The selective activation of the cardiac sarcolemmal sodium-calcium exchanger by plasmalogenic phosphatidic acid produced by phospholipase D

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Abstract Since plasmalogens are the predominant phospholipid of cardiac sarcolemma, the activation of the sodium-calcium exchanger by either plasmenylethanolamine or plasmalogenic phosphatidic acid generated by phospholipase D was explored. Sodium-calcium exchange activity was 7-fold greater in proteoliposomes comprised of plasmenylethanolamine compared to proteoliposomes comprised of only plasmenylcholine. Phospholipase D treatment of proteoliposomes resulted in 1 mol % conversion of plasmenylcholine or phosphatidylcholine to their respective phosphatidic acid molecular species with a concomitant 8-fold or 2-fold activation of sodium-calcium exchange activity, respectfully. Thus, phospholipase D-mediated hydrolysis of plasmalogens to phosphatidic acid may be an important mechanism for the regulation of the sodium-calcium exchanger.

Key words: Plasmalogen; Sodium-calcium exchange; Myocardium; Phosphatidic acid; Phospholipase D

1. Introduction

Cardiac sarcolemmal phospholipids are predominantly comprised of plasmalogens which are substrates for phospholipases that are activated during myocardial ischemia [1–3]. Plasmalogens have unique membrane biophysical properties [4–6]. Compared to their diacyl phospholipid counterpart plasmalogens have been demonstrated to provide a critical membrane environment for the regulation of the cardiac sarcolemmal sodium-calcium exchanger in the presence of the anionic phospholipid, phosphatidylserine [7]. Since anionic phospholipids regulate sodium-calcium exchange activity in plasmalogen based proteoliposomes, the activation of sodium-calcium exchange by plasmalogenic phosphatidic acid was compared and contrasted to that elicited by diacyl phosphatidic acid. The present study demonstrates that plasmalogenic phosphatidic acid is a potent activator of the cardiac sodium-calcium exchanger in comparison to that of diacyl phosphatidic acid. Additionally, the results reported herein demonstrate that the sodium-calcium exchanger is stimulated by plasmenylethanolamine and not phosphatidylethanolamine.

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Abbreviations: FMOC, 9-fluorenylmethoxy-carbonyl; GPC, *sn*-glycero-3-phosphocholine; GPE, *sn*-glycero-3-phosphoethanolamine; PIP_2, phosphatidylinositol-4,5-bisphosphate; 16:0-18:1 plasmenylcholine (PlasCho), 1-*O*-hexadec-1′-enyl-2-octadec-9′-enoyl-GPC; 16:0-18:1 phosphatidylcholine (PhosCho), 1-hexadecanoyl-2-octadec-9′-enoyl-GPC; 16:0-20:4 plasmenylethanolamine (PlasEtn), 1-*O*-hexadec-1′-enyl-2-eicosatetra-5′,8′,11′,14-enoyl-GPE; 16:0-20:4 phosphatidylethanolamine (PhosEtn), 1-hexadecanoyl-2-eicosatetra-5′,8′,11′,14-enoyl-GPE; di-20:0 phosphatidylcholine, 1,2-dieicosanoyl-GPC

2. Materials and methods

2.1. Synthesis and purification of plasmenylcholine

Plasmenylcholine was synthesized by an anhydrous reaction utilizing 1-*O*-hexadec-1′-enyl-GPC and octadec-9′-enoyl chloride as precursors with dimethylaminopyridine as catalyst as previously described [8]. Plasmenylethanolamine was prepared by a similar synthetic scheme as that described for plasmenylcholine except that the primary amine of lysoplasmenylethanolamine was first protected with FMOC prior to the reaction with eicosatetra-5′,8′,11′,14′-enoyl chloride [7]. Synthetically prepared plasmenylcholine and plasmenylethanolamine were determined to be greater than 95% pure by thin-layer chromatography, straight-phase HPLC, reversed-phase HPLC and capillary gas chromatography of the aliphatic constituents. 16:0-18:1 plasmenylcholine and 16:0-20:4 plasmenylethanolamine were quantified by capillary gas chromatography.

2.2. Preparation of cardiac sarcolemmal vesicles

Cardiac sarcolemmal vesicles were prepared from bovine ventricles by a modification of the method described by Slaughter et al. [9] as previously described [10]. Sarcolemmal vesicle preparations were resuspended in 160 mM NaCl, 20 mM MOPS/TRIS (pH = 7.4) and stored at −70°C.

2.3. Reconstitution of cardiac sodium-calcium exchange activity

Sodium-calcium exchange activity was reconstituted in proteoliposomes prepared from bovine cardiac sarcolemmal vesicles and activity was assayed under linear reaction conditions as previously described [11]. All experiments were performed in triplicate with 1–3 different bovine cardiac sarcolemmal vesicle preparations. In experiments with phospholipase D, proteoliposomes were pretreated with 1 U of *Streptomyces chromofuscus* phospholipase D (Calbiochem, San Diego, CA) in 160 mM NaCl, 20 mM MOPS/TRIS (pH = 7.4) for 15 min at 37°C prior to their immediate assay for sodium-calcium exchange activity.

2.4. Electrospray ionization mass spectrometry of proteoliposome choline glycerophospholipids

Phospholipid constituents of the reconstituted proteoliposomes were confirmed utilizing a triple quadrupole mass spectrometer equipped with an electrospray ionization interface as previously described [12]. Tandem mass spectrometry was performed by passage of the mass selected precursor ion from the first quadrupole into the collision cell (typically collision energies were ∼35 eV). Typically, spectra were averaged 3–5 min and processed utilizing ICIS software (Finnigan). Chloroform extracts of the reconstituted proteoliposomes were diluted with 2 volumes of methanol and infused into the ionization chamber at flow rate of 1 μl/min in the presence of 2 nmol/μl of lithium hydroxide.

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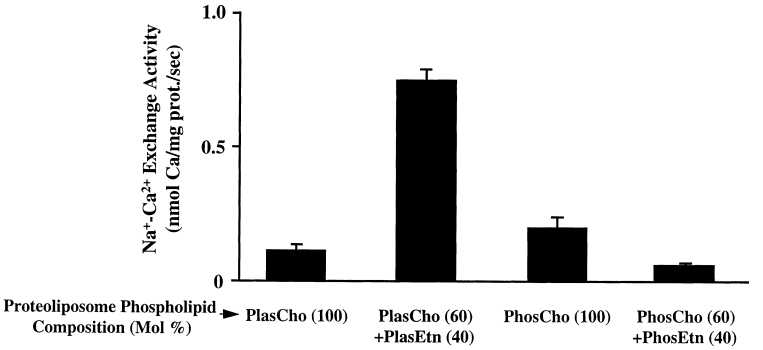

Fig. 1. Sodium-calcium exchange in choline glycerophospholipid proteoliposomes in the presence and absence of ethanolamine glycerophospholipids. Bovine cardiac sarcolemmal vesicles were prepared and vesicle proteins were extracted with sodium cholate. Solubilized proteins were reconstituted with indicated phospholipids by the detergent dilution technique resulting in a final protein/phospholipid ratio of 2 mg vesicle protein/25 mg phospholipid. Sodium-calcium exchange activity was measured as described in Section 2 and was measured as the difference in ^{45}Ca vesicular uptake in the presence or absence of an outwardly directed sodium gradient. Values for sodium-calcium exchange activity are the mean ± S.E.M. for three independent measurements.

2.5. *Quantitation of phosphatidic acid produced by phospholipase D treatment of proteoliposomes*

Phosphatidic acid molecular species generated by phospholipase D treatment were extracted into chloroform utilizing a modified Bligh-Dyer extraction technique [13]. Extracted phosphatidic acids and choline glycerophospholipids were purified by thin-layer chromatography utilizing silica gel G plates and a mobile phase of chloroform/acetone/methanol/acetic acid/water (6/8/2/2/1, v/v) ($R_f = 0.7$ and 0.4, respectively). Silica corresponding to regions containing phosphatidic acids and choline glycerophospholipids was scrapped and extracted in the presence of 20 or 200 μg of arachidic acid into 1.5 ml of chloroform with an aqueous phase comprised of 4.5 ml of methanol/30% acetic acid (3/5, v/v). Following two further extractions of the aqueous phase with 1.5 ml of chloroform, the pooled extracts were dried under nitrogen. The resultant purified phosphatidic acids and choline glycerophospholipids were then quantified and their aliphatic constituents determined following acid methanolysis and sequential capillary gas chromatography of the resultant fatty acid methyl ester and dimethyl acetal derivatives. In brief, capillary gas chromatography was performed on a Hewlett-Packard 5890 chromatograph equipped with a flame ionization detector utilizing a 40 m Supelco SP2330 capillary column and a flow rate of 2 cc/min at 20 psi capillary pressure with a 20:1 split ratio.

2.6. *Miscellaneous assays*

Bovine cardiac sarcolemmal vesicles were assayed for protein content by the method described by Lowry et al. [14]. A modification of the method described by Schaffner and Weismann [15] was performed to quantitate proteoliposomal protein content as previously described [11].

3. Results

3.1. *Reconstitution of cardiac sarcolemmal sodium-calcium exchange activity in proteoliposomes comprised of either plasmalogen or diacyl phospholipid mixtures*

Since plasmalogens are the predominant phospholipid of cardiac sarcolemma and have been demonstrated to enhance cardiac sarcolemmal sodium-calcium exchange activity, the individual roles of plasmenylcholine and plasmenylethanolamine on sodium-calcium exchange activity were determined. The reconstitution of the sodium-calcium exchange activity in proteoliposomes comprised of plasmenylethanolamine and plasmenylcholine was 7-fold greater than that of proteolipo-

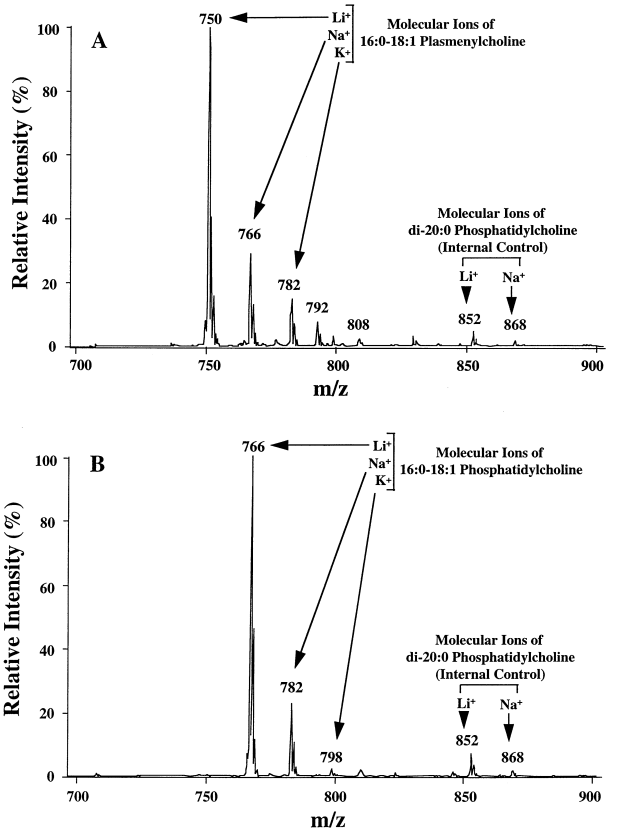

Fig. 2. Electrospray ionization mass spectrometry of choline glycerophospholipids from proteoliposomes reconstituted in either plasmenylcholine or phosphatidylcholine. Proteoliposomes reconstituted with either 16:0-18:1 plasmenylcholine (panel A) or 16:0-18:1 phosphatidylcholine (panel B) for the measurement of sodium-calcium exchange activity as described in Fig. 1 were prepared and were extracted into chloroform prior to analysis of the proteoliposome choline glycerophospholipid molecular species by electrospray ionization mass spectrometry as described in Section 2. During the lipid extraction of proteoliposomes, di-20:0 phosphatidylcholine was added as an internal control for mass spectrometry. In panel A, molecular ions at *m/z* 750, 766, and 782 are the Li^+, Na^+, and K^+ ions, respectively, of 16:0-18:1 plasmenylcholine. In panel B, molecular ions at *m/z* 766, 782, and 798 are the Li^+, Na^+, and K^+ ions, respectively, of 16:0-18:1 phosphatidylcholine. In both panels A and B, molecular ions at *m/z* 852 and 868 are the Li^+ and Na^+ ions, respectively, of di-20:0 phosphatidylcholine. These spectra are representative of analyses of multiple independent preparations of reconstituted proteoliposomes.

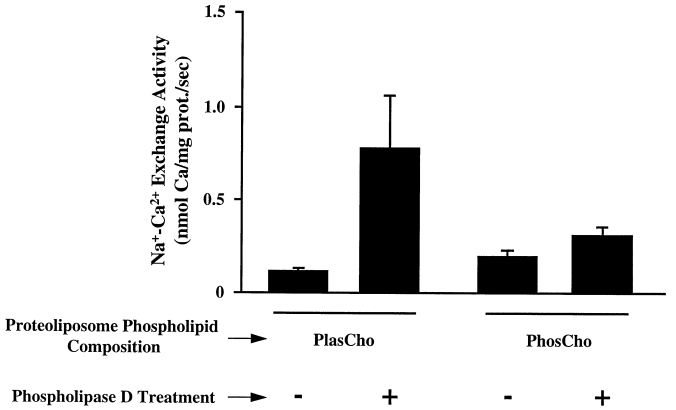

Fig. 3. Sodium-calcium exchange in choline glycerophospholipid proteoliposomes treated with phospholipase D. Sodium-calcium exchange activity was measured as described in Section 2 and was measured as the difference in ^{45}Ca vesicular uptake in the presence or absence of an outwardly directed sodium gradient. Prior to the measurement of sodium-calcium exchange, 500 μl of proteoliposomes comprised of either plasmenylcholine or phosphatidylcholine were incubated with either 1 U of *Streptomyces chromofuscus* phospholipase D in 160 mM NaCl (+) or 160 mM NaCl (−) for 15 min at 37°C. Values for sodium-calcium exchange activity are the mean ± S.E.M. for three independent measurements.

somes comprised of plasmenylcholine alone (Fig. 1). The addition of phosphatidylethanolamine with phosphatidylcholine in the reconstitution system did not enhance sodium-calcium exchange activity to levels comparable to that with plasmenylcholine and plasmenylethanolamine (Fig. 1).

3.2. Phospholipase D treatment of proteoliposomes comprised of either plasmenylcholine or phosphatidylcholine

Since proteoliposomes containing either plasmenylcholine or phosphatidylcholine possessed only modest sodium-calcium exchange activity, these proteoliposomes were characterized for choline glycerophospholipid composition following the reconstitution protocol and were also used for experiments to delineate the role of phospholipase D in modulating the exchange activity. Electrospray ionization mass spectrometry in the positive ion mode of chloroform extracts from proteoliposomes prepared with either 16:0-18:1 plasmenylcholine or 16:0-18:1 phosphatidylcholine revealed that $>95\%$ of the choline glycerophospholipid molecular species in the reconstituted proteoliposomes were the choline glycerophospholipid utilized in the reconstitution (Fig. 2). The predominance of the Li^+ molecular ions of these choline glycerophospholipids is due to the addition of lithium hydroxide to the sample prior to its injection into the mass spectrometer. The presence of minor Na^+ and K^+ molecular ions is due to the exposure of lipid samples to these contaminating monovalent cations prior to their detection by the mass spectrometer. It should be noted that the small amounts of molecular ions at m/z 792 and 808 (Fig. 2A) are the Li^+ and Na^+ molecular ions of 1,2-dioctadec-9′-enoyl-GPC which are minor contaminants of the synthetic 16:0-18:1 plasmenylcholine preparation. Thus, electrospray ionization mass spectrometric analysis confirms the utility of the reconstitution method for the introduction of specific synthetic phospholipid molecular species and the removal of endogenous sarcolemmal phospholipids.

Proteoliposomes comprised of either plasmenylcholine or phosphatidylcholine that were treated with phospholipase D possessed disparate sodium-calcium exchange activities. The treatment of 16:0-18:1 plasmenylcholine-containing proteoliposomes with phospholipase D resulted in a 8-fold increase in sodium-calcium exchange activity (Fig. 3). Only a 2-fold increase was observed in proteoliposomes comprised of 16:0-18:1 phosphatidylcholine following phospholipase D treatment (Fig. 3). Lipid analysis of the proteoliposomes by sequential thin-layer chromatography purification of the choline glycerophospholipids and phosphatidic acids and subsequent iodine staining of the thin-layer chromatography plates demonstrated that only choline glycerophospholipids were present in the untreated proteoliposomes whereas phosphatidic acid was observed in samples prepared from both proteoliposome species following treatment with phospholipase D. A quantitative assessment of the conversion of choline glycerophospholipids to phosphatidic acid was made by capillary gas chromatographic analysis of the acid methanolysis derivatives of the thin-layer chromatography purified choline glycerophospholipids and phosphatidic acids which revealed that phospholipase D treatment resulted in conversion of either 16:0-18:1 plasmenylcholine or 16:0-18:1 phosphatidylcholine in the reconstituted proteoliposomes to their phosphatidic acid catabolites (Figs. 4 and 5, respectively). Comparisons of the integrated areas from the phospholipid aliphatic derivatives (e.g. the dimethyl acetal of palmitaldehyde and oleic acid methyl ester) to that of the internal standard (i.e. the methyl ester of arachidic acid) demonstrated that 1% of the plasmenylcholine and phosphatidylcholine were converted to their respective phosphatidic acid derivatives following the phospholipase D protocol employed (Figs. 4 and 5). Thus, minute conversions in the plasmenylcholine or phosphatidylcholine membrane environment (e.g. 1 mol % hydrolysis by phospholipase D) resulted in disproportionate changes in sodium-calcium exchange activity.

4. Discussion

There have been several reports indicating that cardiac sodium-calcium exchange is affected by its membrane environment and is specifically upregulated or activated by anionic phospholipids [10,16–18]. Earlier studies showed that the stimulation of sodium-calcium exchange by anionic phospholipids is not due to a general surface charge effect but is likely caused by a direct interaction of anionic phospholipids with the sodium-calcium exchange protein [19]. It has been proposed that anionic phospholipids may specifically interact with the endogenous XIP domain on the sodium-calcium ex-

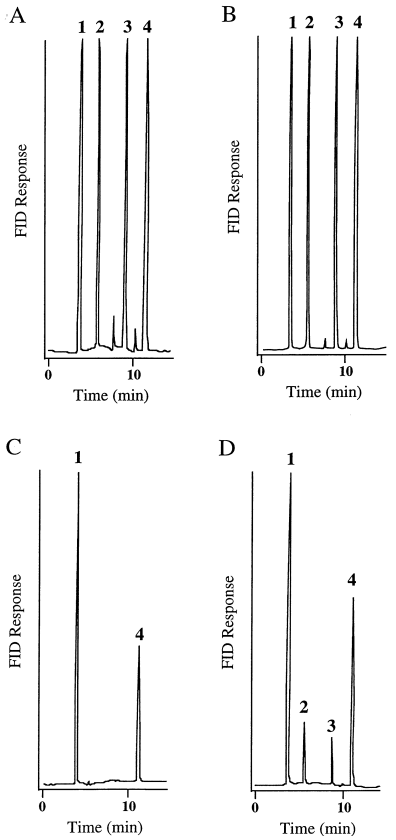

Fig. 4. Capillary gas chromatography of derivatized 16:0-18:1 plasmenylcholine and plasmalogenic phosphatidic acid from proteoliposomes treated with phospholipase D. Proteoliposomes prepared from cardiac sarcolemmal vesicles and reconstituted with 16:0-18:1 plasmenylcholine were subjected to either incubations with phospholipase D (panels B and D) or control incubations with 160 mM NaCl (panels A and C) as described in Fig. 3. Following treatments, phospholipids in the proteoliposomes were extracted, were resolved into their plasmenylcholine and plasmalogenic phosphatidic acid constituents by thin-layer chromatography and the aliphatic groups of these purified phospholipids were converted to their dimethyl acetal and fatty acid methyl ester derivatives by acid methanolysis prior to their analysis by capillary gas chromatography as described in Section 2. Panels A and B are gas chromatograms of the acid methanolysis derivatives of thin-layer chromatography purified plasmenylcholine. Panels B and D are gas chromatograms of the acid methanolysis derivatives of thin-layer chromatography purified plasmalogenic phosphatidic acid. Peaks 1 (R_t = ~3.8 min), 2 (R_t = ~5.7 min), 3 (R_t = ~9.0 min) and 4 (R_t = ~11.4 min) correspond to the solvent peak (petroleum ether), dimethyl acetal of palmitaldehyde, oleic acid methyl ester and arachidic acid methyl ester (from the internal standard, arachidic acid), respectively. Chromatograms are representative of analyses of multiple independent preparations of reconstituted proteoliposomes subjected to identical conditions.

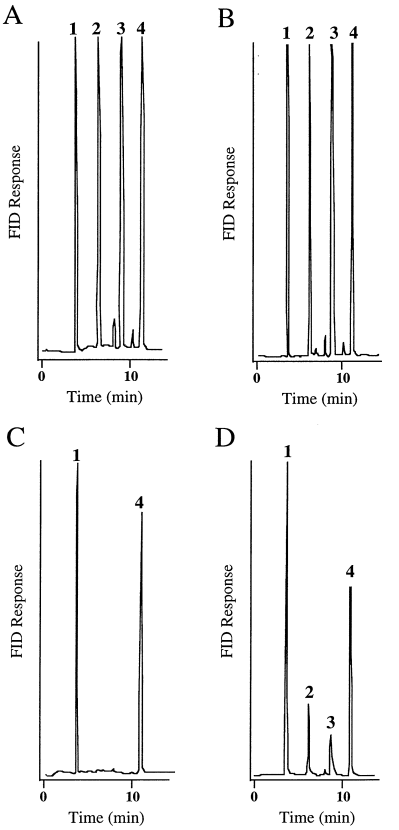

Fig. 5. Capillary gas chromatography of derivatized 16:0-18:1 phosphatidylcholine and diacyl phosphatidic acid from proteoliposomes treated with phospholipase D. Proteoliposomes prepared from cardiac sarcolemmal vesicles and reconstituted with 16:0-18:1 phosphatidylcholine were subjected to either incubations with phospholipase D (panels B and D) or control incubations with 160 mM NaCl (panels A and C) as described in Fig. 3. Following treatments, phospholipids in the proteoliposomes were extracted, were resolved into their phosphatidylcholine and diacyl phosphatidic acid constituents by thin-layer chromatography and the aliphatic groups of these purified phospholipids were converted to their fatty acid methyl ester derivatives by acid methanolysis prior to their analysis by capillary gas chromatography as described in Section 2. Panels A and B are gas chromatograms of the acid methanolysis derivatives of the thin-layer chromatography purified phosphatidylcholine. Panels B and D are gas chromatograms of the acid methanolysis derivatives of the thin-layer chromatography purified diacyl phosphatidic acid. Peaks 1 (R_t = ~3.8 min), 2 (R_t = ~6.4 min), 3 (R_t = ~9.0 min) and 4 (R_t = ~11.4 min) correspond to the solvent peak (petroleum ether), palmitic acid methyl ester, oleic acid methyl ester and arachidic acid methyl ester (from the internal standard, arachidic acid), respectively. Chromatograms are representative of analyses of multiple independent preparations of reconstituted proteoliposomes subjected to identical conditions.

changer which induces a protein conformation that is conducive to transport [10].

Several anionic phospholipids stimulate sodium-calcium exchange activity including phosphatidylserine, phosphatidic acid, and PIP_2 as demonstrated by reconstitution or enzymatic hydrolysis by phospholipases [10,16–18]. With the exception of a recent report from Ford and Hale [7], anionic phospholipid stimulation of sodium-calcium exchange activity in reconstitution experiments have been performed exclusively with diacyl phospholipids. Although diacyl phospholipids, in comparison to plasmalogens, provide a less than optimal environment for an active exchange protein, the anionic phospholipid effect was still demonstrated to be discernable [7]. Additionally, Hilgemann and co-workers have demonstrated that the application of either phospholipase D or ATP and phosphatidylinositol to generate phosphatidic acid or PIP_2, respectively, to giant patches from cardiac myocytes results in an increase in sodium-calcium exchange activity [17,18]. Since the present results demonstrate that phospholipase D hydrolysis of plasmalogens activates sodium-calcium exchange activity and since cardiac myocyte sarcolemma is enriched with plasmalogens [1,20], it is possible that phospholipase D

treatment resulting in enhanced sodium-calcium exchange activity reported in giant patches from cardiac myocytes [17] may involve, at least in part, the hydrolysis of plasmalogens. Furthermore, the hydrolysis of plasmalogens by phospholipase D which is activated during myocardial ischemia [21] may modulate the sodium-calcium exchanger during ischemia.

A striking observation on the effectiveness of controlling the membrane environment during reconstitution using sodium cholate and the detergent dilution method is reported here. We are not aware of any previous studies that have documented the completeness of the exchange of endogenous for exogenous phospholipids during reconstitution. Using electrospray ionization mass spectrophotometric analysis, we were able to demonstrate that the exchange of endogenous for exogenous phospholipids approached 100%. These data show that reconstitution can be used to establish a defined membrane environment.

While anionic phospholipids, like phosphatidic acid, stimulate sodium-calcium exchange activity, the level of stimulation is a function of the type of phospholipid comprising the membrane. Using phosphatidylserine as the stimulating anionic phospholipid, Ford and Hale [7] showed that there was a direct correlation to the proportion of plasmalogens in the membrane and sodium-calcium exchange activity. In the present study, the generation of phosphatidates produced an 8-fold and 2-fold stimulation in plasmalogen and diacyl proteoliposomes, respectively. Furthermore, in contrast to phosphatidylethanolamine, plasmenylethanolamine stimulates sodium-calcium exchange in the absence of anionic phospholipids. It is possible that the mechanism responsible for plasmalogen activation of the sodium-calcium exchanger is similar, in part, to that of the model proposed for amphiphile-mediated activation of the sodium-calcium exchanger proposed by Philipson and Ward [22]. For example, studies using truncated driven nuclear Overhauser enhanced NMR have demonstrated that the polar headgroup of plasmenylcholine is more parallel to the membrane director than that of phosphatidylcholine [6]. Thus, the phosphate in the polar head group of plasmalogens may have an enhanced negative charge that interacts with the XIP domain as compared to that of their diacyl phospholipid counterparts. Additionally, plasmalogens are enriched with unsaturated aliphatic chains at the *sn*-2 carbon of the glycerol backbone which would effect their packing in biological membranes as compared to their more saturated diacyl phospholipid counterparts. Despite these similarities with the amphiphile activation model of the sodium-calcium exchanger, the effect of plasmalogens on sodium-calcium exchange activity likely is mediated by the spatial constraints dictated by the vinyl ether bond which packs plasmalogens in the membrane and directs the polar head group out of the membrane bilayer differently than diacyl phospholipids.

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