1.7	0
Sulfhydryl groups				
Dithiothreitol	5.0	30	15.9 ± 1.0	+20
Glutathione (reduced)	1.0	30	11.0 ± 0.4	−17
MMTS	1.0	30	16.8 ± 1.6	+27
NEM	0.5	30	14.7 ± 4.2	+11
Carboxyl groups				
EDC	2.0	30	13.7 ± 2.2	+4
Tyrosine				
NBD-Cl	0.2	10	17.4 ± 3.3	+32
Tryptophan				
NBS	0.5	30	38.5 ± 4.6	+192
Arginine				
Phenylgyloxal	0.4	30	14.3 ± 1.9	+8
Lysine[b]				
FITC	0.1	4	25.1 ± 4.2	+15
PITC	0.1	4	20.9 ± 6.6	−5
Histidine				
DEP	1.0	1	15.9 ± 0.6	+20
Serine and threonine				
NTCB	0.4	30	13.8 ± 1.0	+5

[a] Sarcolemmal vesicles suspended in 140 mM KCl, 10 mM MOPS (pH 7.4) were loaded passively with 1 mM $^{45}CaCl_2$ as described in Materials and Methods. Control vesicles contained 44.9 ± 2.3 nmol Ca^{2+}/mg protein prior to the efflux period. The presence of modifying reagents in the final concentrations and for the times indicated below did not alter this initial Ca^{2+} load by more than 10%, except in the case of NBS. In this latter case, the initial Ca^{2+} load was 10.3 ± 1.3 nmol Ca^{2+}/mg protein after modification, indicating that these vesicles were highly leaky and could not maintain a Ca^{2+} load. Thus, for comparative purposes, we arbitrarily chose the control value (44.9 ± 2.3 nmol/mg) as an initial load for the NBS experiments.

[b] In the lysine modification experiments, the assay pH was maintained at 10.25. This altered the initial Ca^{2+} load and the efflux rate for control preparations (63.0 ± 6.9 nmol/mg and 21.9 ± 3.1 nmol/mg/2 min, respectively). Therefore, the FITC and PITC results were compared to these appropriate control conditions. (n = 3.)

Na^+-Ca^{2+} exchange but the ion present in the preincubation medium did not alter this stimulation.

The hydroxyl groups of serine and threonine amino acid residues in proteins can be modified with NTCB (Liao & Wadano, 1979). The relatively small inhibition of Na^+-Ca^{2+} exchange after NTCB modification which is shown in Table 2 was maximal for this reagent. It was uninfluenced by a change in ion in the modification medium.

The effects of the various modification protocols on the passive Ca^{2+} permeability of the cardiac sarcolemmal vesicles are presented in Table 3. The concentrations of the modifying reagents and the length of time of modification were maintained as closely as possible to those used in the Na^+-Ca^{2+} exchange experiments (Table 2). However, in some cases the experimental conditions or the limited reagent solubility necessitated final conditions in the passive permeability experiments which only approximated those used in the Na^+-Ca^{2+} exchange reaction. Many of the reagents slightly altered the passive Ca^{2+} permeability of the sarcolemmal vesicles. Only NBS elicited a substantial increase in passive Ca^{2+} loss. The value presented in Table 3 for the effect of NBS on Ca^{2+} permeability likely represents an underestimation. We conclude this because NBS-treated vesicles exhibited a substantial reduction in Ca^{2+} load prior to the addition of efflux medium. The increase in permeability demonstrated after modification with the other reagents was small (≤32% increase over control) and unlikely to significantly affect Na^+-Ca^{2+} exchange measurements.

We also examined the effect of dithiothreitol on passive Na^+ permeability. In the absence of dithiothreitol modification, Na^+ load was (as a per-

cent of the initial load) 87 ± 6%, 68 ± 4%, 53 ± 5%, 45 ± 3% after 0.25, 1, 3, and 5 min, respectively, of passive Na^+ efflux. In vesicles pretreated for 30 min with 5 mM dithiothreitol, Na^+ load was 75 ± 5%, 58 ± 5%, 43 ± 4%, 38 ± 6% after 0.25, 1, 3, and 5 min of efflux, respectively. The passive Na^+ efflux was not significantly different as a function of dithiothreitol, although dithiothreitol treatment did tend to cause slightly greater efflux. The effect was sufficiently small that little influence on Na^+-Ca^{2+} exchange would be expected.

Discussion

Treatment of cardiac sarcolemmal membrane vesicles with dithiothreitol resulted in a significant decrease in Na^+-Ca^{2+} exchange activity. This modification decreased the V_{max} but did not alter the K_m for Ca^{2+} of the Na^+-Ca^{2+} exchange process. The effect was due to modification of the membrane by the reagent and was not due to an artifactual interference in the reaction media. This conclusion is based upon two observations. First, if dithiothreitol was included in the Na^+-Ca^{2+} exchange assay medium without prior preincubation step, no alteration in Na^+-Ca^{2+} exchange activity was observed. Second, if the vesicles were treated with dithiothreitol, centrifuged, resuspended in medium which did not contain the reagent and then measured for Na^+-Ca^{2+} exchange, a similar inhibition was observed.

The inhibition of Na^+-Ca^{2+} exchange by dithiothreitol is not the result of a disruption in the membrane permeability barrier. Dithiothreitol increased passive Ca^{2+} efflux by 2.7 nmol/mg/2 min (20% stimulation over control). This small increase in passive Ca^{2+} flux represents less than 1% of the rate of Na^+-Ca^{2+} exchange (5.06 nmol Ca^{2+}/mg/sec) and is unlikely to significantly alter measurement of Na^+-Ca^{2+} exchange. Large changes in membrane leakiness are necessary before Na^+-Ca^{2+} exchange activity is significantly affected. This is emphasized by the observation that NBS, which increased passive Ca^{2+} efflux nearly 200% over control values (Table 3), inhibited Na^+-Ca^{2+} exchange by only 50% (Table 2).

Information on the site of interaction of dithiothreitol with the sarcolemma was obtained from experiments in which the ionic composition of the modification medium was varied. A common methodological approach in modification experiments is to examine whether the presence of an enzyme substrate in the modification medium can influence the extent of the alteration of enzyme activity (for ref. *see* Freedman, 1978). Such a protocol can suggest whether the modification site is located near the active site for enzyme catalysis. The initial modification step contained either Na^+, as an exchange substrate, or K^+ to maintain osmolarity. In addition, 50 μM EDTA was included in the modification medium to chelate endogenous Ca^{2+}, which could act as a substrate ion. The EDTA would also prevent dithiothreitol from interacting with metal ions to generate free radicals (Strauss, 1984).

The data presented in Fig. 2 demonstrate a large difference in the Na^+-Ca^{2+} exchange reaction after dithiothreitol modification in the presence of K^+ or Na^+ ions. The difference is possibly an underestimation due to our experimental protocol. As described in Materials and Methods, a 10-min exposure of K^+-containing vesicles to Na^+ was necessary after the initial modification step in order to load these vesicles with Na^+ before initiating the Na_i^+-dependent Ca^{2+} uptake reaction. Vesicles were exposed to this step whether initial modification occurred in either a Na^+ or K^+ medium. Some modification may have continued during this Na^+-loading step despite a lowered modifier concentration. Since Na^+ was present, the relative rate of modification will be about the same in both cases. Differences that occurred during initial modification might tend to be masked. Therefore, the differences that we observe between the K^+ and Na^+ vesicles possibly represent a minimal estimate. The difference demonstrated in the dithiothreitol experiments suggests that a Na^+-binding site in the Na^+-Ca^{2+} antiporter protein may be in proximity to a site of dithiothreitol modification. Alternatively, it is possible that Na^+ binding may cause a conformational change in the exchanger which could affect modification of sulfur-containing residues some distance from the active site. Because the Na^+-Ca^{2+} exchange protein has not been characterized at a molecular level, we cannot address this possibility. Use of reduced glutathione led to results similar to those obtained with dithiothreitol (Table 2).

The difference between dithiothreitol modification of Na^+-Ca^{2+} exchange in the absence or presence of Na^+ is not a consequence of an artifact in the experimental protocol. With the exception of one modifier (FITC), only sulfhydryl-modifying reagents exhibited such a Na^+ effect (Table 2). A possibility, however, is that the internal Na^+ load may alter the response of the exchanger after sulfhydryl modification. For example, it sulfhydryl modification changes the intravesicular K_m (Na^+), inhibitory effects might be Na^+-load dependent. Thus, we cannot differentiate whether the presence of Na^+ during modification changes the amount of modification that occurs or whether treatment by dithiothreitol changes the interaction of Na^+ with the exchanger. In either case, the data indicate that

dithiothreitol-sensitive sites somehow interact with Na^+-binding sites.

Two observations suggest that there may be more than one sulfur-containing site on the exchanger which reacts with dithiothreitol. First, the effect of dithiothreitol on Na^+-Ca^{2+} exchange was biphasic as a function of modification time (Fig. 1). The effect on Na^+-Ca^{2+} exchange was mostly completed within the first minute of preincubation. Longer incubation time resulted in further inhibition but at a much slower rate. Second, whereas Na^+ had no significant effect on the modification of Na^+-Ca^{2+} exchange at lower dithiothreitol concentrations, large differences were observed at higher dithiothreitol concentrations as a function of the ion present (Fig. 2).

Sulfhydryl-modifying reagents NEM and MMTS also exhibited differences between the extent of alteration of Na^+-Ca^{2+} exchange activity when Na^+ was present in the modifying medium. However, Na^+ enhanced the inhibition of Na^+-Ca^{2+} exchange by NEM, whereas K^+ enhanced the stimulation of Na^+-Ca^{2+} exchange by MMTS. It is interesting that NEM and MMTS exhibited opposite effects on Na^+-Ca^{2+} exchange. In preliminary experiments with other sulfhydryl modifiers (pCMB, pCMBS and DTNB), Na^+-Ca^{2+} exchange was consistantly inhibited (*data not shown*). The anomalous results with MMTS may be explained on the basis of reagent size. All of the sulfhydryl modifiers with the exception of MMTS incorporate relatively bulky groups at these structurally sensitive locations (Smith & Kenyon, 1974; Smith et al., 1975; Botts et al., 1979). It is possible that the modification of sulfhydryl residues with the relatively small reagent MMTS would have a different steric effect on Na^+-Ca^{2+} exchange, which would result in the observed stimulation. This hypothesis has been proposed to explain a similar dissociation of the action of MMTS from other sulfhydryl modifiers in studies of several purified proteins (Smith & Kenyon, 1974; Smith et al., 1975; Botts et al., 1979). The data obtained with NEM and MMTS suggest that sulfhydryl groups are important in the Na^+-Ca^{2+} exchange process. The experiments discussed above using dithiothreitol and reduced glutathione imply significance for disulfide and/or sulfhydryl groups.

In summary, our data suggest that sulfur-containing residues interact with Na^+-binding sites of the Na^+-Ca^{2+} exchanger. Our conclusions are based on differential modification of Na^+-Ca^{2+} exchange activity in the presence and absence of Na^+. Binding of Na^+ to the exchanger possibly results in conformational changes which alter the accessibility of groups to reagents. It is not clear whether or not the important sites of modification are near Na^+ binding sites. We cannot detect an involvement of carboxyl, tyrosyl, arginyl, histidyl or hydroxyl groups at a Na^+-binding site. In addition, although FITC demonstrated some differences in its effects on Na^+-Ca^{2+} exchange when Na^+ substrate was absent or present, two other lysine modifiers (EAA, PITC) did not exhibit such differences. It is interesting that FITC, MMTS and DEP were the only reagents capable of stimulating Na^+-Ca^{2+} exchange. It may be worthwhile to pursue the mechanisms responsible for this stimulation.

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