Plasmalogen and anionic phospholipid dependence of the cardiac sarcolemmal sodium-calcium exchanger

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Received 17 July 1996; revised version received 8 August 1996

Abstract Although plasmalogens are the predominant phospholipids of cardiac sarcolemma, their physiological role has not been forthcoming. Since the cardiac sarcolemmal sodium-calcium exchanger has been proposed to be regulated by anionic phospholipids, the roles of plasmalogens and anionic phospholipids as regulators of the sodium-calcium exchanger were explored. Reconstituted sodium-calcium exchange activity in plasmalogen-containing proteoliposomes was 10-fold higher than that in control proteoliposomes comprised of only diacyl phospholipids. Additionally, exchange activity in plasmalogen-containing proteoliposomes was regulated by anionic phospholipids. Thus, plasmalogens provide a critical lipid environment in which anionic phospholipids serve as boundary lipids for the regulation of the trans-sarcolemmal sodium-calcium exchanger.

Key words: Plasmalogen; Sodium-calcium exchange; Sarcolemma; Phospholipid; Myocardium

1. Introduction

Plasmalogens are a phospholipid molecular subclass containing a vinyl ether bond that links the sn-1 aliphatic chain to the glycerol backbone and are the predominant phospholipids found in the sarcolemma of myocardium [1]. In comparison to diacyl phospholipids, plasmalogens have unique membrane biophysical properties [2,3], have a greater propensity to facilitate membrane fusion [4], and are selectively hydrolyzed during myocardial ischemia [5–7]. Although the biochemical and biophysical differences between plasmalogens have been identified, the physiological importance of plasmalogens in cardiac sarcolemma remains unresolved. One possible physiological role of sarcolemmal plasmalogens is the regulation of sarcolemmal transmembrane proteins that are responsible for normal myocytic intracellular ion homeostasis. Accordingly, to delineate the role of plasmalogens on sarcolemmal transmembrane protein function, we now report that cardiac sarcolemmal plasmalogens are important regulators of sodium-calcium exchange activity and participate in the regulation of sodium-calcium exchange activity by anionic phospholipids which serve as boundary lipids.

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Abbreviations: FMOC, 9-fluorenylmethoxy-carbonyl; GPC, sn-glycero-3-phosphocholine; GPE, sn-glycero-3-phosphoethanolamine; GPFE, sn-glycero-3-*N*-FMOC-phosphoethanolamine; 16:0-18:1 plasmenylcholine, 1-*O*-hexadec-1′-enyl-2-octadec-9′-enoyl-GPC; 16:0-20:4 phosphatidylethanolamine, 1-hexadecanoyl-2-eicosatetra-5′,8′,11′,14′-enoyl-GPE; 16:0-18:1 phosphatidylcholine, 1-hexadecanoyl-2-octadec-9′-enoyl-GPC

2. Materials and methods

2.1. Synthesis and purification of plasmenylcholine and plasmenylethanolamine

Plasmenylcholine was synthetically prepared and HPLC-purified as previously described [8] by an anhydrous reaction utilizing 1-*O*-hexadec-1′-enyl-GPC (120 mg) and octadec-9′-enoyl chloride (300 mg) as precursors and dimethylaminopyridine (100 mg) as catalyst. The purity of the synthetically prepared 16:0-18:1 plasmenylcholine was determined to be greater than 99% by reversed-phase HPLC, straight-phase HPLC and capillary gas chromatography of the aliphatic constituents. Capillary gas chromatography was also used to quantitate 16:0-18:1 plasmenylcholine.

Plasmenylethanolamine was synthesized utilizing similar strategies to that used for the preparation of plasmenylcholine with the exception that the ethanolamine head group of lysoplasmenylethanolamine was protected with FMOC and deprotected prior to and following, respectively, the acylation of 1-*O*-hexadec-1′-enyl-GPE with eicosatetra-5′,8′,11′,14′-enoyl chloride. Briefly, lysoplasmenylethanolamine was prepared by base methanolysis of 2 g of bovine heart ethanolamine glycerophospholipids and was purified by preparative straight-phase HPLC utilizing a Dynamax-Si column (total column dimensions: 21 mm×600 mm) and a linear gradient over 90 min from chloroform to methanol at 10 ml/min (HPLC System I). Next the ethanolamine head group of lysoplasmenylethanolamine was protected with FMOC by mixing 240 mg lysoplasmenylethanolamine and 540 mg FMOC in 20 ml of anhydrous, distilled chloroform for 2 h at 40°C. The FMOC-protected mixture of lysoplasmenylethanolamines was extracted into chloroform prior to the purification of 1-*O*-hexadec-1′-enyl-GPFE by HPLC utilizing an Alltech C18 column (10 mm×250 mm, 10 μm particle size) and isocratic elution with a mobile phase comprised of methanol/acetonitrile/water (57/23/20, v/v/v) containing 20 mM choline chloride at a flow rate of 9 ml/min. FMOC-protected 1-*O*-hexadec-1′-enyl-2-eicosatetra-5′,8′,11′,14′-enoyl-GPE was then prepared by mixing 40 mg 1-*O*-hexadec-1′-enyl-GPFE, 100 mg eicosatetra-5′,8′,11′,14′-enoyl chloride and 47 mg dimethylaminopyridine in 50 ml of anhydrous, redistilled chloroform at 35°C for 3 h. The reaction was terminated with the addition of 2 volumes of water/methanol (1/1, v/v) and the product, 1-*O*-hexadec-1′-enyl-2-eicosatetra-5′,8′,11′,14′-enoyl-GPFE, was collected in the chloroform phase. Deprotection of 1-*O*-hexadec-1′-enyl-2-eicosatetra-5′,8′,11′,14′-enoyl-GPFE in 1.5 ml of chloroform was initiated by the addition of 1.5 ml of diethylamine and the deprotection reaction was stirred under nitrogen for 2 h at room temperature. Deprotection was continued for another 2 h with 1 ml of additional diethylamine added. Deprotection was terminated by extraction with methanol and water added to form a counterphase. Straight-phase HPLC System I was utilized to purify 16:0-20:4 plasmenylethanolamine followed by further HPLC purification with the same preparative HPLC column but with a mobile phase comprised of hexane/isopropanol/0.005% ammonia hydroxide in water with a gradient from a mobile phase composition of 48.5/48.5/3 to 46.5/46.5/7. The purity of the synthetically prepared 16:0-20:4 plasmenylethanolamine was then determined to be greater than 99% by reversed-phase HPLC, straight-phase HPLC and capillary gas chromatography of the aliphatic constituents. Capillary gas chromatography was also used to quantitate 16:0-20:4 plasmenylethanolamine.

2.2. Preparation of cardiac sarcolemmal vesicles

Cardiac sarcolemmal vesicles were prepared from bovine ventricular tissue as described by Slaughter et al. [9] and modified by Shannon

PII S0014-5793(96)00930-1

et al. [10]. The final sarcolemma vesicle preparation was 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 from cardiac sarcolemmal vesicles and assayed as described by Hale et al. [11]. All experiments were performed in triplicate with 1–3 different bovine cardiac sarcolemmal vesicle preparations. In some experiments, the exchange inhibitory peptide (XIP) was used to inhibit sodium-calcium exchange activity as previously reported [12].

2.4. Miscellaneous assays

Bovine cardiac sarcolemmal vesicle protein content was determined by the method of Lowry et al. [13]. Reconstituted proteoliposome protein content was determined by the method of Schaffner and Weismann [14] as described by Hale et al. [11].

3. Results

3.1. Reconstitution of cardiac sodium-calcium exchange activity in proteoliposomes containing plasmalogens

Since plasmalogens are the major molecular subclass but not the exclusive molecular subclass of phospholipids present in mammalian cardiac myocyte sarcolemma [1], the effect of plasmalogens on sodium-calcium exchange activity in reconstituted proteoliposomes was compared to that of diacyl phospholipids. Previous attempts to reconstitute sodium-calcium exchange activity with diacyl phospholipids in the absence of negatively charged phospholipids (e.g. phosphatidylserine) were unsuccessful [10]. However, reconstitutions with diacyl phospholipids (e.g. 16:0-18:1 phosphatidylcholine and 16:0-20:4 phosphatidylethanolamine) in the presence of cholesterol and phosphatidylserine resulted in the reconstitution of limited sodium-calcium exchange activity (Fig. 1, condition I). In contrast, reconstitutions of sarcolemmal proteins in the presence of the plasmalogens, 16:0-18:1 plasmenylcholine and 16:0-20:4 plasmenylethanolamine, with the diacyl phospholipids, 16:0-18:1 phosphatidylcholine and 16:0-20:4 phosphatidylethanolamine, in the absence of cholesterol and phosphatidylserine resulted in the reconstitution of significantly more sodium-calcium exchange activity which was greater than that observed with diacyl phospholipids even in the presence of cholesterol and phosphatidylserine (e.g. Fig. 1, conditions II vs. I). Also, sodium-calcium exchange activity quadrupled by adding phosphatidylserine and cholesterol to the mixture of diacyl and plasmalogen phospholipids in the reconstitution system (Fig. 1, conditions III vs. I). It is important to note that condition III of Fig. 1 corresponds to the mol percent composition of plasmalogens, diacyl phospholipids and phosphatidylserine that is similar to that found in canine sarcolemma [1] and the resultant reconstituted activity approaches that of native bovine sarcolemma (e.g. Fig. 1, conditions III vs. IV). To confirm that the vesicular ^{45}Ca accumulation observed in proteoliposomes comprised of mixtures of plasmalogen and diacyl phospholipids shown in Fig. 1 was indeed a result of sodium-calcium exchange activity, we measured the ability of XIP to inhibit the exchange process [12]. As shown in Fig. 2, XIP inhibited reconstituted sodium-calcium exchange activity in all proteoliposomes, including those proteo-

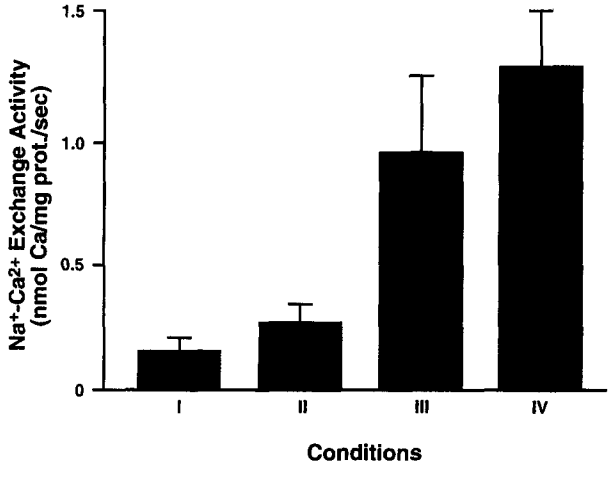

Fig. 1. Sodium-calcium exchange reconstitution experiments with plasmalogen and diacyl phospholipids. Bovine cardiac sarcolemmal vesicle proteins were extracted with sodium cholate. Solubilized vesicle proteins were reconstituted with selected exogenous phospholipids by the detergent dilution method with the final protein/phospholipid ratio being 2 mg vesicle protein/25 mg phospholipid. Sodium-calcium exchange activity was quantified as described in Section 2. Sodium-calcium exchange activity was measured as the difference of ^{45}Ca vesicular uptake in the presence or absence of an outwardly directed sodium gradient. The phospholipid composition of proteoliposomes (in mol percent) were as follows for each condition: condition I = 16:0-18:1 phosphatidylcholine (50), 16:0-20:4 phosphatidylethanolamine (40), phosphatidylserine (5), and cholesterol (5); condition II = 16:0-18:1 plasmenylcholine (30), 16:0-20.4 plasmenylethanolamine (30), 16:0-18:1 phosphatidylcholine (30), and 16:0-20:4 phosphatidylethanolamine (10); and condition III = 16:0-18:1 plasmenylcholine (25), 16:0-20:4 plasmenylethanolamine (30), 16:0-18:1 phosphatidylcholine (25), 16:0-20:4 phosphatidylethanolamine (10), phosphatidylserine (5), and cholesterol (5). For condition IV, sodium-calcium exchange activity was quantified in native bovine sarcolemmal vesicles. Values for sodium-calcium exchange activity are the mean ± S.E.M. for three independent measurements.

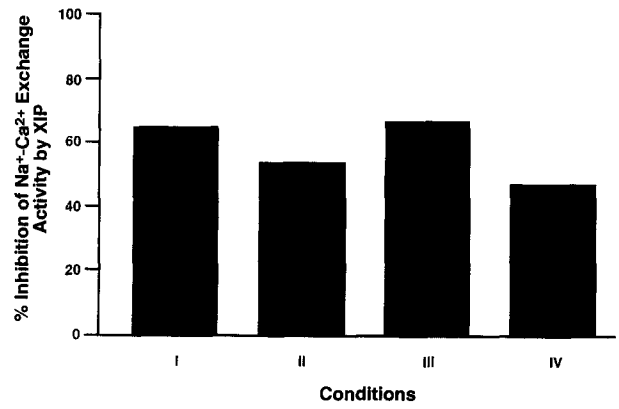

Fig. 2. Inhibition of sodium-calcium exchange activity in reconstituted proteoliposomes by the exchange inhibitory peptide (XIP). Sodium-calcium exchange activity was reconstituted into proteoliposomes as described in Section 2. Sodium-calcium exchange activity was measured in the presence and absence of 5 μM XIP (RRLLFYKYVYRYRAGKQRG) [12]. XIP was present only during the transport assay period (3 s). The proteoliposome phospholipid composition was (in mol%): condition I = 16:0-18:1 phosphatidylcholine (50), 16:0-20:4 phosphatidylethanolamine (40), phosphatidylserine (5), and cholesterol (5); condition II = 16:0-18:1 plasmenylcholine (30), 16:0-20:4 plasmenylethanolamine (30), 16:0-18:1 phosphatidylcholine (30), and 16:0-20:4 phosphatidylethanolamine (10); and condition III = 16:0-18:1 plasmenylcholine (25), 16:0-20:4 plasmenylethanolamine (30), 16:0-18:1 phosphatidylcholine (25), 16:0-20:4 phosphatidylethanolamine (10), phosphatidylserine (5), and cholesterol (5). For condition IV, sodium-calcium exchange activity was quantified in native bovine sarcolemmal vesicles. Values of sodium-calcium exchange inhibition are from three measurements under each condition.

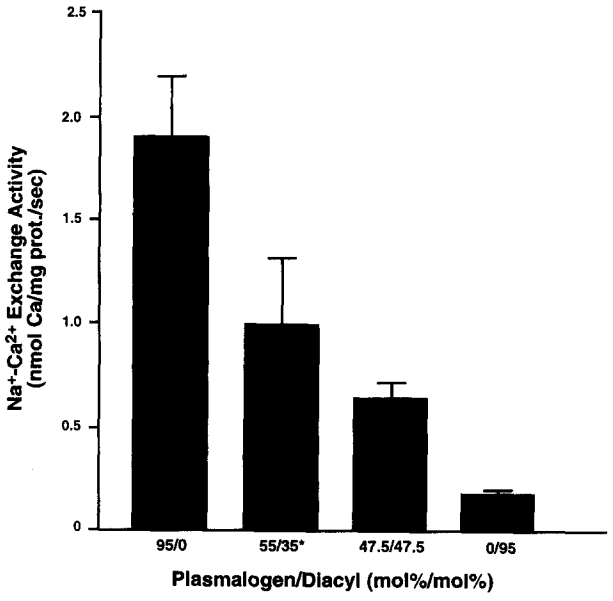

Fig. 3. Sodium-calcium exchange activity in reconstituted proteoliposomes as a function of the plasmalogen/diacyl phospholipid ratio. Sarcolemmal proteins were reconstituted into proteoliposomes, and sodium-calcium exchange activity was measured as described in Section 2. All proteoliposomes contained 5 mol% phosphatidylserine (PS). The ratios of plasmalogen/diacyl are shown on the x-axis and are expressed as mol% plasmalogen/mol% diacyl phospholipid. Plasmalogens in these experiments included 55% 16:0-18:1 plasmenylcholine and 45% 16:0-20:4 plasmenylethanolamine. Diacyl phospholipids in these experiments included 55% 16:0-18:1 phosphatidylcholine and 45% 16:0-20:4 phosphatidylethanolamine. The asterisk indicates 5 mol% cholesterol present in addition to 5 mol% phosphatidylserine and indicated plasmalogen and diacyl phospholipids. Values are the means ± S.E.M. for three measurements.

liposomes containing both plasmalogen and diacyl molecular subclasses of phospholipids as well as those containing diacyl phospholipids alone. Under all conditions, partial inhibition was observed by XIP since reconstitutions result in both right-side-in and inside-out incorporation of the sodium-calcium exchanger protein and since the XIP inhibitory peptide inhibits only at the cytoplasmic side of the exchanger [12].

3.2. *Sodium-calcium exchange activity in proteoliposomes as a function of plasmalogen content*

The role of plasmalogens as lipid activators of sodium-calcium exchange was further explored by reconstituting the sodium-calcium exchanger in vesicles comprised of different mol percents of diacyl and plasmalogen subclasses of phospholipids. Reconstitutions in these experiments contained 5 mol% phosphatidylserine (with the remaining phospholipid comprising 95 mol% of the lipid expressed as plasmalogen/diacyl phospholipid proteoliposome content). These experiments clearly demonstrated that reconstituted sodium-calcium exchange activity increased as the ratio of plasmalogen to diacyl molecular subclasses of phospholipids increased (Fig. 3). When comparing the extremes (i.e. 95/0 versus 0/95), reconstitution in plasmalogen-enriched proteoliposomes yielded a greater than 10-fold enhancement in reconstituted sodium-calcium exchange activity. Taken together, these data show that plasmalogens are important phospholipid regulators of the sarcolemmal sodium-calcium exchanger and demonstrate for the first time that plasmalogens are critical membrane phospholipids that can have profound effects on endogenous plasma membrane proteins.

3.3. *Phosphatidylserine regulates sodium-calcium exchange activity in plasmalogen vesicles*

The previous demonstration that anionic phospholipids enhance sodium-calcium exchange [10,15] has led to a model of phospholipid regulation of sodium-calcium exchange which includes a neutral lipid bilayer containing microenvironments enriched with anionic phospholipids that interact with the positively charged XIP domain of the exchanger protein [10]. Accordingly, we examined the effect of phosphatidylserine in proteoliposomes containing plasmalogens in the absence of diacyl phospholipids. The addition of phosphatidylserine (5 mol%) to plasmalogen vesicles enhanced the observed sodium-calcium exchange activity nearly 2-fold (Fig. 4). Thus, the presence of phosphatidylserine appears to enhance sodium-calcium exchange activity irrespective of the molecular subclass of the neutral phospholipid. Additionally, reconstitutions in vesicles comprised of plasmalogens and phosphatidylserine enhanced sodium-calcium exchange nearly 6-fold over that in vesicles comprised of diacyl phospholipids and phosphatidylserine (Fig. 4). It should also be noted that data in Fig. 1 demonstrate that phosphatidylserine and cholesterol enhance the activity in the presence of mixtures of diacyl and plasmalogen phospholipids.

4. Discussion

The sodium-calcium exchanger is predominantly found in the plasma membrane of excitatory cells and is a critical com-

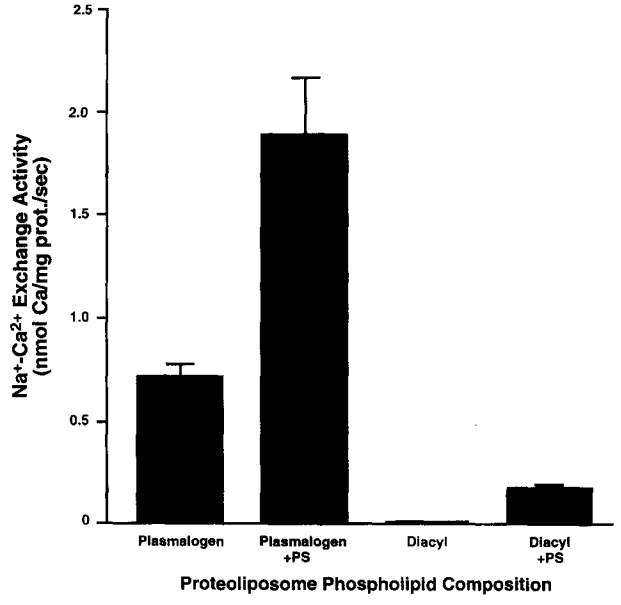

Fig. 4. The effect of phosphatidylserine on reconstituted cardiac sodium-calcium exchange activity. Sodium-calcium exchange activity was reconstituted into proteoliposomes with selected concentrations of phospholipids and quantified as described in Section 2. The proteoliposome phospholipid composition was (in mol%): plasmalogen = 16:0-18.1 plasmenylcholine (60) and 16:0-20:4 plasmenylethanolamine (40); plasmalogen + PS = 16:0-18:1 plasmenylcholine (55), 16:0-20:4 plasmenylethanolamine (40) and phosphatidylserine (5); diacyl = 100% phosphatidylcholine (from [10]); and diacyl+PS = 16:0-18:1 phosphatidylcholine (55), 16:0-20:4 phosphatidylethanolamine (40), and phosphatidylserine (5).

ponent of excitation-secretion as well as excitation-contraction coupling in these cells. Plasmalogens are the predominant phospholipids found in cardiac sarcolemma and have been proposed to have a role in myocytic ion homeostasis [16]. Although plasmalogens are the major phospholipid found in cardiac sarcolemma and the sodium-calcium exchanger is enriched in the sarcolemma, the cardiac sarcolemmal sodium-calcium exchanger has been previously reconstituted exclusively with diacyl phospholipids (e.g. [10,15]). The present results clearly demonstrate the preferential reconstitution of the sodium-calcium exchanger in phospholipid vesicles comprised of plasmalogens as compared to diacyl phospholipids and underscore the importance of plasmalogens in the regulation of endogenous plasma membrane protein function.

The demonstration that phosphatidylserine increases sodium-calcium exchange activity in plasmalogen vesicles supports and extends the model of regulation of sodium-calcium exchange activity by membrane phospholipids proposed by Hale and coworkers [10]. This expanded model includes anionic phospholipids (such as phosphatidylserine) in the presence of neutral diacyl or plasmalogen phospholipids serving as boundary lipids that promote a more active conformation of the exchange protein through interactions with the cationic XIP site. Furthermore, the enhanced sodium-calcium exchange activity in proteoliposomes comprised of plasmalogens and phosphatidylserine as compared to diacyl phospholipids and phosphatidylserine suggests that the biophysical conformation of plasmalogen vesicles provides an enhancement of sodium-calcium exchange activity either directly or indirectly through alterations in anionic phospholipid membrane dynamics in plasmalogen vesicles.

In the present study, we evaluated the ability of selected lipids (i.e. plasmalogens, diacyl phospholipids, and anionic phospholipids) to modulate cardiac sodium-calcium exchange activity. An important issue that remains unanswered is the role of the lipid environment specifically on the transported substrate ions. Mechanistically, it is possible that alterations in the phospholipid membrane environment changes substrate affinity perhaps via conformational alterations or even direct involvement with the substrate binding site. Unfortunately, current yields of synthetic plasmalogens are not amenable to producing sufficient reconstituted proteoliposomes for such experiments. This issue may be better addressed using electrophysiological techniques.

The role of membrane lipids in the modulation of transmembrane protein function is well established [17–19]. It has been suggested that one role of plasmalogens is in the regulation of intracellular ion homeostasis [16]. However, the role of plasmalogens in critical subcellular membrane pools has not been established. The results herein suggest that endogenous sarcolemmal plasmalogens have a critical role in the regulation of sarcolemmal sodium-calcium exchange. Furthermore, changes in the composition of other phospholipids (e.g. anionic phospholipids) in plasmalogen-based membrane pools could have profound effects on sodium-calcium exchange. Additionally, since plasmalogens are selectively hydrolyzed during myocardial ischemia [5–7], it is possible that alterations in sarcolemmal plasmalogen content during ischemia may contribute to ischemia-induced alterations in calcium metabolism [20] through effects on sarcolemmal sodium-calcium exchange.

Acknowledgements: This research was supported by HL 42665 from NIH (D.A.F.), Research Career Development Award, HL 03316 from NIH (D.A.F.), and the American Heart Association (C.C.H.).

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